Topology of a Human Equilibrative, Nitrobenzylthioinosine (NBMPR)-sensitive Nucleoside Transporter (hENT1) Implicated in the Cellular Uptake of Adenosine and Anti-cancer Drugs*

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The human equilibrative nucleoside transporter hENT1, the first identified member of the ENT family of integral membrane proteins, is the primary mechanism for the cellular uptake of physiologic nucleosides, including adenosine, and many anti-cancer nucleoside drugs. We have produced recombinant hENT1 in Xenopus oocytes and used native and engineered N-glycosylation sites in combination with immunological approaches to experimentally define the membrane architecture of this prototypic nucleoside transporter. hENT1 (456 amino acid residues) is shown to contain 11 transmembrane helical segments with an amino terminus that is intracellular and a carboxyl terminus that is extracellular. Transmembrane helices are linked by short hydrophilic regions, except for a large glycosylated extracellular loop between transmembrane helices 1 and 2 and a large central cytoplasmic loop between transmembrane helices 6 and 7. Sequence analyses suggest that this membrane topology is common to all mammalian, insect, nematode, protozoan, yeast, and plant members of the ENT protein family.

Nucleoside transporters play key roles in physiology and pharmacology (1). Uptake of exogenous nucleosides, for example, is a critical first step of nucleotide synthesis in tissues such as bone marrow and intestinal epithelium and certain parasitic organisms that lack de novo pathways for purine biosynthesis (2, 3). The same transport mechanisms function as drug transporters and mediate uptake of many synthetic nucleoside analogs used in cancer (and viral) chemotherapy (2). Nucleoside transporters also control the extracellular concentration of adenosine in the vicinity of its cell surface receptors and regulate processes such as neurotransmission and cardiovascular activity (1–3). Adenosine itself is used clinically to treat cardiac arrhythmias, and nucleoside transport inhibitors such as dipyridamole, dilazep, and drafazine function as coronary vasodilators. In mammals, plasma membrane transport of nucleosides is brought about by members of the concentrative, Na+-dependent (CNT) and equilibrative, Na+-independent (ENT) nucleoside transporter families (1–3). CNTs are expressed in a tissue-specific fashion; ENTs are present in most, possibly all, cell types.

Two ENT isoforms have been identified in human and rat tissues (4–7). Human (h) and rat (r) ENT1 and ENT2 (456–457 amino acid residues) transport both purine and pyrimidine nucleosides, including adenosine, and are distinguished functionally by a difference in sensitivity to inhibition by NBMPR: hENT1 and rENT1 are potently inhibited by NBMPR (K i 1–10 nM) and have the functional designation equilibrative-sensitive (es), while hENT2 and rENT2 are unaffected by micromolar concentrations of NBMPR and have the functional designation equilibrative-insensitive (ei) (4–7). They also differ in sensitivity to inhibition by vasodilator drugs (hENT1 > hENT2 > rENT1 = rENT2) and by the ability of hENT2 and rENT2 to transport nucleobases as well as nucleosides (1, 3, 7, 8). ENTs are widely distributed in other eukaryotes, including insects, nematodes, protozoa, yeast, and plants and do not appear to be present in prokaryotes (3).

The predicted membrane topology of hENT1, the first identified member of the ENT family, contains 11 putative transmembrane helices (4). Binding domains for NBMPR and vasodilator drugs, which compete with permeant for the substrate-binding site at the extracellular surface, comprise a region of the h/ENT1 protein (amino acid residues 100–231) encompassing putative transmembrane helices 3–6 (9, 10). A residue in the same structural domain of rENT2 (Cys140) is responsible for substrate-protectable inhibition of the transporter by PCMBS (11). In protozoa, mutations of Gly183 in transmembrane helix 5 of Leishmania donovani LdNT1.1 result in altered substrate specificity and drug resistance to tubercidin (12). A mutant form of TbAT1 from Trypanosoma brucei brucei that confers resistance to melaminophenyl arselenicals contains six amino acid substitutions in different transmembrane helices and loops of its sequence (13). While these studies have been successful in identifying functionally important roles for transmembrane helices 3–6 and other re-
gions, there is currently no experimentally based model of ENT topology. Such information is essential to provide a structural basis for further molecular and mechanistic studies of ENT transporter function.

