Communication

Molecular Determinants of Substrate Selectivity in Na\(^+\)-dependent Nucleoside Transporters*

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In mammalian cells, the salvage of purine and pyrimidine nucleosides is mediated by both facilitated and Na\(^+\)-dependent nucleoside transporters. These transporters play important roles in the transmembrane flux of therapeutic nucleoside analogs, which are widely used in the treatment of cancer and viral infections. The N1, N2, and N3 Na\(^+\)-dependent nucleoside transporters differ in terms of their transport selectivity for purine and pyrimidine nucleosides. N1 is purine-selective, N2 is pyrimidine-selective, and N3 is broadly selective. To identify structural domains involved in substrate binding and molecular determinants responsible for distinct transport selectivity, chimeric transporters were made from the cloned rat N1 and N2 transporters. Of the 14 transmembrane domains (TM) of N1 and N2, transplanting TM8–9 of N1 into N2 converted N2 from a pyrimidine- to a purine-selective transporter. Transplanting only TM8 generated a chimera with characteristics similar to the N3 transporter that has yet to be cloned. These data suggest that TM8–9 confer substrate selectivity and may form at least part of a substrate-binding site in Na\(^+\)-dependent nucleoside transporters.

Nucleosides and nucleoside analogs are increasingly being developed and used in the treatment of cancer, viral infections, and cardiac arrhythmias (1–3). Notable examples of therapeutic nucleoside analogs include cytosine arabinoside, cladribine, azidothymidine, and 2',3'-dideoxyinosine used in the treatment of cancer and human immunodeficiency virus infection. The endogenous nucleoside, adenosine, exerts profound cardiac effects and is used in the treatment of cardiac arrhythmias (3). In mammalian cells, transmembrane flux of nucleosides and nucleoside analogs is mediated by both equilibrative and Na\(^+\)-dependent nucleoside transporters (4–7). Consequently the distribution and functional characteristics of these transporters play important roles in determining the absorption, disposition, and elimination of nucleoside drugs (5, 6). Nucleoside transporters present in tumor cells and in the vicinity of purinergic receptors may also represent important gene targets for drug therapy (3, 7).

The equilibrative nucleoside transporters mediate passive downhill transport of nucleosides and function bidirectionally in accordance with the concentration gradient of the substrate. Equilibrative nucleoside transporters exhibit a broad substrate selectivity for both purine and pyrimidine nucleosides and appear to be ubiquitous in mammalian cells. They have been further classified into two subtypes (es and ei) according to their sensitivity to inhibition by nitrobenzylthioinosine (5–7). The human es type transporter, hENT1, is recently cloned and has been implicated in the cellular uptake of some chemotherapeutic drugs (8).

Na\(^+\)-dependent nucleoside transporters mediate active uphill transport of nucleosides into cells by coupling to the inwardly directed Na\(^+\) gradient across the plasma membrane. These transporters have been demonstrated in a variety of tissues including intestinal and renal epithelia, hepatocytes, choroid plexus, and cultured leukemia cells (9–14). Na\(^+\)-dependent nucleoside transporters exhibit distinct transport selectivity for purine and pyrimidine nucleosides and have been classified into several subtypes based on their substrate selectivity. The N1 (or cif) Na\(^+\)-dependent nucleoside transport system is purine-selective; the N2 (or cii) system is pyrimidine-selective; and the N3 (or cib) system is broadly selective (or nonselective). Uridine and adenosine are transported by all known Na\(^+\)-dependent nucleoside transport systems. The unique features of Na\(^+\)-dependent nucleoside transporters such as their ability to mediate uphill nucleoside transport, their distinct transport selectivity for purine and pyrimidine nucleosides, and their presence in many critical organs suggest that they may play special physiological and pharmacological roles in mammalian cells.

Recently, a purine-selective (N1 subtype) nucleoside transporter, SFNT, and a pyrimidine-selective (N2 subtype) transporter, rCNT1, were cloned from rat by expression cloning in Xenopus laevis oocytes (9, 10). The cloned N1 (659 amino acids) and N2 (648 amino acids) transporters share 64% identity and are both predicted to possess 14 putative transmembrane domains. These two cloned transporters are shown to be involved in the cellular uptake of nucleoside drugs including adenosine, cladribine, azidothymidine, and 2',3'-dideoxyctydine (9, 15–17). More recently, the human N1 and N2 homologs (hSPNT1 and hCNT1) have been cloned (18, 19). The human homologs show high sequence homology to their rat analogs (81% identity for N1 and 83% identity for N2) and display few functional differences from the rat clones (18, 19).

