Molecular Cloning and Characterization of NKT, a Gene Product Related to the Organic Cation Transporter Family That Is Almost Exclusively Expressed in the Kidney*

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We have identified a gene product (NKT) encoding an apparently novel transcript that appears to be related to the organic ion transporter family and is expressed almost exclusively in the kidney. Analysis of the deduced 546-amino acid protein sequence indicates that NKT is a unique gene product which shares a similar transmembrane domain hydropathy profile as well as transporter-specific amino acid motifs with a variety of bacterial and mammalian nutrient transporters. Nevertheless, the overall homology of NKT to two recently cloned organic ion transport proteins (NLT and OCT-1) is significantly greater; together these three gene products may represent a new subgroup of transporters. The NKT was characterized further with respect to its tissue distribution and its expression during kidney development. A 2.5-kilobase transcript was found in kidney and at much lower levels in brain, but not in a number of other tissues. Studies on the embryonic kidney indicate that the NKT transcript is developmentally regulated with significant expression beginning at mouse gestational day 18 and rising just before birth, consistent with a role in differentiated kidney function. Moreover, in situ hybridization detected specific signals in mouse renal proximal tubules. NKT was mapped by linkage disequilibrium to mouse chromosome 19, the same site with a role in differentiated kidney function. Moreover, in situ hybridization detected specific signals in mouse renal proximal tubules. NKT was mapped by linkage disequilibrium to mouse chromosome 19, the same site with a role in differentiated kidney function. Moreover, in situ hybridization detected specific signals in mouse renal proximal tubules. NKT was mapped by linkage disequilibrium to mouse chromosome 19, the same site with a role in differentiated kidney function. Moreover, in situ hybridization detected specific signals in mouse renal proximal tubules. NKT was mapped by linkage disequilibrium to mouse chromosome 19, the same site with a role in differentiated kidney function.

A complementary DNA from rat kidney (OCT-1), which has the functional characteristics of organic cation uptake in the basolateral membrane of renal proximal tubules has been recently isolated (8). At the present time, only one nucleotide sequence (NLT) with significant homology to OCT-1 has been reported (9). NLT is a transporter protein of unknown substrate(s) present in the sinusoidal (basolateral) domain of hepatocytes. Increased expression of NLT at the time of birth correlates with the maturation of enterohemepatic circulation. NLT is also present in the kidney although at a lower level than in liver. Organic anions, such as bilirubin and bromosulfophthalein, have been postulated as potential substrates for NLT, although this remains to be determined.

We report here the cloning and the molecular characterization of a transcript encoding a novel protein (NKT) apparently related to the recently identified OCT-1 and NLT. The gene product is almost exclusively expressed in kidney.

MATERIALS AND METHODS

Reverse Transcription and PCR Amplification—We have previously reported a method to selectively represent mammalian protein-coding regions based on statistically designed primer sets (1). This method is based on the distribution frequency of nucleotide combinations (k-tuples) in certain genetic subsets, and the combined ability of primer pairs, based on these oligonucleotides, to detect genes. Total RNA was prepared from various mouse tissues (brain, heart, placenta, lung, liver, spleen, kidney, and stomach) using the guanidinium thioctate-cesium chloride method (10). First strand cDNA was synthesized using a commercial kit (Life Technologies, Inc., Gaithersburg, MD). A 50-μl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 0.5 mM each dNTP, 10 μg/ml oligo(dT)₁₂₋₁₈, 5 μg total RNA, 200 units of Moloney murine leukemia virus (reverse transcriptase) was incubated 60 min at 37 °C, followed by PCR amplification. In hot start PCR microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) a 20-μl reaction mixture containing 2 μl of solution from the first reaction (the final concentration of buffer components was 50 mM KCl, 1.425 mM MgCl₂, 1 μM of each primer (Life Technologies, Inc.), 12.5 μM of each dNTP, 0.5 μM [³²P]dATP, and 1 unit of Taq DNA polymerase (Perkin-Elmer) was used. The reaction mixture was subjected to 35 PCR thermocycles at 94 °C for 30 s to denature, 50 °C for 30 s for annealing, and 72 °C for 30 s for extension, followed by 5 min at 72 °C. For analysis of the PCR products, the samples were electrophore-
sed in 6% sequencing-grade gels, DNA bands were visualized by autoradiography.

