Subcellular Distribution and Membrane Topology of the Mammalian Concentrative Na⁺-Nucleoside Cotransporter rCNT1*

Received for publication, January 18, 2001, and in revised form, May 25, 2001
Published, JBC Papers in Press, May 25, 2001, DOI 10.1074/jbc.M100518200

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The rat transporter rCNT1 is the archetype of a family of concentrative nucleoside transporters (CNTs) found both in eukaryotes and in prokaryotes. In the present study we have used antibodies to investigate the subcellular distribution and membrane topology of this protein. rCNT1 was found to be expressed predominantly in the brush-border membranes of the polarized epithelial cells of rat jejunum and renal cortical tubules and in the bile canalicular membranes of liver parenchymal cells, consistent with roles in the absorption of dietary nucleosides, of nucleosides in the glomerular filtrate, or of nucleosides arising from the action of extracellular nucleotidases, respectively. The effect of endoglycosidase F treatment on wild-type and mutant rCNT1 expressed in Xenopus oocytes revealed that the recombinant transporter could be glycosylated at either or both of Asn⁶⁰⁵ and Asn⁶⁴³, indicating that its C terminus is extracellular. In contrast, potential N-glycosylation sites introduced near the N terminus, or between putative transmembrane (TM) helices 4 and 5, were not glycosylated. The deduced orientation of the N terminus in the cytoplasm was confirmed by immunocytochemistry on intact and saponin-permeabilized Chinese hamster ovary cells expressing recombinant rCNT1. These results, in conjunction with extensive analyses of CNT family protein sequences using predictive algorithms, lead us to propose a revised topological model, in which rCNT1 possesses 13 TM helices with the hydrophilic N-terminal and C-terminal domains on the cytoplasmic and extracellular sides of the membrane, respectively. Furthermore, we show that the first three TM helices, which are absent from prokaryote CNTs, are not essential for transporter function; truncated proteins lacking these helices, derived either from rCNT1 or from its human homolog hCNT1, were found to retain significant sodium-dependent uridine transport activity when expressed in oocytes.

Nucleoside transport across cell membranes plays important roles both in prokaryotic and in eukaryotic organisms. For example, uptake is essential for nucleotide synthesis via salvage pathways in cells, such as mammalian bone marrow cells and many protozoan parasites of mammals, which lack de novo biosynthetic pathways (1, 2). In humans, transporters also provide the route of cellular uptake of many nucleoside analog drugs used in anti-viral or anti-cancer therapies (1, 3). By modulating levels of extracellular adenosine, nucleoside transport also plays a key role in regulating many physiological processes in mammals, including coronary vasodilatation, renal vasoconstriction, lipolysis, and platelet aggregation (4).

Transport in mammals is mediated both by Na⁺-independent (equilibrative) (c) and by Na⁺-dependent (concentrative (c)) processes (1, 2, 5). Equilibrative transport processes are found in most cell types (1, 2). They exhibit similar broad substrate selectivities for purine and pyrimidine nucleosides but can be divided into two classes, designated es and ei, by virtue of their respective sensitivity and insensitivity to inhibition by nonmolar concentrations of NBMPR (nitrobenzylthioinosine or 6-((4-nitrobenzylthio)-9-β-D-ribofuranosylpyrurine) (1, 2, 5). In contrast the concentrative transport processes, which have been functionally identified in specialized tissues such as intestinal and renal epithelia, liver, and choroid plexus, are typically insensitive to NBMPR and have been divided into three major classes on the basis of their permeant selectivities (1, 2). The cit (concentrative, insensitive to NBMPR and accepts thymidine as a permeant) processes accept pyrimidine nucleosides and adenosine, although the latter is a poor substrate. The cif (concentrative, insensitive to NBMPR and accepts formycin B as a permeant) processes accept purine nucleosides and uridine. The cib (concentrative, insensitive to NBMPR, accepts a broad range of permeants) processes accept both purine and pyrimidine nucleosides. A number of minor, NBMPR-sensitive, concentrative transport processes have also been identified (2, 6).

cDNA clones encoding proteins that mediate cit, cif, and cib transport have been isolated recently from a number of mammalian species (7–12). These ~650-residue proteins comprise a

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* This work was supported by the Medical Research Council and the Biotechnology and Biological Sciences Research Council of the United Kingdom, the National Cancer Institute of Canada, with funds from the Canadian Cancer Society, the Alberta Cancer Board, and the Natural Sciences and Engineering Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Other abbreviations used are: NBMPR, nitrobenzylthioinosine (6-((4-nitrobenzylthio)-9-β-D-ribofuranosylpyrurine); CNT, concentrative nucleoside transporter; TM, transmembrane; CHO, Chinese hamster ovary; GLUT, glucose transporter; GST, glutathione S-transferase; PBS, phosphate-buffered saline; contig, group of overlapping clones.

