Molecular Determinants of Specificity for Synthetic Nucleoside Analogs in the Concentrative Nucleoside Transporter, CNT2*

Received for publication, December 16, 2005, and in revised form, June 27, 2006. Published, JBC Papers in Press, July 13, 2006, DOI 10.1074/jbc.M513421200

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Members of the concentrative nucleoside transporter (CNT) family (SLC28) mediate the transport of naturally-occurring nucleosides, and nucleoside analog drugs across the plasma membrane of epithelial cells. Each of the three CNT family members has a distinct specificity for naturally occurring nucleosides, and residues that contribute to the specificity of each transporter have been identified. In contrast, the molecular determinants of specificity for synthetic nucleoside analogs are not known. In this study, we take advantage of the large species differences that exist between human and rat CNT2 (hCNT2 and rCNT2) in their ability to transport the nucleoside analog drug cladribine, 2CdA, (rCNT2 > > > hCNT2) to identify the critical domains and amino acid residues that contribute to the observed difference in specificity between CNT2 orthologs. Using chimeric proteins of human and rat CNT2, we determined that the C-terminal half of CNT2 contained the determinants of 2CdA selectivity. We replaced key residues in the C terminus of hCNT2 with the equivalent residue in rCNT2. One residue in the C-terminal portion of CNT2 was found to significantly contribute to 2CdA selectivity: hCNT2-S354A. This mutant caused an increase of 5–6-fold over hCNT2. The 2-chloro pharmacophore, rather than the 2'-deoxyribose was responsible for the reduced 2CdA uptake by hCNT2. Our data are consistent with a model in which an increased capability for hydrogen bonding in critical amino acids that reside in the C terminus of rCNT2 contributes to its enhanced selectivity for 2CdA.

The concentrative nucleoside transporter (CNT,2 SLC28) family consists of Na⁺-dependent influx transporters located on the plasma membrane of many different epithelial cell types (1, 2). There are three members of the CNT family, and each has a distinct specificity for naturally occurring nucleosides: CNT1 prefers pyrimidine nucleosides, CNT2 prefers purine nucleosides, but also transports uridine, and CNT3 transports both purine and pyrimidine nucleosides (3–5). Because of their unique specificity and their reasonably high sequence identity (e.g. 62.7% for CNT1 and CNT2) the molecular determinants of substrate specificity has been a subject of great interest. Studies using chimera transporters and site-directed mutagenesis have revealed large structural domains and key residues responsible for specificity of the naturally occurring nucleosides (6–8). These studies have mapped critical amino acid residues responsible for purine and pyrimidine specificity. Notably, switching two sets of amino acid residues in CNT1 to their equivalent residues in CNT2 is sufficient to change the specificity of CNT1 to that of CNT2 (8, 9).

In addition to the large specificity differences among paralogs of concentrative nucleoside transporters, there are striking specificity differences among species orthologs particularly with respect to synthetic nucleoside analogs. Evolutionary pressures exist on orthologs to maintain their specificity for naturally occurring nucleosides; however, such pressures do not exist for synthetic nucleoside analogs. Thus, striking species differences may exist among orthologs in their specificity for drugs. For example, whereas human and rat CNT2 (hCNT2 and rCNT2, respectively) have very similar specificities for naturally occurring nucleosides, there are notable specificity differences for nucleoside analogs (10). As shown in Fig. 1A, hCNT2 and rCNT2 exhibit comparable rates of transport for the naturally occurring nucleoside, inosine, whereas the orthologs differ dramatically in their ability to transport the nucleoside analog, cladribine (2CdA), a drug used in the treatment of various leukemias and lymphomas (10, 11). In particular, rCNT2 transports 2CdA at a substantially greater rate than does hCNT2 (Fig. 1, A and B). Similar species differences in transport rates between human and rat CNT2 have been previously observed for the anti-viral nucleoside analog adenosine arabinoside (Ara-A) (10).

The goal of this study was to identify the molecular determinants of substrate specificity for the nucleoside analog drug 2CdA by taking advantage of the observed difference in rate of transport of 2CdA between human and rat CNT2 orthologs. Because the two CNT2 orthologs are ~80% identical at the amino acid level, identifying key residues and domains that are responsible for the specificity differences is experimentally tractable using molecular biology methods. Such information will provide an understanding of the critical structural regions responsible for specificity for nucleoside analogs in CNT2.

In this study, we identified the region of the protein most likely involved in the selectivity of 2CdA (as well as Ara-A and
The mutation sequences were confirmed by DNA sequencing according to the mutation, and were synthesized by Invitrogen.

**EXPERIMENTAL PROCEDURES**

**Construction of Human and Rat CNT2 Single Mutants**—Human and rat CNT2 cDNA were subcloned into the amphibian high expression vector pOX (12). Human and rat CNT2 were used as templates to create the mutations described under “Results.” Mutants were created via site-directed mutagenesis, with the following PCR conditions: 94 °C for 2 min (1 cycle); 94 °C for 1 min, 55 °C for 1 min, 68 °C for 8 min (16 cycles); 72 °C for 8 min, 4 °C ∞ (1 cycle). Primers were custom designed according to the mutation, and were synthesized by Invitrogen. The mutation sequences were confirmed by DNA sequencing at the UCSF Biomolecular Recourse Center.