Cloning and Sequencing—Bands from these gels that were only present in the kidney were cut using a razor blade and DNA was dissolved in water and subsequently precipitated in a solution of 0.5 M sodium acetate (pH 6) and 2.5 volumes of ethanol. DNA in the pellet was reamplified using the same primer pair and PCR conditions. The amplified material was examined in a low-melting point 2% agarose gel, and a commercial kit (TA Cloning[Trade], Invitrogen, San Diego, CA) was employed to clone the PCR products. Positive clones (screened by blue-white changes) were grown in 1 ml of LB broth and plasmid DNA was isolated and then sequenced on a 373A DNA fluorescence automated sequencer. Sequence homology searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service (11, 12).

RNA Blot Analysis—Total RNA was extracted from several mouse tissues (see above), as well as from mouse embryonic kidney from several developmental stages as has been previously described (10). Total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a nylon membrane. In addition, human multiple tissue Northern blots I and II were purchased from Clontech (Palo Alto, CA). The probe used for hybridization was the 332-base pair fragment from the NKT cDNA clone originally isolated from the differential display gels. The probe was labeled with [32P] using a random oligonucleotide labeling kit (Pharmacia). The final washes were carried out at 80°C. Blots were exposed to x-ray film with an intensifying screen for 3 days at ~80°C.

Rapid Amplification of 5'-cDNA and 3'-cDNA Ends (5'- and 3'-RACE)—Adaptor-ligated mouse kidney double-stranded cDNA ready for use as template in 5'- and 3'-RACE was purchased from Clontech. Gene-specific primers for 5'- and 3'-RACE reactions were designed based on the sequence of the 332-base pair fragment from the NKT cDNA originally obtained from the differential display gels. RACE reactions were performed using Clontech’s Advantage[Trade] KlenTaq Polymerase Mix, 0.5 ng of template, 50 µM of each dNTP, 0.2 µM of the adapter primer (AP), and 0.2 µM of either the 5' or 3' gene-specific primer (5'- and 3'-RACE respectively). The PCR products obtained were cloned and sequenced as has been previously described.

In Situ Hybridization—Mouse kidney was collected, rinsed in phosphate-buffered saline, and then fixed in ice-cold freshly prepared 4% paraformaldehyde phosphate-buffered saline for 1 h. They were then rinsed in 0.9% NaCl and dehydrated through a graded series of ethanol and embedded in paraffin. 7-µm sections were mounted on slides, dewaxed, pretreated, and prehybridized as described in Wedden et al. (13). Probes and RNA probes labeled with [α-35S]UTP (Amersham) were produced with T7 RNA polymerase and HindIII-linearized PCR II-NKT. Hybridization was done overnight at 50°C. Post-hybridization treatments were as follows: (i) two washes in 50% formamide, 2 × SSC, 20 mM mercaptoethanol (FSM) at 60°C for 30 min, (ii) digestion with 10 µg/ml RNase A in 4 × SSC, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA at 37°C for 30 min, and (iii) two washes in FSM at 60°C for 45 min. Slides were then dipped in Kodak NTB-2 emulsion and exposed for 10 days. Slides were then stained in 5 µg/ml Hoechst 33258 dye in water for 2 min, followed by rinsing 2 min in water. The slides were viewed under epifluorescence optics.

Chromosomal Localization—Primers were designed to amplify a region corresponding to the 3′-untranslated region of NKT in order to test for single strand conformation polymorphisms (SSCPs) between mouse strains. These were analyzed as described previously (14). Briefly, oligonucleotides were radiolabeled with [32P]ATP using polynucleotide kinase and genomic DNAs from a series of mouse strains were amplified using standard protocols (anneal at 55°C for 1 min, extend at 72°C for 2 min, and denature at 94°C for 1 min for 40 cycles, with a final extension at 72°C). 2 µl of the amplified product was added to 8.5 ml of the probe, dissolved at 94°C for 5 min, and immediately placed onto ice. 2 µl of each reaction was loaded on a 6% nondenaturing acrylamide sequencing gel and electrophoresed in 0.5 × TBE buffer for 2–3 h at 40 watts in a 4°C cold room. The primer pair with the sequences CAGACGCTGCAATTCAAGAAT (forward) and CTTCGGAATGTTGAGGTGAGAA (reverse) identified polymorphisms on C57BL/6J and Mus musculus, and was used to analyze DNA prepared from the BSS backcross (15) (Fig. 7). The strain distribution pattern was analyzed using the Map Manager Program (16).