One approach to test the two-dimensional orientation of integral membrane proteins is to identify sites of N-glycosylation. During protein synthesis, attachment of N-linked oligosaccharides to nascent polypeptide occurs on Asn residues in the motif Asn-X-Ser/Thr, where X can be any amino acid except Pro (14, 15). Due to strict compartmentalization of enzymes, N-glycosylation is carried out exclusively on the luminal side of the endoplasmic reticulum, which is topologically equivalent to the extracellular side of the protein. Thus, it is possible to identify exofacial and endofacial segments of the protein from the N-glycosylation profile. Here, we have produced recombinant hENT1 in *Xenopus* oocytes and used native and engineered *N*-glycosylation sites in combination with immunological studies of native h/ENT1 in erythrocytes and ventricular myocytes to experimentally define the topology of this prototypical nucleoside transporter protein. When combined with computer-based sequence analyses, the results provide a unified model of membrane architecture for all eukaryotic ENT nucleoside transporters.

**EXPERIMENTAL PROCEDURES**

**Computer Predictions of Membrane Topology**—The locations of transmembrane helices in hENT1 were predicted by analysis of the amino acid sequence using the hidden Markov model procedure of Sonnhammer et al. (16) as implemented in the computer program TMHMM (version 2.0) as well as computational analysis of the protein and used for putative transmembrane helices using the TMAP procedure of Persson and Argos (17) and the neural network approach (PHDhtm) of Rost et al. (18). Analyses were performed on the 34 members of the ENT protein family listed in Table I of Ref. 3. Based on the computer-based sequence analyses, the results provide a unified model of membrane architecture for all eukaryotic ENT nucleoside transporters.

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**Enzymic Deglycosylation—Oocyte membranes (10 μg of total protein) were heated to 65 °C for 5 min in 0.5% SDS, cooled to room temperature, and digested with N-Glycosidase F (Roche Molecular Biochemicals) according to the manufacturer’s instructions.** Identically treated samples omitting enzyme were used as controls.

**Competitive ELISA—Antibodies (anti-rENT1 292–307) for competitive ELISA were raised in rabbits against a synthetic peptide corresponding to hENT1 residues 254–272 by established methods (23).** Samples of the antiserum (diluted 1:50) were incubated for 90 min with serial dilutions of intact erythrocytes or of unsealed peripheral blood-depleted erythrocyte membranes (24), as previously described for topological studies on GLUT1 (23). The amount of free antibody remaining in 100-μl samples of the supernatants after centrifugation was then assessed by ELISA using microtitre plates coated with 2 μg/well protein-depleted membranes.

**Immunocytochemistry and Immunoblotting—Antibodies (anti-rENT1 292–307) for immunocytochemistry were raised against a glutathione S-transferase fusion protein bearing rENT1 residues 227–290 and affinity-purified on a column of immobilized cellulose binding domain fusion protein bearing the same rENT1 fragment.** The antibodies labeled a single band of ~65 kDa on immunoblots of rat heart membranes (data not shown). Freshly isolated rat ventricular myocytes fixed with 2% paraformaldehyde in 50 mM HEPES, pH 7.5, and Triton X-100 in PBS to permeabilize the cell membranes, followed by blocking with 10% donkey serum in PBS. Cells were then incubated overnight at 4 °C with 10 μg/ml affinity-purified antibodies in PBS containing 2% BSA. Where indicated, antibodies were pretreated with an equal concentration by weight of cellulose binding domain fusion protein for 1 h before use. After washing in PBS, samples were incubated for 1 h at 20 °C with a 1:100 dilution of an fluorescein isothiocyanate conjugate of donkey anti-rabbit IgG (Chemicon) in PBS containing 1% BSA and 1.5% donkey serum. After further washes in PBS, samples were mounted in Vectashield mounting medium and examined using a Leica TCS NT laser scanning confocal microscope and LEICA software.