**Construction of Chimeric Proteins**—To create the chimeric proteins HHRR and RRHH, a NarI site was introduced via site-directed mutagenesis at amino acid position 318 of hCNT2 and 319 of rCNT2. The introduction of the NarI site was confirmed by sequencing. The human and rat CNT2 containing the NarI mutation were digested with NarI and SpeI at 37 °C for 2 h, and then the digest was loaded into a 1% agarose gel, and the bands were run until separated. The bands were then excised from the gel, and the DNA was isolated using the Qiagen gel extraction kit. The gel-extracted bands were then ligated to the appropriate fragments in a ligation reaction using T4 DNA ligase enzyme (New England Biolabs). The ligation reaction was then transformed into DH5α Escherichia coli cells, and colonies were picked and sent for sequence confirmation. Creation of the chimeric proteins RRRH, and HHHR involved the introduction of an AatII site (instead of NarI) at positions 529 and 530 of hCNT2 and rCNT2, respectively. The AatII site (which caused an I529T substitution in hCNT2) initially rendered all the chimeric proteins nonfunctional, but after the chimeras were generated, the AatII site was reversed back to the native sequence via site-directed mutagenesis, and function was restored. AatII, NarI, and SpeI were all obtained from New England Biolabs.

**Functional Screening of Variants and Chimeras in Xenopus laevis Oocytes**—X. laevis oocytes were chosen as an expression system because they have previously been used to characterize the specificity of naturally occurring nucleosides (9). Oocytes are also easily maintained, can be injected individually, and express membrane proteins very well. pOX plasmids containing CNT2 were linearized with PvuI (New England Biolabs), and cRNA was synthesized in vitro from the linearized plasmids by RNA transcription kits from either the mCAP RNA capping kit (Stratagene) or mMESSAGE mMACHINE T3 kit (Ambion). Healthy stage V and stage VI X. laevis oocytes were injected with 30–50 ng of capped cRNA transcribed in vitro with T3 RNA polymerase. Spectrophotometry was used to determine the concentration of cRNA, and an aliquot of each RNA preparation was run on a 1% agarose gel to ensure that the RNA was not degraded. Injected oocytes were stored in modified Barth’s solution at 18 °C (changed one or two times daily) for 2–3 days of expression before uptake studies. Seven to nine oocytes were incubated in Na+ buffer containing 0.1 μM [3H]2CdA and 1 μM unlabeled substrate. The uptake of radiolabeled inosine, cladrine, 2-chloroadenosine, 2’-deoxyadenosine, adenosine arabinoside, and Fluorobin was measured (all radiolabeled compounds were purchased from Moravek). Oocytes were incubated in radiolabeled substrate for 30–40 min. Uptake was terminated by the removal of buffer containing the radioligand, and the oocytes were washed five times in ice-cold choline buffer. Oocytes were then individually lysed by the addition of 100 μl of 10% SDS, and the radioactivity associated with each oocyte was determined by scintillation counting. Uptake of all substrates in oocytes expressing each construct was determined in 7–9 oocytes from a single frog. The functional studies were repeated in oocytes from at least one other frog. Data are presented as pmol of substrate/oocyte/30 min uptake, and the error bars indicated are ± S.E. Uninjected oocytes incubated with the same reaction mix were used as a control.

**Curve Fitting and Statistics**—The 2CdA kinetic curves for human and rat CNT2 were generated by fitting the data to a Michaelis-Menten curve fit, which was done by GraphPad Prism 4 Software. For the statistical tests, the mean value of the...
mutant in question was compared with the mean value of the reference CNT2, and not any of the other mutants. The $p$ values for the single mutants were also calculated by GraphPad Prism 4 software using an unpaired Student’s two-tailed $t$ test.

**Transmembrane Domain Modeling and Alignment**—The models of the individual transmembrane domains (TMDs) were created using the program HelicalWheel, and the alignment of human rat, and rabbit CNT2 was generated using ClustalW. Both programs are available as part of the SACS software package for UCSF.