Xenopus Oocyte Microinjection and Transport Measurement—Xenopus oocyte injection was performed as described previously (28). Manually defolliculated oocytes were injected with 40–50 ng of rat kidney medulla mRNA or NKT cRNA. Five days after injection, the uptake of radioisotope-labeled substrates was determined. For analyzing urea transport (positive control), 2.74 µCi of [14C]urea/ml and 1 mM urea were added to the uptake solution containing 200 mM mannitol, 2 mM KC1, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 5 mM Tris (pH 7.4).

Uptake was stopped by washing the oocytes with ice-cold uptake solution containing unlabeled urea. Washed oocytes were dissolved in 10% SDS and radioactivity was counted in a scintillation counter. For organic anion and cation uptake, the same procedure was followed except that the uptake solution contained 100 mM NaCl, 2 mM KC1, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 5 mM Tris (pH 7.4). The uptake was stopped by washing the oocytes with ice-cold choline solution (100 mM choline, 2 mM KC1, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 5 mM Tris, pH 7.4) and the radioactivity was counted as described above.

RESULTS

Isolation of NKT cDNA—Using a new approach to selectively represent mammalian protein-coding regions (1), we identified a novel cDNA with a kidney-specific pattern of expression (Fig. 1). This clone is referred to as NKT cDNA.

NKT cDNA Nucleotide and Primary Amino Acid Sequence—The NKT cDNA is 2161 nucleotides in length and contains both a consensus polyadenylation signal (AATAAA), and a nucleotide poly(A) tract defining the 3′ end of the clone (Fig. 2). The open reading frame is 1638 nucleotides long and encodes a protein of 546 amino acids. The deduced primary amino acid sequence of NKT is shown in Fig. 2. The deduced amino acid sequence was separately confirmed from an 84-cDNA clone amplified from mouse kidney mRNA. The AUG located in nucleotide position 182 has the strongest translation initiation consensus sequence according to Kozak’s rules and was tentatively assigned as the first codon (17). An analysis of the primary amino acid sequence using the Kyte and Doolittle algorithm predicts 11 α-helical transmembrane spanning domains (18) (Fig. 3).

These sequence data were identified as likely transmembrane domains using the Eisenberg algorithm (19). The rather large polypeptide sequence was separately confirmed from a cDNA very similar in sequence to the cDNA clone. A novel NKT cDNA clone is derived from mouse kidney RNA, and is referred to as NKT cDNA.
Cys-122 that may be involved in the formation of disulfide bridges. In addition, two hydroxyl amino acids (Ser-265 and Ser-270) located in the large intracellular loop between putative transmembrane domains six and seven represent potential targets for kinase C phosphorylation (20, 21).

**Computer Searches and Conserved Motifs—Comparison of the deduced peptide sequence of this protein with those found in available data banks revealed that NKT is a novel gene**
product related to the family of nutrient transport proteins from eukaryotes and bacteria, including, the mammalian facilitated glucose transporters, the yeast transporters for maltose, lactose, and glucose, and the proton driven bacterial transporters for arabinose, xylose, and citrate. Computer-based homology searches of GenBank, EMBL, and SwissProt data bases indicated that our cloned cDNA has not been previously described. The databasesearches indicated the NKT cDNA clone shares the greatest homology with the rat organic cation transporter (OCT-1) and a rat liver-specific transporter (NLT) of still undetermined substrate specificity. These transporters were found to be 30 and 35% identical to NKT at the amino acid level (Fig. 4). These proteins are also homologous to a group of sugar transport proteins including the human glucose transporters, the 

\[ \text{FIG.} \ 3. \  \text{Kyte-Doolittle hydropathy analysis of NKT using a window setting of 21 amino acids.} \]

Hydrophobic regions corresponding to putative transmembrane spanning domains are numbered. There may be an additional potential transmembrane spanning domain between domains 2 and 3.