**For immunoblotting, oocyte membranes (2 μg of total protein) were resolved on 12% SDS-polyacrylamide gels (25).** The electrophoresed membranes were transferred to polyvinylidene difluoride membranes and probed with affinity-purified anti-hENT1 254–272 (22). Blots were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) and developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Blots were performed at least twice to ensure reproducibility.

**RESULTS**

The topology of hENT1 predicted by hydrophathy analysis of the amino acid sequence using the algorithm of Eisenberg et al. (26) contains 11 putative transmembrane helices with an amino terminus (residues 1–12) that is intracellular and a carboxyl terminus (residues 452–456) that is extracellular (Fig. 1) (10). Transmembrane helices are linked by short (~15 residue) hydrophilic regions, except for those connecting transmembrane helices 1 and 2 (loop A), and transmembrane helices 6 and 7 (loop H), which contain 41 and 66 residues, respectively. Loop A is predicted to be extracellular and contains an N-glycosylation acceptor site at Asn258. Loop H is proposed to be intracellular and contains the remaining two hENT1 N-glycosylation acceptor sites at Asn777 and Asn288. In the present study, we have combined computer predictions of membrane topology with glycosylation scanning mutagenesis and immunological approaches to provide an in-depth analysis of the membrane topology of hENT1 and other ENT family members.

**Computer Predictions of Membrane Topology**—Application of the TMHMM algorithm to the hENT1 amino acid sequence predicted an 11-transmembrane helix topology, with cytosolic and extracellular amino and carboxyl termini, respectively (Fig. 2). This method employs a seven-state hidden Markov model for membrane proteins, and although it is applied to individual sequences, it performs with an accuracy comparable with that for multiple sequence alignment methods, correctly

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Topology of Nucleoside Transporter hENT1

Fig. 1. Topological model of hENT1. Membrane-spanning α-helices predicted from the hydrophathy profile of hENT1 according to Eisenberg et al. (30) are numbered and shown in darkened circles (10). Regions of hENT1 investigated in the present study are shown as gray circles. The three potential glycosylation sites of the protein are indicated by solid arrows. The solid star represents the endogenous N-glycosylation site (Asn48), whereas the open star indicates a novel glycosylation site engineered by point mutation. N-Glycosylation tags that were attached independently at either end of the protein are represented by open arrows. Loop insertions are shown by rectangular boxes at positions where they were introduced. To generate a constant loop size of 34 residues, segments of 19–22 amino acids derived from the native glycosylation loop A were inserted into the middle of loops B, D, and E.

Fig. 2. Transmembrane helix predictions for the ENT family of transporters. Computer predictions of membrane topology were performed on 34 mammalian, insect, nematode, protozoan, yeast, and plant members of the ENT protein family as described under “Experimental Procedures.” The solid line represents the sequence of hENT1, with the location of the natural glycosylation site indicated by an arrow. The locations of the 11 segments previously predicted from hydropathic analysis to be transmembrane helices (10) are shown as the open, numbered boxes along the sequence, while the approximate locations of insertions and deletions in the aligned sequences of the ENT family are shown by triangles. The locations of transmembrane helices predicted using the TMHMM algorithm are shown beneath the representation of hENT1 as cross-hatched rectangles. The results of analyses of the aligned sequences by the TMAP and PHDhtm methods are illustrated as black and dotted rectangles, respectively. Segments predicted by the three algorithms to be intracellular or extracellular are indicated by “i” and “o,” respectively.

