Na\(^{+}\)-dependent purine nucleoside transporter from human kidney: cloning and functional characterization

Wang, Juan, Sheng-Fang Su, Mark J. Dresser, Marci E. Schaner, Carla B. Washington, and Kathleen M. Giacomini. Na\(^{+}\)-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. Am. J. Physiol. 273 (Renal Physiol. 42): F1058–F1065, 1997.—Many purine nucleosides and their analogs are actively transported in the kidney. Using homology cloning strategies and reverse transcriptase-polymerase chain reactions, we isolated a cDNA encoding a Na\(^{+}\)-dependent nucleoside transporter, hSPNT1, from human kidney. Functional expression in Xenopus laevis oocytes identified hSPNT1 as a Na\(^{+}\)-dependent nucleoside transporter that selectively transports purine nucleosides but also transports uridine. The Michaelis constant (K\(_{m}\)) of uridine (80 µM) in interacting with hSPNT1 was substantially higher than that of inosine (4.5 µM). hSPNT1 (658 amino acids) is 81% identical to the previously cloned rat Na\(^{+}\)-nucleoside transporter, SPNT, but differs markedly from SPNT in terms of its primary structure in the NH\(_2\) terminus. In addition, an Alu repetitive element (~282 bp) is present in the 3'-untranslated region of the hSPNT1 cDNA. Northern analysis revealed that multiple transcripts of hSPNT1 are widely distributed in human tissues including the kidney. In contrast, rat SPNT transcripts are absent in kidney and highly localized to liver and intestine. The hSPNT1 gene was localized to chromosome 15. This is the first demonstration of a purine nucleoside transporter in human kidney.

EXPERIMENTAL PROCEDURES
cDNA cloning and analysis. Two nondegenerate primers, spnt1 (5’-TGATGTTTCTCTACTATCTCGCTTGTTG-3’) and spnt2 (5’-CTGTTCTGTTTCTCTACTATCTCGCTTGTTG-3’), derived from rat SPNT cDNA, corresponding to conserved regions VMSILYGLGV and LCGFANLTSIGITLG (3), were used in PCR to amplify sequences of nucleoside transporters from human kidney cDNA under the following conditions: 94°C for 1 min, 50°C for 1.5 min, 72°C for 2 min, 30 cycles followed by a final 15-min incubation at 72°C. A PCR product of 0.8 kb (hNT1) was obtained, and DNA sequencing of hNT1 showed 88% identity to rat SPNT cDNA. To obtain the 5’ and 3’ portions of the full-length cDNA, 5’-RACE and 3’-RACE systems for rapid amplification of cDNA ends (GIBCO-BRL) were used according to the manufacturer’s protocol. A 1.6-kb 3’-RACE product was obtained after two rounds of amplification with the adapter primer and nested primers derived from hNT1. The sequence of this 1.6-kb

PURINE NUCLEOSIDES and their analogs are being widely used and developed for the treatment of cardiac disease, cancer, and viral infections. Despite extensive studies of the therapeutic activity of purine nucleosides, little is known about the renal handling of these compounds. Nephrotoxicity is one of the limiting toxicities of some purine nucleoside analogs and has been observed in deoxycoformycin and tubercidin therapy in humans (6, 7). Thus understanding the mechanisms by which the kidney transports purine nucleosides is essential in rational drug therapy and development. Previous clinical studies indicate that purine nucleosides are actively transported in the human kidney (13). In mammalian cells, several subtypes of Na\(^{+}\)-dependent secondary active nucleoside transporters have been described including a purine-selective nucleoside transporter, N1 (20), a pyrimidine-selective nucleoside transporter, N2 (20, 23), and several broadly selective nucleoside transporters (N3 and N4) (8, 24). Recently, an N1-type transporter, SPNT, was cloned from a rat liver cDNA library (3), and an N2-type transporter, rCNT1, was cloned from a rat intestine cDNA library (11). Interestingly, Northern analysis (3) and reverse transcriptase-polymerase chain reaction (RT-PCR) studies in our laboratory (data not shown) demonstrated that the mRNA transcript of SPNT was not expressed in the rat kidney, indicating that this transporter does not play a role in the renal transport of purine nucleosides in the rat.