Despite the extensive studies on the kinetic properties of these transporters and their interactions with nucleoside drugs, little is known about the structural elements and molecular mechanisms that underlie the functional properties of Na\(^+\)-dependent nucleoside transporters. In this study, we focus on delineating the structural domains involved in substrate binding and the molecular determinants responsible for the distinct substrate selectivity of N1 and N2 nucleoside transporters. By constructing a series of chimeric N1/N2 transporters and analyzing their transport selectivity for purine or pyrimidine nucleosides, structural elements contributing to substrate binding and selectivity are revealed. Knowledge
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about the substrate-binding domains of Na\(^+\)-dependent nucleoside transporters and the determinants of their substrate selectivity should benefit the design of nucleoside drugs with improved membrane permeability and the development of nucleoside transporter inhibitors with improved potency and specificity.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric Transporters**—The cDNAs of wild-type N1 and N2 transporters were isolated by reverse transcriptase polymerase chain reaction from mRNA prepared from rat IEC-6 cells and rat intestine, respectively. The cDNAs were then subcloned into pGEM-T vector (Promega) under the control of T7 promoter. The predicted amino acid sequence of N2 is identical to the published sequence of rCNT1. The predicted amino acid sequence of N1 is identical to the published sequence of SPNT except for an Ala to Gly substitution at residue 419. The A419G substitution was also observed by other workers and was attributed to polymorphism in rats (16).

The Genetics Computer Group software (Wisconsin Package, version 8) was used to align the nucleotides and the deduced amino acid sequences of N1 and N2. Chimeras were constructed by using both native restriction enzyme sites and engineered sites introduced through site-directed mutagenesis (Sculptor<sup>TM</sup> in vitro mutagenesis system, Amaresham Corp.). Junctions were made within homologous regions, generally in stretches of identical amino acid sequences between the wild-type transporters. No foreign amino acid was introduced in any chimeras construct. The sequence of each chimera was confirmed by automated DNA sequencing in the Biochemical Resource Center at the University of California at San Francisco.

**Transport Assays in X. laevis Oocytes**—cRNA of each chimera was synthesized and injected into defolliculated oocytes as described previously (18). Uptake was measured on groups of 10 oocytes 48–56 h post-injection at 25 °C in 150 μl of transport buffer (2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4) containing either 100 mM NaCl or 100 mM choline chloride and the respective 3H-labeled nucleoside (Moravek Biochemicals). The kinetic parameter (apparent K<sub>m</sub> values) were determined by nonlinear least square fits of substrate/velocity profiles to the Michaelis-Menten equation using Kaleidagraph (version 3.0, Synergy Software). Because of the intrinsic variability in the expression level of the transporters between batches of oocytes, the data are generally expressed as the means ± S.E. from a representative experiment performed in the same batch of oocytes. However, experiments were repeated at least twice in separated batches of oocytes.

**RESULTS AND DISCUSSION**

The cDNAs of rat N1 transporter (SPNT) and rat N2 transporter (rCNT1) were cloned by reverse transcriptase polymerase chain reaction. The 14 putative transmembrane domains of transporter (rCNT1) were cloned by reverse transcriptase polymerase chain reaction. The 14 putative transmembrane domains of Na\(^+\)-dependent nucleoside transporters and the determinants of their substrate selectivity were repeated at least twice in separated batches of oocytes. Because uridine and adenosine are transported by all known Na\(^+\)-dependent nucleoside transporters, the native N3 transporter when its sequence becomes available.