The abbreviations used in transporter acronyms are: e, equilibrative; c, concentrative; s and i, sensitive and insensitive to inhibition by NBMPR, respectively; f, formycin B (nometabolized purine nucleoside); t, thymidine; g, guanosine; b, broad selectivity.

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single family of transporters, unrelated to the equilibrative transporter family of mammals or to other known transporter families, which we have designated the CNT (concentrative nucleoside transporter) family. Members of the family with cit-type activity are designated CNT1, those with cif-type activity are designated CNT2, and those with cib-type activity are designated CNT3. Genome sequencing projects have revealed that CNT family members are present in other eukaryotes, including Drosophila melanogaster and Caenorhabditis elegans, and also in prokaryotes. The best characterized example of a bacterial CNT family member is the Escherichia coli nucleoside transporter NupC, which differs from its eukaryote homologs not only in being considerably smaller (~400 residues) but also in catalyzing the symport of nucleosides with protons rather than with sodium ions (13).

The existence of multiple CNT family members in mammals presumably reflects a variety of physiological roles for these transporters. Gaining a better understanding of these roles will require a knowledge not only of the detailed kinetic properties of the transporters, but also of their tissue and subcellular distributions. Northern blotting analyses have shown the expression of CNT1 and CNT2 mRNAs in many tissues (1), but in most cases it is uncertain whether these findings reflect the presence of physiologically significant amounts of transport proteins; results from Western blotting have so far revealed the presence of the rat cit-type transporter rCNT1 in liver, kidney, and intestine, and the cif-type transporter rCNT2 in liver and kidney (14, 15). Similarly, although site-directed mutagenesis experiments are beginning to reveal regions of the transporters which may be involved in substrate recognition (16, 17), their interpretation is very much dependent upon a knowledge of the arrangement of the proteins in the membrane. Initial hydrodynamic analysis of the cit-type transporter rCNT1 from rat jejunum, which was then the only eukaryote CNT family member to have been identified, tentatively suggested that this protein possessed between 10 and 14 transmembrane (TM) α-helices, and it was predicted that both the N and C termini of the protein were located on the cytoplasmic side of the membrane (7). However, no direct evidence for this topology has so far been presented, and no predictions of the topology of the now extended CNT family, including shorter bacterial members, have been made.

The aim of the present investigation was to gain a greater understanding of both the physiological roles and the structure-function relationships of a eukaryotic CNT protein. To this end polyclonal antibodies were raised against fragments of the rat cit-type transporter, rCNT1. The tissue and subcellular distributions of the transporter were assessed in a range of rat tissues by Western blotting and immunocytochemistry. Transporter topology was investigated by using the antibodies to detect recombinant rCNT1 following its production in CHO cells or in Xenopus oocytes. In combination with endoglycosidase F treatment, production in the latter system enabled us to assess the glycosylation status both of the wild-type transporter and of a series of glycosylation mutants. The resultant information, together with topological analysis of multiple CNT family protein sequences now available, has allowed us to predict a much refined model for the arrangement of rCNT1 in the membrane.

EXPERIMENTAL PROCEDURES

Creation and Removal of Potential Glycosylation Sites—Glycosylation sites were introduced into or removed from rCNT1 using a QuikChange™ site-directed mutagenesis kit (Stratagene) and the plasmid pQQH (7) as template. After mutagenesis of the potential glycosylation sites at Asn605 and/or Asn643 (BamHI-XhoI cassettes (477 base pairs) encoding the mutated C-terminal region of the protein were used to replace the corresponding region in pQQH, yielding the constructs pSRH3 (N605T), pSRH4 (N643T) and pSRH5 (N605T/N643T). Similarly, after mutation of Glu6 to Thr to introduce a potential glycosylation site in the N-terminal region of the protein at Asn9, a 532-base pair NdeI-EcoRI fragment bearing the G67 mutation was used to replace the corresponding region of pSRH5, yielding the construct designated pSRH22.

Potential N-linked glycosylation sites were also introduced into rCNT1 by insertion of the 33-residue extracellular loop of human GLUT4, which contains the site of glycosylation of this transporter at Asn38 (18). To generate this loop, oligonucleotide primers were used to amplify by polymerase chain reaction the region encoding residues 467–500 from human GLUT4 cDNA and to incorporate the flanking KpnI sites. The latter were used to insert the loop region into a unique KpnI site that had been introduced by mutagenesis immediately downstream of the nucleotides encoding Arg9 of rCNT1 in pSRH5. The presence of KpnI sites flanking the introduced a glycine-threonine sequence at each end of the GLUT4 sequence, yielding an insert that was 37 residues long.