Recently, a human homolog of rCNT1, termed hCNT1, was cloned from human kidney, providing the first evidence that pyrimidine-selective transporters are present in human kidney (17). However, it is not known whether the purine-selective transporters are present in human kidney. In this study, we report the cloning and functional characterization of a Na\(^{+}\)-dependent purine-selective transporter hSPNT1 in human kidney. The many unique features of hSPNT1 suggest that this transporter may play a critical role in the specific uptake and salvage of purine nucleosides in human kidney and other human tissues. This study provides the first molecular evidence of a Na\(^{+}\)-purine nucleoside transporter in humans.
fragment overlapped with hNT1 and contained a 15-bp poly(A)\(^+\) tail. A 1.0-kb 5′-RACE product was obtained after two rounds of amplification with the anchor primer and nested primers. This 1.0-kb fragment overlapped with hNT1 and contained a 5′ untranslated sequence as indicated by alignment with rat SPNT cDNA. The full clone was obtained by RT-PCR using a primer spanning nucleotides 10–33 of the 5′-RACE product and a primer before the poly(A)\(^+\) tail of the 3′-RACE product. The full-length cDNA fragment, termed hSPNT1, was subcloned into pGEM-T vectors (Promega) and oriented under the control of the T7 promoter. At least three clones from independent PCR reactions were sequenced. The open-reading frames of all sequenced clones were identical except for a T-to-C change at position 124 in one clone, which corresponded to a change of proline-22 to leucine-22. Either Ex-Taq (TaKaRa Shuzo, Japan) or Pfu (Stratagene) DNA polymerase was used in PCR to increase the fidelity of the reactions. DNA was sequenced at the Biochemical Resource Center at the University of California, San Francisco, using an automated DNA sequencer (Applied Biosystems). BLAST network at the National Center for Biotechnology Information was used in database searching, and the Genetics

Fig. 1. Nucleotide and deduced amino acid sequences of hSPNT1. Start of the coding sequence is defined by the first ATG downstream of two in-frame stop codons. The 14 putative transmembrane domains are underlined. The Alu repetitive element in the 3′-untranslated region is indicated in bold.
Computer Group software package (Wisconsin Package) was used to analyze nucleotides and the deduced amino acid sequences.

Expression in Xenopus laevis oocytes and nucleoside uptake assays. hSPNT1 cDNA was synthesized and injected into defolliculated oocytes as previously described (25). Uptake experiments were carried out with the respective [3H]labeled nucleoside (Moravek Biochemicals) 48–56 h postinjection at 25°C in transport buffer containing 100 mM NaCl or 100 mM choline chloride. In kinetic studies, the values for apparent Michaelis constant (Km) and maximal rate of uptake (Vmax) were determined by fitting the data to the Michaelis-Menten equation by nonlinear regression. The IC50 was determined by fitting the data to the equation V = V0/(1 + (IC50/C)), where V is the rate of uptake of inosine in the presence of the inhibitor, V0 is the rate of uptake of inosine in the absence of inhibitor, I is the inhibitor concentration, n is the slope, and IC50 is the half-maximal inhibitory concentration. Assuming a competitive mechanism of inhibition, the inhibition constant (Ki) was determined by the equation

KI = IC50/(1 + C/Km)

where C represents the concentration of inosine, and Km represents the apparent Km of inosine uptake.

Northern blot analysis. A biotin-labeled antisense RNA probe of hSPNT1 corresponding to amino acid residues 27–300 was synthesized and hybridized to a commercial human multiple tissue blot (Clontech) at 68°C overnight and detected as previously described (25). In addition, 3 µg of human small intestine poly(A)+ RNA was fractionated on a formaldehyde-agarose gel, transferred to nylon membrane (Ambion), and hybridized to the probe. The quality and quantity of the poly(A)+ RNA of each tissue loaded on the blot was checked by stripping the membrane and reprobing with a human β-actin cDNA probe.

Chromosome localization. Chromosome localization was performed by Research Genetics using radiation hybrid mapping methods (5, 22). A GeneBridge-4 panel containing 93 radiation hybrid clones of human and hamster cells was screened by PCR in a 96-well cycle plate under standard screening conditions. The primers (sense, 5′-GAGGCCAGGCAGGGAAATCCATGTGTAAGGTGAGAGCGCAATGGTGCACGGCCATCTCATC-3′; antisense, 5′-CTCTCCCTCTCGTATGGGAGGGCCAGTGTCATC-3′) used in the reaction were derived from the 5′ region of the hSPNT1 cDNA. A single hSPNT1 gene-specific PCR product, which was further confirmed by DNA sequencing, was generated when using human genomic DNA as a template, whereas no product was detected when using hamster genomic DNA as the template. The presence or absence of this gene marker in each hybrid cell line was scored by the presence or absence of the PCR product from three independent PCR reactions. The scores were then linked to the database of Whitehead radiation hybrid framework map at the MIT Center for Genome Research, and the position of the gene marker was localized on the framework map.