Predicting the entire topology for 77% of the sequences in a standard dataset of 83 proteins with known topology (16). The predicted locations of the 11 transmembrane helices were almost identical to those previously deduced from hydropathic analysis (4) (Fig. 1). Similarly, application of the TMAP (17) and PHDhtm (18) algorithms to the aligned sequences of hENT1 and 33 other mammalian, insect, nematode, protozoan, yeast, and plant ENT family members (3) also led to the prediction of a common 11-transmembrane helix topology (Fig. 2). TMAP scans alignments for peaks in propensity curves for the hydrophobic and terminal regions of the transmembrane sequence spans, whereas PHDhtm uses a neural network trained with experimentally determined structures to predict transmembrane helices. Additional support for the locations of the proposed transmembrane helices was provided by the observation that both insertions and deletions were present in the aligned sequences in each of the proposed loops linking the transmembrane helices, except for loop C between transmembrane helices 4 and 5 (Fig. 2). The predicted shortness of loop C, the fact that it never naturally contains an insertion or deletion, and the observation that it contains a highly conserved Pro, suggest that it may play an important structural role in the ENT family. ENTs predicted to have an 11-transmembrane helix topology included T. brucei brucei TbaT1, which previously has been suggested to have a 10-transmembrane helix architecture (13).

hENT1 Glycosylation Variants—Initially, hENT1 Asn48, Asn277, and Asn288 were mutated to Gln to create the three single-site mutants N48Q, N277Q, and N288Q. Glycosylation-defective mutant N48Q was then used as the recipient for introduction of novel N-glycosylation acceptor sites into the different locations of hENT1 shown in Fig. 1. For optimal glycosylation, each novel site was well spaced (greater than 11 residues) from its nearest transmembrane helix, and engineered loops were greater than 30 residues in size (27). Thus, artificial glycosylation sites were introduced into loops B, D, E, and H, and at the amino and carboxyl termini, resulting in constructs N48Q/loop-B, N48Q/loop-D, N48Q/loop-E, N48Q/Q246N, N48Q/N-tail, and N48Q/C-tail, respectively. The remaining external loop (loop C) was not targeted, for reasons described above. RNA transcripts for hENT1 and each of the glycosylation variants were expressed in Xenopus oocytes and assayed for nucleoside transport activity (10 μM uridine influx) as described under “Experimental Procedures” (4, 22). N48Q/loop-B and N48Q/loop-E displayed lower levels of transport activity than hENT1, N48Q, N227Q, N288Q, N48Q/Q246N, N48Q/loop-D, N48Q/N-tail, or N48Q/C-tail and required a longer incubation period (30 min versus 5 min) to obtain comparable levels of cellular uptake. Nevertheless, all nine engineered transporters were functional, demonstrating that the native conformation was generally retained in all constructs and that the mutated transporters reached the cell surface. Representative influx values, corrected for endogenous nucleoside uptake measured in control water-injected oocytes, were 1.96 ± 0.06, 0.47 ± 0.06, 0.79 ± 0.09, 1.00 ± 0.17, 1.65 ± 0.11, 0.74 ± 0.06, 0.44 ± 0.06, and 1.87 ± 0.14 pmol/oocyte min⁻¹ for hENT1, N48Q, N227Q, N288Q, N48Q/Q246N, N48Q/loop-D, N48Q/N-tail, and N48Q/C-tail, respectively, and 1.39 ± 0.20 and 0.77 ± 0.22 pmol/oocyte min⁻¹ for N48Q/loop-B and N48Q/loop-E, respectively. N48Q was also functional in Saccharomyces cerevisiae (28). The glycosylation status of wild-type hENT1 and each of the mutants was then examined by immunoblotting of oocyte membrane samples before and after treatment with N-Glycosidase F.

Immunoreactivity of Wild-type Recombinant hENT1—Blots of membranes from oocytes producing wild-type hENT1 exhibited two characteristic immunobands: a diffuse major band about 50 kDa (aglyco form) (Fig. 3A) and a sharp minor band at about 50 kDa (aglyco form) (Fig. 3A). Similar to the native glyco form of the human erythrocyte es transporter (29, 30), treatment with N-glycosidase F eliminated the 55-kDa band and increased the amount of immunostaining at 50 kDa. Since intracellular membranes (endoplasmic reticulum and Golgi) as well as plasma membrane were present in the preparation, the small amount of aglyco hENT1 seen in the absence of N-glycosidase F possibly reflects newly synthesized transporter not yet modified by sugar addition. The apparent molecular mass of aglyco hENT1 was in close agreement with that predicted from its amino acid sequence (50.2 kDa), and blots of control membranes from water-injected oocytes produced no immunoreactive bands, demonstrating the specificity of the antibodies used in the study.