Construction of N-terminal Truncations of rCNT1 (rCNT1/5’D3) and hCNT1 (hCNT1/5’D3)—DNAs for wild-type rCNT1 and hCNT1 (GenBank™ accession U62968) were subcloned into the vector pGEM-HE (19) for enhanced expression in Xenopus oocytes. Deletion of the regions encoding the first 173 residues of rCNT1 and the first 174 residues of hCNT1 was performed by a deletion polymerase chain reaction strategy using forward primer (5′-GATGAAATTAATGCTGAGCGGGCCCTGAGCAGCTCAGCGGCTGCTAGCACGGGCTGAGCAGCTGACG-3′) and reverse primer (5′-GATGAAATTAATGCTGAGCGGGCCCTGAGCAGCTGACG-3′) designed to contain a start codon (underlined) as well as an insert sequence corresponding to the site of truncation. Because of sequence identity between rCNT1 and hCNT1 in this region, the same primer was used to construct both rCNT1/5’D3 and hCNT1/5’D3. The reverse primer corresponded to a region within the vector multiple cloning site (AAGGATCCCCGGGGAATTGATCTG-CAAGATTG).

Topology of a Mammalian Concentrative Nucleoside Transporter
FIG. 1. Effects of endoglycosidase F (endo F) treatment on the electrophoretic mobility of recombinant rCNT1 and hCNT1. Membranes were prepared from oocytes injected with water (lanes A and B) or with 10 ng of mRNA encoding rCNT1 (lanes C and D) or hCNT1 (lanes E and F). After treatment with (+) or without (−) endo F, samples (5 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis, electrophroblotted onto nitrocellulose paper, and then stained with 2 μg/ml affinity-purified anti-rCNT1505–524. The mobilities of standard proteins of known molecular mass are indicated by the arrows.

gels, electrophroblotted onto nitrocellulose, incubated with 2 μg/ml primary antibody overnight and then with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Roche Molecular Biochemicals). Blots were developed with chemiluminescent substrate (Roche Molec-
antibody overnight and then with horseradish peroxidase-conjugated

FIG. 2. Western blot analysis of the distribution of rCNT1 in rat tissues. 70-μg samples of membranes prepared from rat brain (B), heart (H), small intestine (I), kidney (K), liver (L), skeletal muscle (M), spleen (S), and testis (T) were subjected to SDS-polyacrylamide gel electrophoresis, electrophroblotted onto nitrocellulose paper, and then stained with 2 μg/ml affinity-purified anti-rCNT1505–524. The mobilities of standard proteins of known molecular mass are indicated by the arrows.

For immunocytochemical investigation of kidney, the organ was perfused with PBS followed by 4% paraformaldehyde in PBS until the tissue surface was blanched evenly. After overnight incubation in 0.5 M sucrose at 4 °C sections were quenched by incubation with 1 mg/ml sodium tetraborohydride and blocked using an avidin/biotin blocking kit (Vector Laboratories). Sections were stained as described above, except that anti-rCNT121–78 was used at a concentration of 2.2 μg/ml followed by biotinylated goat anti-rabbit IgG (Sigma) in combination with fluorescein isothiocyanate-conjugated Extravidin™ (Sigma) to amplify the signal. Parallel sections were stained with 10 μg/ml control rabbit IgG or with antiserum raised against GST or against residues 477–492 of rat GLUT1, each at a dilution of 1:100. Sections were incubated subsequently for 1 h with goat anti-rabbit IgG FITC-conjugate (Sigma), washed, then mounted in Vectashield medium.

For immunocytochemical investigation of liver and small intestine, sections were embedded in Tissue-Tek® O.C.T. Compound and frozen in liquid Biochemicals). Blots were developed with chemiluminescent substrate (Roche Molec-
antibody overnight and then with horseradish peroxidase-conjugated