RESULTS

Nucleotide and deduced amino acid sequences of hSPNT1. Excluding the poly(A)+ tail, the exact length of the hSPNT1 cDNA is 2,459 bp with an open-reading frame of 1,977 bp. The open-reading frame encodes a protein of 658 amino acids and is flanked by 59-bp 5′-untranslated region (UTR) and 423-bp 3′-UTR (Fig. 1). The predicted initiation codon is preceded by a Kozak consensus sequence (A/G/XATG) (12). Two in-
frame upstream stop codons further suggest that the ATG at position 60 is the translation initiation site. The encoded protein has a calculated molecular mass of 72 kDa and an isoelectric point of 7.93. Hydropathy analysis of the primary amino acid sequence suggested the presence of 14 putative membrane-spanning segments. The NH$_2$ and COOH termini were predicted to be intracellular, and the 14 putative transmembrane domains were assigned by a combination of Kyte-Doolittle hydropathy analysis (14), application of the positive-inside rule (21), and multiple sequence alignment analysis of hSPNT1 and its related rat transporters rCNT1 and SPNT. There are six possible N-linked glycosylation sites (asparagine-238, -538, -600, -605, -624, and -653). However, none of these sites is predicted to be extracellular; therefore, none will be glycosylated, if the membrane topology prediction of hSPNT1 is correct. There are six potential protein kinase C phosphorylation sites (serine-5, -36, -198, -376, and -522 and threonine-604). Except for serine-376, all of the other sites are predicted to be intracellular and therefore may be substrates of protein kinase C. The 3'-UTR of hSPNT1 contains an Alu repetitive element (nucleotides 2177–2458) (Fig. 1). Alignment with Alu consensus sequences shows that it shares the highest identity (92%) with the Alu-Sb subfamily, one of the several subfamilies of human Alu genes.

hSPNT1 is one amino acid shorter than rat SPNT and shares 81% identity with the rat liver SPNT. The most divergent region between hSPNT1 and rat SPNT resides in the NH$_2$-terminal region. Less than 50% identity was observed in the first 63 amino acids located in the predicted NH$_2$-terminal region, whereas more than 84% identity was observed in the remaining regions (amino acids 64–658). An important difference in the NH$_2$ terminus is that the rat SPNT possesses an ATP/GTP binding motif (GXXXXGKT), whereas hSPNT1 does not (Fig. 2). Comparison with protein

Fig. 3. Expression of hSPNT1 in X. laevis oocytes. A: cRNA dose-dependent uptake. Oocytes were injected with 1, 5, 10, 20, 30, and 40 ng of hSPNT1 cRNA. Uptake of 10 µM of [H]inosine was measured at 25°C in presence of sodium or choline. B: time course of [H]inosine uptake. C: effects of nucleoside and nucleoside analogs on [H]inosine uptake in oocytes injected with hSPNT1 cRNA. Uptake was determined in sodium buffer in presence and absence (as control [ctrl]) of 1 mM of various compounds (U, uridine; I, inosine; C, cytidine; T, thymidine; G, guanosine; A, adenosine; HP, hypoxanthine; FB, formycin B). D: uptake of [H]inosine, [H]uridine, and [H]thymidine. In B, C, and D, oocytes were injected with 20 ng of cRNA or water. Each value represents the mean ± SE from 8–10 oocytes.
sequences in the database shows that hSPNT1, similar to SPNT, shares significant homology with the pyrimidine-selective transporter hCNT1 and its rat homolog rCNT1 (Fig. 2) (3, 11, 17).

Functional expression and characterization of hSPNT1. The uptake of inosine, a model purine, in X. laevis oocytes 2 days after injection was dependent on the injected dose of cRNA in a saturable manner (Fig. 3A). Because maximal expression was obtained at cRNA doses of 10–40 ng, a 20-ng dose of cRNA was used in all subsequent studies to ensure maximum expression. Compared with water-injected oocytes, a 35-fold increase in the uptake of [3H]inosine (at 30 min) was observed in cRNA-injected oocytes. [3H]inosine uptake driven by the Na\(^+\) gradient (extracellular concentration kept constant at 100 mM) was linear up to 3 h (Fig. 3B).