Wild-type N1 transports inosine but not thymidine (Fig. 2A). The N1-mediated Na\(^+\)-dependent uridine uptake is fully inhibited (i.e., to the uptake level in the water-injected oocytes) by the common substrates, uridine and adenosine, as well as by the model purine nucleosides, inosine and guanosine. The model pyrimidine nucleosides, cytidine and thymidine, only partially inhibit the uptake (Fig. 3A). In contrast, wild-type N2 transports thymidine but not inosine (Fig. 2B). N2-mediated Na\(^+\)-dependent uridine uptake is fully inhibited by the model pyrimidine nucleosides and the common substrates but not by the model purine nucleosides (Fig. 3B). Chimera T8–9 exhibited both a substrate selectivity and an inhibition profile similar to those of the wild-type N1 (Fig. 2C and 3C), suggesting that TM8–9 contain domains responsible for the distinct purine selectivity. Interestingly, the requirement of Na\(^+\) in the transport process seemed less stringent in oocytes expressing Chimera T8–9 (Fig. 2C). Replacement of Na\(^+\) with choline only partially inhibited inosine uptake. The reason for this observation is unknown.

Chimera T8 exhibited a broad substrate selectivity, transporting both inosine and thymidine (Fig. 2D). The transport activity was fully inhibited by all of the naturally occurring purine and pyrimidine nucleosides (Fig. 3D). Broadly selective Na\(^+\)-dependent nucleoside transporters have been described and classified as N3 subtype nucleoside transporters (12, 20), but to date no typical N3 transporter has been cloned. It would be interesting to compare the sequence of Chimera T8 to that of the native N3 transporter when its sequence becomes available.

Chimera T9 was a low activity transporter with a novel substrate selectivity. When expressed in oocytes, Chimera T9 induced a 4–5-fold increase in Na\(^+\)-dependent uridine uptake (0.72 ± 0.11 pmol/oocyte/30 min for cRNA-injected versus 0.15 ± 0.02 pmol/oocyte/30 min for water-injected; mean ± S.E.) without a significant increase in the uptake of inosine or thymidine. However, the Na\(^+\)-dependent uridine uptake was inhibited to the basal level by all nucleosides at 1 mM including...
inosine and thymidine (data not shown), suggesting that all nucleosides can serve as inhibitors to this process.

To exclude the possibility that the introduction of any N1 sequence into N2 would alter its substrate selectivity, a chimera (Chimera T11) in which the TM11 and the preceding intracellular loop of N2 were replaced with those of N1 was generated. Chimera T11 maintained the substrate selectivity of N2 (data not shown), suggesting that replacing regions other than TM8–9 does not affect the substrate selectivity of N2.

In summary, these data suggest that TM8–9 confer the minimal domain requirement for the distinct substrate selectivity of N1 and N2 nucleoside transporters and may form at least part of a substrate-binding site in these transporters. Further changes within TM8–9 may alter the fitness of the binding pocket, thereby generating nucleoside transporters with novel properties that are not observed with either wild-type transporter (e.g. Chimera T8 and Chimera T9). However, it is also possible that TM8–9 themselves may not be directly involved in the binding and/or selectivity but may influence the transport selectivity through indirect interactions with other sites in the transporter protein. Sequence alignment revealed that 11 amino acids differ in the 63 amino acids spanning the

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**FIG. 2.** Uptake of 3H-labeled nucleosides by wild-type N1 (A), wild-type N2 (B), Chimera T8–9 (C), and Chimera T8 (D). X. laevis oocytes were injected with water (control) or 20 ng of cRNA. Two days after injection, uptake was measured on groups of 10 oocytes at 25 °C in 150 μl of transport buffer (2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4) in the presence of Na⁺ (100 mM NaCl, black bars) or the absence of Na⁺ (100 mM choline chloride, white bars) and the respective 3H-labeled nucleoside (10 μM). Each value represents the mean ± S.E. of data obtained in 8–10 oocytes from one representative experiment of at least three experiments of similar results (i.e. the same substrate selectivity pattern for each transporter).

**FIG. 3.** Effects of naturally occurring nucleosides on [3H]uridine uptake mediated by wild-type N1 (A), wild-type N2 (B), Chimera T8–9 (C), and Chimera T8 (D). Oocytes were injected with water or 20 ng of cRNA. [3H]Uridine uptake was determined in Na⁺-containing buffer in the absence of inhibitors in cRNA- and H₂O-injected oocytes (controls) or the presence of 1 mM of various nucleoside inhibitors in cRNA-injected oocytes. Each value represents the mean ± S.E. of data obtained in 8–10 oocytes from one representative experiment of several experiments of similar results (i.e. the same inhibition pattern for each transporter). The inhibitors are the common substrates uridine (U) and adenosine (A), the model pyrimidine nucleosides, cytidine (C) and thymidine (T), and the model purine nucleosides, guanosine (G) and inosine (I).
The initial velocities of uptake of each nucleoside were determined at 6–8 concentration points ranging from 1 to 200 μM. For each concentration point, the initial velocity (mean ± S.E.) was determined from data obtained from 8–10 oocytes. The apparent $K_m$ values and their S.E. values were determined by nonlinear least square fits of concentration/velocity profiles to the Michaelis-Menten equation using Kaleidagraph (version 3.0, Synergy Software). Each $K_m$ value in the table represents the $K_m$ (mean ± S.E.) from one representative experiment of at least two experiments in which the mean values were not significantly different. Dashes indicate no significant uptake of the respective nucleoside and hence no available $K_m$ values.