Computer Predictions of Membrane Topology—The locations of TM helices in CNT family members were predicted by analysis of individual amino acid sequences using the hidden Markov model procedure of Sonnhammer et al. as implemented in the computer program TMHMM (version 1.0) (23). In addition, multiple sequence alignments were analyzed for putative TM helices using the TMAP procedure of Persson and Argos (24) and the neural network approach (PHDhtm) of Rost et al. (25). Analyses were performed on the following 18 members of the concentative nucleoside transporter family: rCNT1 (GenBank™ accession U10279), hCNT1 (U62968), pkCNT1 (AF096976), rCNT2 (U25055), mCNT2 (AF078953), hCNT2 (AF036109), hCNT3 (AF132298), P27E11.1 (AF016413), P27E11.2 (AF016413), NUPC_HELPY (AE006623), YUTK_BACSU (U299120), YEIM_HAEIN (Swiss-Prot accession P44742), YEIM_ECOLI (P33024), YEIJ_ECOLI (P33021), YXJA_BACSU (P42312), NUPC_ECOLI (P33031), NUPC_BACSU (P39141), NUPC_STREP (open reading frame present in contig 188 from the Streptococcus pyogenes genome sequencing project, Oklaho-

RESULTS AND DISCUSSION

Tissue Distribution of rCNT1—To investigate the distribution of rCNT1 in rat tissues, polyclonal antibodies (anti-rCNT1505–524) were raised against a synthetic peptide corresponding to the region containing residues 505–524 of the protein. This region is hydrophilic and exhibits only 60% identity to the homologous transporter rCNT2. The specificity of anti-rCNT1505–524 was confirmed by its ability to recognize recombinant rCNT1 and hCNT1 (not shown in sequence in this region) but not rCNT2 or hCNT2 on Western blots of membranes from Xenopus oocytes producing these proteins (Fig. 1 and data not shown). Western blotting of membrane fractions prepared from eight rat tissues showed strongly immunoreactive bands with an apparent size between 60 and 66 kDa in small intestine, kidney, and liver, but essentially no immunoreactivity of heart, skeletal muscle, spleen, or testis membranes (Fig. 2). A faint cross-reactive band with an appar-
and the rCNT1 glycosylation site mutants N605T, N643T, and N605T/N643T. Membranes were prepared from oocytes injected with 10 ng of mRNA encoding rCNT1 (lanes A and B) or the mutants N605T (lanes C and D), N643T (lanes E and F), or N605T/N643T (lanes G and H), or with water (lanes I and J). After treatment with (+) or without (−) endoglycosidase F, Western blots of samples (5 μg of protein) were stained with 2 μg/ml affinity-purified anti-rCNT1505–524. The mobilities of standard proteins of known molecular mass are indicated by the arrows.

**Fig. 5.** Effects of endoglycosidase F (endo F) treatment on the electrophoretic mobilities of recombinant wild-type rCNT1 and the rCNT1 glycosylation-site mutants N605T/N643T/Q6T and N605T/N643T/Q200DGLUT4. Membranes were prepared from oocytes injected with 10 ng of mRNA encoding wild-type rCNT1 (lanes A and B) or the mutants N605T/N643T/Q6T (lanes C and D) or N605T/N643T/200DGLUT4 (lanes E and F). After treatment with (+) or without (−) endoglycosidase F, Western blots of samples (5 μg of protein) were stained with 2 μg/ml affinity-purified anti-rCNT1505–524. The mobilities of standard proteins of known molecular mass are indicated by the arrows.

**Subcellular Distribution of rCNT1**—The subcellular location of rCNT1 was examined by confocal immunofluorescence microscopy. Preliminary experiments revealed that paraformaldehyde fixation prevented recognition of the transporter by anti-rCNT1105–524; however, frozen tissue sections, fixed with acetone postsectioning, retained immunoreactivity and so were used for studies of intestine and liver. Specificity of rCNT1 staining was indicated by the absence of staining when preimmune serum was used (Fig. 3, insets).

**Intestine**—In sections of rat jejunum, strong staining for rCNT1 was observed at the brush-border membrane of the absorptive epithelial cells, whereas no staining was apparent on the basolateral membranes (Fig. 3, A and B). Staining of the absorptive epithelial cells was apparent from the base of the crypts of Lieberkühn to the tips of the villi, but was absent from the goblet cells. The location of rCNT1 is consistent with the reported presence of both cit- and cif-type sodium-linked nucleoside transport processes in rat and human brush-border membrane vesicles (26, 27). In contrast, nucleoside (formycin B) transport across the basolateral membrane of rabbit jejunal cells lacks sodium dependence and is probably mediated by an es-type transporter (28). The abundance of rCNT1 at the enterocyte brush border, revealed by immunocytochemistry, suggests its involvement in the absorption of luminal nucleosides derived from the diet and from cells shed from the villus tip. Although enterocytes have been reported in some studies to possess the capacity for de novo nucleotide synthesis, the energetically favorable salvage pathways are likely to be of particular importance for such rapidly dividing cells (29).