Inhibition studies with various purine and pyrimidine nucleosides demonstrated that [3H]inosine (12 µM) uptake was almost completely inhibited by (1 mM) adenosine, guanosine, and uridine but only slightly by cytidine and thymidine (Fig. 3C). Formycin B, a purine derivative, also significantly inhibited the uptake activity; however, it appeared to have a lower inhibition potency than the other purines. Hypoxanthine, a nucleobase, did not inhibit the uptake (Fig. 3C). Uptake studies with [3H]thymidine demonstrated that hSPNT1 did not transport thymidine at a significant level (Fig. 3D). These data suggest that hSPNT1 is a functional human Na\(^+\)-dependent purine-selective nucleoside transporter that belongs to the N1 subtype and differs

Fig. 4. Michaelis-Menten studies of inosine uptake (A), uridine uptake (B), and IC\(_{50}\) studies of inosine uptake in presence of adenosine (C) and 2'-deoxyadenosine (D) at various concentrations in hSPNT1 cRNA-injected oocytes. Each point represents the mean ± SE (n = 8-10) from one representative experiment. Apparent K\(_{m}\) and V\(_{max}\) values were determined by fitting the data to the Michaelis-Menten equation. Apparent IC\(_{50}\) and K\(_{i}\) values were obtained by fitting the data to the equations V = V\(_{o}\)[1 + (I/IC\(_{50}\))]\(^n\) and K\(_{i}\) = IC\(_{50}\)(1 + C/K\(_{i}\)), respectively. All fittings were carried out using a nonlinear fitting routine of Kaleidagraph.
from the previously characterized brush border membrane transporter, N4, and the recently cloned N2 subtype, hCNT1, in human kidney (8, 17).

Although hSPNT1 exhibits purine selectivity, it also transports the pyrimidine, uridine (Fig. 3D). To investigate whether there was a kinetic difference between the purine and pyrimidine transport processes, we examined the initial rates of uptake of inosine and uridine. Uptake of both nucleosides was saturable (Fig. 4, A and B). The \( K_m \) of inosine was 4.5 ± 1.0 \( \mu M \), whereas that of uridine was 80 ± 10 \( \mu M \). The \( V_{max} \) of inosine was 1.9 ± 0.1 pmol · oocyte \(^{-1} \) · 10 \(^{-1} \) min \(^{-1} \), whereas that of uridine was 5.3 ± 0.2 pmol · oocyte \(^{-1} \) · 10 \(^{-1} \) min \(^{-1} \) (Fig. 4, A and B). These data suggest for the first time that the N1 transporters have a higher affinity (18-fold) for inosine than for uridine and therefore may primarily transport purines under physiological conditions in which low concentrations of nucleosides (i.e., less than micromolar concentrations) are found.

The inhibition potency of adenosine and 2'-deoxyadenosine was determined by IC \(_{50} \) studies. At an inosine concentration of 12 \( \mu M \), an IC \(_{50} \) of 23 ± 3 \( \mu M \) and a \( K_m \) of 6 ± 1 \( \mu M \) were obtained for adenosine (Fig. 4C). Under identical conditions, an IC \(_{50} \) of 110 ± 26 \( \mu M \) and a \( K_m \) of 30 ± 7 \( \mu M \) were obtained for 2'-deoxyadenosine (Fig. 4D). In addition, we observed that the Na \(^+ \) -dependent uptake of the analog of 2'-deoxyadenosine, \([^3\text{H}]2\text{-chloro-2'}\text{-deoxyadenosine}, \) was enhanced approximately twofold over that in water-injected oocytes (data not shown), suggesting that 2'-deoxyribo-purine nucleosides may be permeants of hSPNT1.

Tissue distribution of hSPNT1 mRNA. Transcripts of 4.4, 2.6, 2.4, and 1.6 kb were identified in Northern blotting studies (Fig. 5). The 4.4-kb transcript was present in all tissues tested (heart, liver, skeletal muscle, kidney, intestine, pancreas, placenta, brain, and lung), with the strongest signal in the heart and the weakest in the lung. The 2.6- and 2.4-kb bands were relatively weaker than the 4.4-kb band and were observed in heart, liver, skeletal muscle, kidney, and pancreas. The 2.6-kb but not the 2.4-kb band was seen in the intestine. In addition, a strong 1.6-kb transcript was detected in heart and skeletal muscle. Excluding the poly(A) tail, the exact length of the hSPNT1 cDNA is 2,459 bp. The 3'-RACE product in the cloning process contained a 15-bp poly(A) tail, suggesting that the 3'-UTR sequence of hSPNT1 cDNA is complete. However, the poly(A) tail in the mRNA transcript of the hSPNT1 can be much longer. In addition, the sequence of the 5'-UTR may be incomplete because of the limitations of the 5'-RACE method, and additional sequences may be present at the 5' end of the hSPNT1 cDNA. For these reasons, the mRNA transcript of hSPNT1 must be longer than 2,459 bp. It is likely that the mRNA transcript at 2.6 kb represents the transcript of hSPNT1. The presence of multiple transcripts may be a result of alternatively spliced transcripts of the hSPNT1 gene or the coexistence of closely related isoforms.