<table>
<thead>
<tr>
<th></th>
<th>Uridine</th>
<th>Inosine</th>
<th>Thymidine</th>
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<tbody>
<tr>
<td>Wild-type N1</td>
<td>34 ± 17</td>
<td>15 ± 1.6</td>
<td>—</td>
</tr>
<tr>
<td>Wild-type N2</td>
<td>22 ± 7.9</td>
<td>—</td>
<td>4.9 ± 1.7</td>
</tr>
<tr>
<td>Chimera T8–9</td>
<td>3.6 ± 0.6</td>
<td>6.3 ± 2.4</td>
<td>—</td>
</tr>
<tr>
<td>Chimera T8</td>
<td>20 ± 2.7</td>
<td>24 ± 3.6</td>
<td>14 ± 4.9</td>
</tr>
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</table>

TM8–9 of rat N1 and N2. Nine of the same substitutions are also conserved in the human N1 and N2 transporters. The importance of these residues in determining the substrate selectivity of N1 and N2 transporters needs to be investigated by mutation studies.

Our effort to construct functional reciprocal chimeras with regions containing TM8–9 of N2 transplanted into N1 was not successful. Two constructs exhibited no transport activity when expressed in oocytes. In structure-function studies with chimeric proteins, the function of chimeras is often lost due to reasons that may include protein misfolding, functional impairment, or improper plasma membrane targeting (21–23). Further studies are needed to investigate why these reciprocal constructs are not functional.

The apparent transport affinities ($K_m$) of wild-type N1 and N2 and Chimeras T8–9 and T8 toward their substrates are measured and compared (Table I). Chimera T8, which displayed a broad N3 transport selectivity, exhibited apparent $K_m$ values for uridine, inosine, and thymidine similar to those of the wild-type N1 and N2 transporters (Table I). Interestingly, Chimera T8–9, which maintained the transport selectivity of the wild-type N1, exhibited increased apparent transport affinities for both uridine ($K_m = 3.6 \mu M$) and inosine ($K_m = 6.3 \mu M$) as compared with those of wild-type N1 (34 μM for uridine and 15 μM for inosine) (Table I). In transport kinetic analysis, the Michaelis constant $K_m$ reflects not only substrate affinity for the binding site but is also influenced by rate constants of substrate translocation and dissociation that occur subsequent to recognition (22). Therefore, the observed affinity changes of Chimera T8–9 may reflect changes in any of these three processes. The apparent maximal rate of transport ($V_{\text{max}}$) reflects the efficiency by which each substrate is translocated and is greatly influenced by the expression level (22). Consequently, direct comparison of $V_{\text{max}}$ between independent experiments is not meaningful. However, under identical expression conditions, the observed single point transport activity for uridine usually followed the order of N2 > Chimera T8 > Chimera T8–9 ≥ N1.

In the present study, we identified a discrete region (TM8–9) as the structural determinant for the distinct transport selectivity of the N1 and N2 Na⁺-dependent nucleoside transporters. TM8–9 may form at least part of a substrate-binding site in these nucleoside transporters. Furthermore, we presented examples (Chimera T8 and Chimera T9) of engineering transporters with novel substrate selectivity from known transporters. Because the three-dimensional structures of membrane proteins remain difficult to achieve and the structure-function relations of Na⁺-cotransporters are largely unknown (24–27), this study using chimeric transporters provides insight into the structure-function relationship of the Na⁺-dependent nucleoside transporters. Information from such studies may benefit the design of nucleoside drugs with improved membrane permeability, targeting, and disposition characteristics.

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