**Liver**—In liver, rCNT1 was found only in the hepatic parenchymal cells and was restricted to the bile canalicular membranes, being absent from the basolateral membranes (Fig. 3C). The liver is the main nondietary source of nucleosides for cells, such as leukocytes and bone marrow cells, which lack de novo purine nucleotide biosynthetic pathways (30). Nucleoside transporters in the canalicular membrane may reduce physiologically undesirable losses of nucleosides in bile. Such nucleosides may either be released directly into bile or generated by degradation of released nucleotides by nucleoside triphosphatase diphosphohydrolases and 5′-nucleotidase, which in hepatocytes are most abundant in the canaliculal membrane (31). Although kinetic studies with canalicular membrane vesicles have provided evidence for the presence of a cif-type adenosine transport process (31, 32), the presence of cif-type processes has not been reported previously. Whether rCNT1 functions for the uptake of pyrimidine nucleosides and adenosine across the canalicular membrane remains unclear; although the protein transports uridine and adenosine with similar affinity, its turnover number for adenosine is much lower than that for uridine (11). Sodium-dependent adenosine transport is reportedly absent from hepatocyte basolateral membrane vesicles (32), although sodium-dependent uridine transport has been described (33), and these membranes have been shown by Western blotting to be enriched in the cif-type nucleoside transporter rCNT2 (14). Interestingly, partially purified basolateral membranes were reported to be impoverished in rCNT1 relative to unfraccionated hepatocyte membranes (14), an observation consistent with our demonstration by immunocytochemistry that this transporter is confined to the bile canalicular domain of hepatocytes.

**Kidney**—To preserve tissue morphology it was necessary to fix the kidney by perfusion with paraformaldehyde, precluding use of anti-rCNT1105–524 for immunocytochemistry. Instead, anti-rCNT121–78 antibodies were employed. These yielded a pattern of staining on Western blots of rat tissues essentially identical to that observed for anti-rCNT1105–524 (data not shown) but were capable of recognizing the transporter in paraformaldehyde-fixed tissue. Their specificity was confirmed by the absence of immunoreactivity in sections incubated either with anti-GST antiserum or with control rabbit IgG (Fig. 3). Immunoreactivity was found only on the brush-border surface of epithelial cells in cortical tubules (arrow in Fig. 3D), a distinctive location that was apparent from a comparison with the staining pattern observed using antibodies against the glucose transporter GLUT1, which is known to be confined to the basolateral membranes of cortical tubular cells (Fig. 3F, arrow) (34). The observed subcellular location of rCNT1 is consistent with the kinetic demonstration of both cit- and cif-type nucleoside transport activities in rat renal brush-border membrane preparations (35). In this location the transporter is likely to play an important role in purine conservation, via reabsorption of filtered nucleosides, and may also influence other physiologically important phenomena because adenosine is known to exert potent effects on many aspects of renal function including vasoconstriction and renin release (36).

**Glycosylation States of rCNT1 and hCNT1**—Although rCNT1 was originally predicted not to be N-glycosylated (7), the differences in the electrophoretic mobility of the immuno-
reactive material observed in samples from different rat tissues and the broadness of the immunoreactive bands in intestinal and kidney samples (Fig. 2) suggested that the transporter was glycosylated. To assess this possibility, recombinant rCNT1 and hCNT1 produced in *Xenopus* oocytes were subjected to treatment with endoglycosidase F. In both cases, the electrophoretic mobility of the bands stained with anti-rCNT1 on Western blots was increased such that their apparent molecular mass decreased from 60–63 kDa (in some experiments two closely spaced bands could be resolved; see Fig. 5) to 56 kDa (Fig. 1). Interestingly, endoglycosidase treatment also increased the intensity of the immunoreactive bands, suggesting not only that both proteins were glycosylated but also that the presence of N-linked oligosaccharide interfered with recognition by the antibodies.

The polypeptide sequence of rCNT1 contains three potential N-linked glycosylation sites, at asparagines 543, 605, and 643. Asn543 is located in a hydrophobic segment of the sequence which is predicted to be membrane-spanning and so is unlikely to be glycosylated. To determine whether one or both of the other two potential glycosylation sites were glycosylated, Asn605 and Asn643 were mutated individually and simultaneously to threonine residues. Neither the single (N605T or N643T) nor the double (N605T/N643T) mutants showed apparent differences in uridine transport activity compared with wild-type rCNT1 when expressed in *Xenopus* oocytes; mediated uridine uptake activities were 123.5 ± 10.9, 109.6 ± 8.5, and 90.6 ± 9.4% of wild-type values, respectively. This observation suggested that all three mutant proteins were inserted correctly into oocyte membranes.