Chromosomal localization. The scores for the presence of hSPNT1 gene marker in the 93 radiation hybrid cell lines were obtained and linked to the database of Whitehead framework map of these hybrid cells. hSPNT1 is assigned to chromosome 15, 3.25 centiRays (~880 kb) from the framework marker WI-4772 (linkage odds greater than 1,000:1, \( P < 0.05 \)). This corresponds approximately to chromosome 15q13–14 on the cytogenetic map.

DISCUSSION

Previous clinical studies indicate that purine nucleosides are actively transported in the human kidney. In particular, the active tubular transport of adenosine and 2'-deoxyadenosine in humans has been described by Kuttesch and Nelson (13). However, little is known about the molecular mechanisms involved in the active transport of purines in the human kidney.

Using consensus sequences from known cloned transporters, we isolated a cDNA encoding a Na \(^+ \) -dependent purine-selective nucleoside transporter, hSPNT1, from human kidney. Functional studies suggest that hSPNT1 transports purines selectively (Fig. 3, C and D) and interacts with both ribo- and deoxyribo-purine nucleosides (Fig. 4, C and D). In addition, hSPNT1 transports the pyrimidine, uridine, with a lower affinity than that of inosine (Fig. 4, A and B). Consistent with our findings, Che and co-workers (3) reported an adenosine \( K_m \) value of 6 \( \mu M \) for the rat liver SPNT, identical to the \( K_m \) of adenosine (6 \( \mu M \)) for hSPNT1. They also reported a minor SPNT-mediated transport of thymidine with a \( K_m \) of 13 \( \mu M \) (3). In contrast, no detectable hSPNT1-mediated thymidine transport was observed (Fig. 3D). Since \( V_{max} \) varies between batches of oocytes and is...
dependent on the level of expression, it is difficult to compare transport capacities between human and rat clones. Nonetheless, the data suggest that there may be some functional differences between the rat and the human transporters.

Several unique structural features markedly distinguish hSPNT1 from rat SPNT. First, the deduced NH$_2$-terminal amino acid sequence of hSPNT1 is considerably different (less than 50% identity) from that of rat SPNT despite the overall high sequence homology (81% identical). An important difference in this region is that the rat SPNT possesses an ATP/GTP binding motif, whereas hSPNT1 does not (Fig. 2). These data suggest that it is possible that different mechanisms may be involved in the regulation, targeting, and activation of these proteins. Second, an Alu repetitive element is found in the 3′-UTR of the hSPNT1 cDNA. Alu repetitive elements are short, interspersed DNA sequences that are unique to primates and comprise 5% of human genomic DNA. Recently, Alu sequences have been found to function as estrogen receptor-dependent transcriptional enhancers and as a silencer in the Wilms’ tumor gene 1 (2, 9). Proteins that bind to the Alu element and Alu RNA have been identified in human cells (4, 10). Thus it is possible that hSPNT1 can be regulated through an Alu-dependent pathway.

Strong signals of multiple hSPNT1 transcripts of different sizes (4.4, 2.6, 2.4, and 1.6 kb) were detected in human kidney as well as in heart, skeletal muscle, liver, intestine, and pancreas. In contrast, a single 3.4-kb transcript of hCNT1 was detected in human liver, intestine, and pancreas. In contrast, a single human kidney as well as in heart, skeletal muscle, different sizes (4.4, 2.6, 2.4, and 1.6 kb) were detected in the extracellular fluids surrounding its receptors, and multi-transcripts of hSPNT1 exist in these organs (1, 16, 19). The findings that cardiac effects and has been shown to prevent skeletal muscle ischemic necrosis (1, 16, 19). Adenosine also has profound effects of adenosine. The biochemical and clinical consequences of 2′-deoxycoformycin in refractory lymphoproliferative malignancy. Blood 57: 406–417, 1981.