Identification of the Native N-Glycosylation Site Defines Loop A as Extracellular—To determine which of the three potential sites in hENT1 is actually glycosylated, the electrophoretic
human erythrocyte oocytes. Thus, disruption of the glycosylation sequence at Asn 48 abolishes –glycosylation of the protein, identifying Asn 48 as the only endogenous glycosylation site of wild-type hENT1. Previous studies have shown that the glycosylation site of the native human erythrocyte transporter is located near one of its ends (30). We have now identified this site as Asn48, which is close to the amino terminus, a result that agrees with our topology model in Fig. 1, which places loop A extracellularly.

N-Glycosylation Tagging Reveals an Intracellular Amino Terminus—We fused to the amino terminus of N48Q a sequence of 13 amino acids (MTNRLDMSQNVSM) from loop A encoding the native N-glycosylation site of hENT1. The new acceptor site was 14 amino acids away from transmembrane helix 1. On immunoblotting, this fusion construct, N48Q/N-tail, gave a single band similar to that of N48Q. No detectable increase in protein mass was observed, and the mobility of the protein was unaffected by N-glycosidase F digestion, indicating that the amino-terminal tag had not been glycosylated (Fig. 3B). This suggested that the amino terminus is in the cytoplasm, consistent with the orientation predicted by our topographical model. With the demonstration that Asn48 is extracellular, a cytosolic location for the amino terminus supports the prediction that the intervening hydrophobic region spans the lipid bilayer only once, giving rise to transmembrane helix 1.

The Orientation of Loop B Is Extracellular—To test the orientation of loop B (between transmembrane helices 3 and 4), we created in this region of N48Q by replacing Gln246 with Asn48, which is close to the amino terminus, a result that agrees with our topology model in Fig. 1, which places loop A extracellularly. The electrophoretic mobility of the immunoband of this double mutant N48Q/Q246N was very similar to that of N48Q both before and after N-glycosidase F treatment (Fig. 3B), indicating that loop B faces the extracellular side of the membrane. Support for this interpretation comes from studies of rENT2 where we have identified a unique Cys residue (Cys140) that is responsible for inhibition of this transporter by extracellular PCMBs (11). Because Cys140 is located in the exofacial half of transmembrane helix 4, it is likely that the loop between transmembrane helices 3 and 4 in rENT2 is also extracellular. Since all ENTs share a common membrane topology, loop B of hENT1 must also be exofacial.

The Central Hydrophilic Region (Loop H) Faces the Cytosol—Asn277 and Asn288, located in the carboxyl-terminal half of loop H, are not utilized by the glycosylation machinery (Fig. 3A). To verify that the first half of this large hydrophilic region is also extracellular, a new glycosylation acceptor site was created in this region of N48Q by replacing Gln246 with Asn246, thereby generating a glycosylation sequence that is responsible for inhibition of this transporter by extracellular PCMBs (11). Because Cys140 is located in the exofacial half of transmembrane helix 4, it is likely that the loop between transmembrane helices 3 and 4 in rENT2 is also extracellular. Since all ENTs share a common membrane topology, loop B of hENT1 must also be exofacial.

FIG. 4. Establishment of the sidedness of antibody binding by competitive ELISA. The ability of intact erythrocytes (○) and unsealed erythrocyte membranes (□) to compete for a limiting amount of anti-hENT1254–272 with hENT1 in 2-µg samples of erythrocyte membranes bound to the surface of a microtitre plate well was assessed as described under “Experimental Procedures.” Results shown are mean ± S.E. for triplicate measurements.
bound antigen, reaching >75% inhibition at the highest concentration of membranes employed (50 μg). In contrast, intact erythrocytes containing equivalent amounts of integral membrane proteins had no effect. The loop H epitope for the antibodies is therefore exposed on the cytoplasmic side of the human erythrocyte membrane.