Western blot analysis of the mutant rCNT1 molecules after their production in *Xenopus* oocytes indicated that the mutations had changed the electrophoretic mobility of the proteins (Fig. 4). Although the wild-type rCNT1 migrated as two closely spaced, major bands with apparent molecular masses of 63 and 60 kDa, mutation of either asparagine 605 or 643 caused the
resulting recombinant transporters (N605T, N643T) to migrate as a single major band with an apparent molecular mass of 60 kDa. However, these mutants proved still to be glycosylated because treatment with endoglycosidase F increased their electrophoretic mobility further, such that they migrated with an apparent molecular mass of 56 kDa, identical to that of the deglycosylated wild-type rCNT1. Moreover, simultaneous mutation of both residues resulted in a transporter (N605T/N643T) that was unaffected by treatment with endoglycosidase F and migrated with an apparent molecular mass of 56 kDa, identical to that of the deglycosylated wild-type transporter. A more rapidly migrating species with an apparent molecular mass of 50 kDa was also evident on Western blots of deglycosylated rCNT1 samples. The relative amounts of this and of the 56-kDa species varied between experiments (cf. Figs. 1 and 5), and it is possible that these bands represent different conformers of the deglycosylated transporter in SDS. Similar behavior is exhibited by other hydrophobic membrane proteins, including the mammalian glucose transporter GLUT1 which, after enzymic deglycosylation, migrates as 46-kDa and 38-kDa species (37).

Probing rCNT1 Topology via Introduction of Novel Glycosylation Motifs—The results described above showed that the C-terminal region of rCNT1 is glycosylated and thus must be exposed at the extracellular face of the plasma membrane. If the transporter spans the membrane an even number of times, the N terminus would similarly be exposed at the extracellular surface of the plasma membrane rather than at the cytosolic surface as originally predicted (7). To distinguish between these possible locations for the N terminus, Gln6 was mutated to Thr in the aglyco mutant (N605T/N643T) of rCNT1, thereby introducing a potential glycosylation site at Asn4. The mutant protein N605T/N643T/Q6T retained 41.3 ± 9.0% of the uridine transport activity of the wild-type protein when produced in Xenopus oocytes, suggesting that it was inserted properly into the oocyte membrane during biosynthesis; however, unlike the wild-type transporter its mobility on SDS-polyacrylamide gel electrophoresis was unaffected by treatment with endoglycosidase F, indicating that the potential glycosylation site at Asn4 had not become glycosylated (Fig. 5); migration of both the mutant and deglycosylated wild-type rCNT1 as doublets probably reflects the presence of a conformer not completely unfolded by SDS.

Lack of glycosylation at Asn4 is consistent with a cytoplasmic location of the N terminus, as originally predicted for rCNT1 (7). Additional information about the protein topology in the N-terminal half of the transporter sequence was sought by introducing another potential glycosylation site in the predicted cytoplasmic loop connecting TM4 and TM5 (residues 198–204), a region that contains four contiguous positively charged residues (KHHR) and so is likely to be exposed at the membrane surface. Because of the predicted shortness of the endogenous loop, the potential glycosylation site was introduced in the form of a 37-residue segment containing the N-linked glycosylation site of human GLUT4. This GLUT4 exofacial loop region has previously been used successfully for glycosylation-scanning mutagenesis studies of the topology of human GLUT1 (38). It was inserted after Arg200 in the middle of the TM4–5 loop of the aglyco mutant (N605T/N643T) of rCNT1, immediately after the stretch of positively charged residues, to place the relevant asparagine residue sufficiently far from the flanking TM helices to act as an efficient glycosylation acceptor site (39). The recombinant mutant protein (N605T/N643T/200DGLUT4) retained 64.6 ± 10.0% of the uridine transport activity of the recombinant wild-type protein when produced in Xenopus oocytes, suggesting that it was inserted properly into the oocyte membrane during biosynthesis.

![Diagram of rCNT1 topology](http://www.jbc.org/Downloaded from)

**FIG. 8. Topographical model of rCNT1.** Potential membrane-spanning α-helices are numbered, and the endogenous sites of N-glycosylation are indicated by asterisks. Sites at which glycosylation motifs were introduced (Asn4 and Arg200) and at which rCNT1 was truncated are indicated by arrows. Hatched and open boxes indicate the regions against which antibodies were raised. The positions of basic (Arg, Lys, His), acidic (Asp, Glu), and polar but uncharged residues (Ser, Thr, Gln, Asn) are indicated by open circles (○) containing plus (+) signs, open circles (○) containing minus (−) signs, and by darkened circles (●), respectively.
acrylamide gel electrophoresis was unaffected by treatment with endoglycosidase F, indicating that the introduced glycosylation site was not glycosylated (Fig. 5). This result is compatible with the original prediction (7) that the region containing residues 198–204 of rCNT1 is exposed at the cytoplasmic surface of the oocyte membrane.