Tissue Distribution and Expression of NKT mRNA—A single transcript of about 2.5 kb was observed in a mouse multiple tissue RNA blot (Fig. 5A). The transcript was most abundant in the kidney, but was also detected in very low levels in the brain. NKT transcript was not present in mouse heart, placenta, lung, liver, spleen, or stomach. In human mRNA blots, a single transcript of similar size (2.5 kb) was also observed in kidney. No signal was detected in a large number of human non-kidney tissues (Fig. 5A). In order to determine the temporal pattern of expression of the NKT gene during kidney development, we carried out RNA blot analysis of total RNA extracted from mouse kidneys at various stages of development. As shown in Fig. 5C, NKT transcripts appeared shortly before birth (fetal day 18) and were present at relatively high levels in the adult. Apparently the NLT gene becomes transcriptionally active close to the time of birth and remains active throughout adulthood.
In situ hybridization using sense and antisense cRNA on mouse kidney paraffin sections showed that the most intense signal was present in kidney cortex, following a pattern characteristic of proximal tubular localization (Fig. 6). There was no detectable signal in the glomeruli, distal tubules, or collecting ducts. RNA blot analysis done in mouse microdissected kidney also showed an intense signal in the cortex, a moderate signal in outer stripe of the outer medulla, a faint signal in inner stripe, and no signal in inner medulla (Fig. 5B), once more consistent with a proximal tubular distribution.

Chromosomal Localization of the NKT Gene—SSCP analysis was used to map the chromosomal localization of NKT (14, 23). Two primer pairs corresponding to non-overlapping regions of the 3'-untranslated region of NKT were analyzed and found to identify SSCP's between mouse species (see "Materials and Methods" and Fig. 7). The BSS interspecific backcross was genotyped and the strain distribution pattern, which were identical for the two primer pairs, was analyzed using the Map Manager program. NKT was found to map to chromosome 19 with a LOD likelihood score of 27.1. No recombinants were found between NKT and D19Mit32 in 94 progeny; NKT is therefore the most proximal gene mapped on chromosome 19 on the BSS cross. This is the very site to which a number of unknown murine mutations have been mapped (see "Discussion").

Evaluation of Microinjected NKT cRNA Capacity to Transport in Xenopus Oocytes—Since TEA and PAH are the prototypes for organic cation and organic anion transporters, respectively (8, 24), and the organic anion transporter was reported to transport PAH by exchanging with intracellular a-ketoglutarate (30), we examined these possibilities with NKT cRNA-injected oocytes under different conditions. The uptake of [14C]urea into rat medulla mRNA-injected oocytes was used as a positive control. Uptake of [14C]urea (1 mM) into rat medulla mRNA-injected oocytes resulted in a 4-fold increase above that of water control level. This is consistent with a previous study reported by You and co-workers (29). Nevertheless, NKT cRNA-injected oocytes did not demonstrate a significant amount of transport of either PAH and TEA under 100 μM concentration (Fig. 8a). When the concentration of these substrates (and, in addition, cimetidine) were increased to 1 mM (Fig. 8b), still no transport was observed. Next, we preincubated 100 μM a-ketoglutarate for 30 min before the uptake of 100 μM PAH was measured (Fig. 8c); however, we were not able to show any transport activity. Our results suggest that NKT cRNA-injected oocytes do not demonstrate sig-
significant transport for the substrates tested, at least under the conditions employed here (see "Discussion").

**DISCUSSION**

Renal tubular cells are responsible for the reabsorption and secretion of numerous substrates. These cells are highly polarized with unique species of transporters localized to either the basolateral or the apical domains of their plasma membranes. Using a new approach to selectively represent mammalian protein-coding regions (1), we have identified a novel transport protein which, by Northern analysis, is almost exclusively ex-
pressed in the kidney.