The location of loop H was also assessed by immunofluorescence microscopy of fixed rat ventricular myocytes before and after permeabilization of the plasma membrane. Myocytes were chosen for study because the heart contains an abundance of the rat homolog of hENT1, rENT1. Fig. 5A shows that fusion protein antibodies against loop H of rENT1 (anti-rENT1227–290) strongly stained the surface membranes of detergent-permeabilized rat ventricular myocytes, with particular staining of the t-tubules (which are continuous with the plasma membrane). They were then stained with anti-rENT1227–290 that had been pretreated with an equal concentration by weight of a CBD fusion protein bearing residues 277–290 of rENT1 (B). Bound primary antibodies were visualized using an fluorescein isothiocyanate conjugate of donkey anti-rabbit IgG. Bar, 25 μm.

Fig. 5. Orientation of the central hydrophilic region of rENT1. Rat ventricular myocytes were fixed with paraformaldehyde and then treated with (A, B) or without (C) 0.1% Triton X-100 to permeabilize the plasma membrane. They were then stained with anti-rENT1227–290 (A, C) or with anti-rENT1227–290 that had been pretreated with an equal concentration by weight of a CBD fusion protein bearing residues 277–290 of rENT1 (B). Bound primary antibodies were visualized using an fluorescein isothiocyanate conjugate of donkey anti-rabbit IgG. Bar, 25 μm.

**DISCUSSION**

Glycosylation scanning mutagenesis was used to determine experimentally the orientation of seven key topographical landmarks of recombinant hENT1 membrane architecture (three in the amino-terminal half of the protein, the central cytoplasmic loop, three in the carboxyl-terminal half of the protein) and, in so doing, define the overall topology of the transporter as a whole. Together with computational approaches, the results obtained for the amino terminus (intracellular), loops A, B, D, and E (extracellular), loop H (intracellular), and the carboxyl terminus (extracellular) validated the topology model of hENT1 shown in Fig. 1 and were consistent with antibody studies, which independently established that loop H of native h/rENT1 was cytoplasmic. Sequence analyses predicted a common 11-transmembrane helix membrane architecture for hENT1 and other eukaryote ENT family members, setting the stage for detailed analysis of ENT structure and function in regions of the transporter implicated in inhibitor binding and nucleoside and nucleotide drug translocation. Since some protozoan ENTs are likely to be proton-dependent (3), our topology model will also have relevance to molecular studies directed toward identification of transporter domains and amino acid residues involved in cation coupling.

In their membrane topology, ENTs are strikingly similar to members of the GLUT family of sugar transporters, although the latter are predicted to have an additional transmembrane segment at the carboxyl terminus. For example, the human glucose transporter GLUT1 has a cytoplasmic amino terminus, a large glycosylated loop connecting transmembrane helices 1 and 2, and a large cytoplasmic loop connecting transmembrane helices 6 and 7 (23). Although the ENTs and the GLUTs, which belong to the major facilitator superfamily of proteins (31), show no obvious sequence similarities, they do share some functional similarities. For example, in human erythrocytes that possess both hENT1 and GLUT1, the potent sugar transport inhibitors cytochalasin B and phloretin weakly inhibit uridine fluxes, while conversely the nucleoside transport inhibitor dipyridamole weakly inhibits sugar transport (32). Moreover, adenosine inhibits hexose transport in these cells in a concentration-dependent manner (32). These observations suggest similarities between the substrate/inhibitor-binding sites of the two transporters. However, it remains to be seen whether these functional and topological similarities reflect similar helix-packing arrangements and molecular mechanisms in the two groups of transporters and a possible common evolutionary origin.

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