**Probing rCNT1 Topology Using Site-specific Antibodies—** The observation that an rCNT1 mutant (N605T/N643T/Q6T) bearing a potential N-glycosylation site at Asn4 was not glycosylated when produced in *Xenopus* oocytes suggested that the protein N terminus was cytoplasmically exposed. To obtain direct evidence for this topology, CHO-K1 cells were transiently transfected with a pCDNA3 construct encoding rCNT1 and then subjected to immunocytochemistry using anti-rCNT1 antibodies. As shown in Fig. 6A, strong staining was evident in the plasma membrane when paraformaldehyde-fixed cells were incubated with antibody in the presence of saponin, which permeabilizes membranes. In contrast, little staining was evident in cells treated with antibody in the absence of saponin (Fig. 6B). The specificity of the antibodies was demonstrated by the lack of staining of permeabilized nontransfected cells (Fig. 6C). These results confirm that the N-terminal region of rCNT1 is cytoplasmically oriented.

**Computer Predictions of the Membrane Topology of Concentrative Nucleoside Transporters—** Demonstration that the C-terminal region of rCNT1 is extracellular and that the N-terminal region is cytoplasmic conflicted with the original prediction (7) that this transporter possesses an even number of TM helices. These findings, together with the recent identification of many related eukaryotic and prokaryotic CNTs, prompted a reanalysis of rCNT1 and its homologs using more sophisticated computer algorithms designed to predict membrane protein topology. Two of the algorithms employed (TMAP (24) and PHDhtm (25)), perform TM helix predictions on multiple sequence alignments, thereby significantly improving the accuracy of prediction. The third algorithm, TMHMM, predicts both the locations and orientations of TM α-helices in individual sequences using a seven-state hidden mark of model for membrane proteins (23).

The results of applying these predictive methods to 18 members of the CNT family are summarized in Fig. 7. The N-terminal regions of the nine eukaryotic transporters examined were predicted by the TMHMM algorithm to be cytoplasmic, a finding consistent with the experimental evidence for the cytoplasmic location of the N terminus of rCNT1. In contrast, the N-terminal regions of the nine prokaryotic transporters examined were predicted to be extracellular. These results are consistent with absence in the prokaryotic transporters of the region corresponding to residues 1–175 of rCNT1, which is strongly predicted by all three algorithms to contain three TM helices (TM helices 1–3; Fig. 7). An extracellular location for the N termini of the prokaryote proteins is also consistent with the “positive-inside rule” (40) because the hydrophilic region following the first putative TM helix of these transporters carries a large positive charge.

All three algorithms predicted the presence in both prokaryotic and eukaryotic transporters of an additional nine shared TM helices (Fig. 7, TM helices 4–6 and 8–13) in the region corresponding to residues 175–600 of rCNT1. Prediction by the TMAP algorithm that the putative helices labeled 8 and 9 form a single TM helix probably reflects the shortness of the hydrophilic region linking them (Fig. 8).

The locations of the 12 TM helices within rCNT1 predicted by application of the three algorithms described above match closely with 12 of those originally proposed by Huang et al. (7) on the basis of hydropathic analysis. However, if the N and C termini of rCNT1 lie on opposite sides of the membrane, the transporter must contain either one or possibly three additional TM helices. The most likely location for an additional TM helix is the region corresponding to residues 290–330 of rCNT1. Although a TM helix was not predicted by the TMAP or PHDhtm algorithm, it was strongly predicted by the TMHMM algorithm in several of the eukaryotic CNT proteins (e.g. mCNT2) and weakly predicted in others, including rCNT1 (Fig. 7, open rectangle on the rCNT1 helix prediction). The presence of a TM helix at this location (Fig. 7, TM helix 7) would result in a transporter with 13 membrane-spanning helices and a topology compatible with the experimentally determined locations of the N and C termini of rCNT1. A diagram of the revised 13-TM helix topology is shown in Fig. 8. Although a 15-TM helix topology would also be compatible with the experimental results and cannot be excluded, the additional two TM helices were not strongly predicted by any of the algorithms. For example, neither the TMAP nor PHDhtm algorithm predicted a TM helix corresponding to that tentatively identified as TM helix 6 by Huang et al. (7) (Fig. 7, TM helix 6’), and a TM helix in this region was predicted by the TMHMM algorithm for only 3 out of the 18 CNT sequences used in the analyses. Similarly, a TM helix in the region corresponding to residues 480–510 in rCNT1 was only weakly predicted, and in just a few of the sequences, by the TMHMM algorithm (Fig. 7, open rectangle on the rCNT1 helix prediction).