The sequence analyses of NKT suggest that it belongs to a recently identified subgroup of transport proteins. One member of this subgroup (OCT-1) has been shown to translocate hydrophobic and hydrophilic organic cations of different structures over the basolateral membrane of renal proximal tubules and hepatocytes (8). OCT-1 is currently considered a new prototype of polyspecific transporters likely to be important in drug elimination, although presently little is known of its specific role in various tissues. Xenobiotics and their metabolites are transported mainly by the organic anion (PAH) and organic cation transport systems, and there exist substrates that interact with both the transporter for organic anions and that for organic cations (2, 3). Neither transporter appears to detect the degree of ionization in bulk solution, and they also accept nonionizable substrates (4). Since these two transport systems (cationic and anionic) share so many common functional features, it is possible that they may also resemble each other at the molecular level. Functional expression of renal organic anion transport in *Xenopus laevis* oocytes injected with rat kidney poly(A) mRNA has shown that the active species with respect to PAH transport was in the range of 1.8 to 3.5 kb (24).

The size of NKT (2.5 kb) is within this range. Deduced amino acid sequence analysis showed that four cysteine residues are conserved among NKT, NLT, and OCT-1. Previous studies of the effect of N-ethylmaleimide (NEM), an irreversible sulfhydryl modifying reagent, on the transport of organic cations in the renal basolateral membrane imply that inactivation involves the binding of at least four molecules of N-ethylmaleimide per active transport unit. This is most consistent with the presence of four sulfhydryl groups at this site. The capability of organic cations to alter the susceptibility to sulfhydryl modification suggests that these groups may have a dynamic role in the transport process (25). For these and other reasons already discussed, NKT was considered to be a strong candidate for the important task of drug elimination by the kidney, a major function of the organ.

PAH and TEA are the prototype substrates of organic cation and organic anion transporters. Therefore, we tested these possibilities by measuring the uptake of the radiolabeled PAH, TEA as well as cimetidine into *Xenopus* oocytes. However, under the conditions we employed (including measurements in the presence of α-ketoglutarate (a-KG)), we were unable to show any transport activity. At present it is unclear whether this negative result was due to suboptimal conditions for NKT.
transport (despite robust transport in the positive control), poor expression or insertion of an inactive (incompletely processed) transporter protein, or because NKT might transport other substrates than those we have examined so far. Expression of NKT protein in other mammalian cells such as COS-7 cells may be required to answer these questions.

Assuming NKT is a transporter, the aforementioned data raises the possibility that it may have significantly different substrates from OCT-1. The low overall homology of the NKT sequence to the hexose transporters argues against its participation in sugar transport. When considering possible substrates from OCT-1. The low overall homology of the NKT sequence to the hexose transporters argues against its participation in sugar transport. When considering possible substrates for NKT, it is of importance to keep in mind that the expression of NKT appears to be kidney-specific, or at least preferential to that observed for OCT1. Confirmatory evidence for proximal tubular distribution was also obtained by Northern blot analysis with positive signals obtained in cortex and outer stripe, but not inner stripe and inner medulla. But unlike OCT-1 and NLT, transcripts were also detected in brain (mouse but not human), while no signal was found in liver (NLT and OCT-1) or small intestine (OCT-1). Of special interest is that NKT is expressed preferentially in the kidney and that its expression is developmentally regulated. The NKT transcripts appear shortly before birth. Studies of gene expression during kidney development have shown that genes appearing late in kidney development or at birth represent markers for highly differentiated kidney tubular cells, and these markers are often lost during neoplastic transformation. Therefore, NKT cDNA in addition to being related to organic ion transporters represents a new molecular marker for the terminally differentiated nephron.

SSCP analysis was used to localize NKT to mouse chromosome 19, tightly linked to D19Mit32. The human homologs of genes in this region such as Gstp1 and Adrbk1 map to 11q13 (27). Since subchromosomal linkage relationships are conserved in many cases between mouse and man, this result suggests that the human homolog of NKT will be found in this region. A number of interesting mouse mutations have been mapped to the proximal portion of chromosome 19, including several that affect neurological function or development (neuromuscular degeneration (nmd), muscle deficient (mdf), Dancer (Dc), deafness (dn)) or bone development (osteochondrodystrophy (ocd), osteosclerosis (oc)). Whether NKT plays a role in these murine mutations awaits further analyses.

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