**Role of the N-terminal Region of Eukaryote CNTs—** The eukaryote CNTs are considerably larger proteins than their prokaryote homologs. In particular, they possess an extensive N-terminal hydrophilic domain that, as described above, we have shown to be intracellular, followed by three predicted TM helices that are absent from the prokaryote transporters. Moreover, the eukaryote and prokaryote transporters differ in their ion dependence; all of the vertebrate CNTs characterized so far have been shown to be sodium symporters (1, 2, 6), whereas the archetypical prokaryote CNT, NupC from *E. coli*, is a proton symporter (13). To investigate the functional significance, if any, of these structural differences between prokaryote and eukaryote CNTs we therefore examined the effects of truncating rCNT1 and its human homolog hCNT1. The first 173 or 174...
residues, respectively, of these proteins, corresponding to the hydrophilic N-terminal region and first three putative TM helices (Fig. 8), were removed by a polymerase chain reaction deletion strategy. Fig. 9 shows that the activities of the truncated proteins, designated rCNT1/5 D3 and hCNT1/5 D3, respectively, were reduced by -97 and 94% compared with their respective wild-type counterparts. Nonetheless, they clearly retained significant uridine transport activity when expressed in Xenopus oocytes. It therefore appears that despite their presence in transporters from eukaryotes as divergent as nematodes and mammals, neither the intracellular N-terminal domain nor the first three putative TM helices of these CNTs are essential for nucleoside transport activity. Moreover, the fact that the residual activities of rCNT1/5 D3 and hCNT1/5 D3 were abolished when sodium ions in the transport medium were replaced by choline (Fig. 9) indicates that these regions of the eukaryote CNTs are also not essential for the sodium dependence of transport. Their functional role(s) therefore remain unclear.

Conclusions—The subcellular locations of rCNT1 revealed in the present study suggest that this protein plays important roles in the absorption of dietary nucleosides, in their reabsorption from the glomerular filtrate, and in their retrieval from bile. Although pyrimidine nucleosides are likely to be physiologically the most important substrates, rCNT1 can also transport adenosine, albeit with lower efficiency. Transport may therefore also be important in regulating adenosine-mediated physiological processes.

To understand the molecular mechanism of nucleoside transport, it will be important to elucidate the three-dimensional structure of the transporter and to identify the regions involved in substrate recognition. As a first step toward this goal, in the present study we have investigated the topology of the protein in the membrane and have shown that, in contrast to previous predictions, the protein spans the membrane an odd number of times. The most likely number of membrane-spanning helices is 13, but we do not exclude a 15-TM helix model. Interestingly, the prokaryote transporters, which lack the first three putative TM helices of their eukaryotic counterparts, are thus predicted to span the membrane an even number of times, most likely 10. If so, both their N and C termini would be located on the extracellular side of the cytoplasmic membrane. This topology contrasts with the prediction from genomic analyses that membrane protein topologies with cytoplasmic N and C termini are strongly preferred in most organisms so far examined (41).

Although the additional TM helices required in a 15-TM helix model for rCNT1, and other eukaryote CNTs are not strongly predicted by any of the computer algorithms employed, this may reflect the fact that these algorithms are intrinsically poor at detecting TM helices possessing hydrophilic/charged residues. Such helices might well be involved in forming the substrate translocation pathways of transporters. It is perhaps noteworthy that the two regions identified as potential additional TM helices in a 15-TM helix model contain highly conserved sequence motifs: (G/A/S)X(I/V)XX(1/3)X/IYXPPPXXXGXXTVFG (residues 235–254 of rCNT1) and (A/G)XXXGXXXXNEFVAYXXLXY (residues 486–507 of rCNT1), respectively. Whatever their location, this high sequence conservation suggests that these regions represent secondary structural elements. Current research in our laboratory is aimed at probing the topology of the C-terminal half of rCNT1 and of the homologous bacterial nucleoside transporter NupC, to ascertain whether or not these regions lie outside or in the membrane.

Acknowledgment—We thank J. Smith for providing sections of perfused rat kidney.
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Ritzel, Maurice P. Gallagher, Peter J. F. Henderson, Carol E. Cass, James D. Young and 
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J. Biol. Chem. 2001, 276:27981-27988. 
doi: 10.1074/jbc.M100518200 originally published online May 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100518200

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