**RESULTS**

**Human and Rat Chimera Transporters of CNT2**—To narrow down the region of CNT2 that was responsible for 2CdA selectivity, we constructed a series of chimeric proteins of human and rat CNT2. We constructed two sets of chimeric proteins, and named them with four letters consisting of H (hCNT2 sequence) and R (rCNT2 sequence), with each letter representing approximately one quarter of the protein. To construct the first chimera pair, a NarI site was introduced approximately halfway through the sequence of human and rat CNT2, and the subsequent chimeras HHRR and RRHH were generated. Both chimeras form functional proteins, and are able to transport inosine (Fig. 2). HHHR shows a 2CdA uptake very similar to that of rCNT2, whereas RRHH takes up 2CdA very poorly, similar to hCNT2. These results suggest that the determinants of 2CdA selectivity are located within the C-terminal half of the protein. A second set of chimeras was generated following the introduction of a second site (AatII), HHRR and RRHH. HHRR and RRHH also formed functional proteins, as they were able to take up the model substrate inosine similar to human and rat CNT2. However, HHRR and RRHH both had low uptakes of 2CdA suggesting that there were determinants of 2CdA selectivity on either side of the AatII splice site, or in the third and fourth “quarters” of CNT2. Secondary structures of the chimeras are shown to the right of the uptake with red representing rat sequence, and white representing human sequence.

**Examination of Pharmacophores in 2CdA Responsible for Specificity Differences between rCNT2 and hCNT2**—There are two modifications between the parent compound adenosine and the nucleoside analog drug 2CdA: a chloro modification on the 2-position of the base, and a 2′-deoxy modification on the sugar (Table 1). The two intermediate compounds are 2-chloroadenosine and 2′-deoxyadenosine. To determine the pharmacophore (2′-deoxyribose or 2-chloroadenine) responsible for the specificity differences of hCNT2 and rCNT2, we studied the uptake of 2-chloroadenosine and 2′-deoxyadenosine in oocytes expressing each CNT2 ortholog as well as the chimeras, HHRR and RRHH. Whereas the transport rate of 2′-deoxyadenosine was comparable to that of inosine in both human and rat CNT2 orthologs, the rate of transport of 2-chloroadenosine was substantially reduced in oocytes expressing hCNT2 and the chimera, RRHH (Fig. 3). In contrast, the rate of transport of 2-chloroadenosine was not reduced in oocytes expressing rCNT2 and the chimera, HHRR. These data suggest that the 2-chloro group of 2CdA, rather than the 2′-deoxyribose, is the pharmacophore responsible for the reduced preference of hCNT2 for 2CdA.

**Ara-A and Fludarabine Determinants Are Also Located in the C-terminal Half of CNT2**—Ara-A is an antiviral nucleoside analog that is transported by rCNT2, but only very poorly by hCNT2, similar to the species difference that exists with 2CdA (10). We tested Ara-A, and the structurally similar anti-leukemia drug Fludarabine (Table 1), with our chimeric proteins HHRR and RRHH. We found that both compounds showed the same selectivity pattern as 2CdA, with rCNT2 and HHRR transporting both compounds much better than hCNT2 and RRHH (Fig. 4). These results are consistent with the determinants of selectivity of several synthetic analogs of adenosine being different between human and rat CNT2.

**Selection of Candidate Amino Acid Residues for Site-directed Mutagenesis**—The chimera studies suggested that the critical amino acids responsible for specificity for 2CdA reside in the C-terminal region of CNT2; however, there are 51 amino acid residues which differ between the rat and human CNT2.
orthologs. All of these would be potential candidates for mutagenesis studies identifying key amino acid residues. To refine our selection of candidate residues for mutagenesis we used the rabbit ortholog of CNT2 (rbCNT2), which exhibits sequence similarity to both human and rat CNT2 in this region (13). As shown in Fig. 5A, rbCNT2 shows a very similar uptake profile to hCNT2 with respect to the uptake of 2CdA relative to inosine. It differs in its preference for 2CdA from rCNT2. Based on these findings, we chose residues for site-directed mutagenesis that were the same in human and rabbit CNT2, but were different in rCNT2 (Fig. 5B). In addition, we mutated residue A354 (Fig. 5B), which was chosen based on its proximity to residues that have previously been shown to be relevant for specificity for naturally occurring nucleosides (9, 14). Residues in the C-terminal tail were not considered good candidates for mutation because they would not likely be close to any substrate recognition portion of the protein.

**Functional Analysis of Mutant CNT2 Transporters**—After selecting the candidate amino acid residues, we performed site-directed mutagenesis on hCNT2 to replace the human amino acid residues with the respective residue of rCNT2. The single mutants of hCNT2 listed in Table 2 were constructed, and along with hCNT2-reference, were incubated with 2CdA, and the uptake values were normalized to that of hCNT2-reference. The mean of 3 trials along with the standard error is listed in Table 2. Several mutant transporters exhibited slightly enhanced or no change in 2CdA uptake relative to hCNT2, whereas hCNT2-S345A resulted in a greater than 5 fold increase in the uptake of 2CdA relative to hCNT2-reference.

We constructed a rat single mutant, rCNT2-A346S (the equivalent residue of hCNT2-S345A in rat), to examine
whether the transport of 2CdA would be reduced. The single mutant rCNT2-S440A, significantly decreased the uptake of 2CdA relative to rCNT2-reference, without a significant change to inosine uptake (Fig. 6). The top panel of Fig. 6 shows the mean uptake of inosine and 2CdA in the three trials, and the error bars represent the S.E. of the three mean uptakes. In each of the three trials, the difference between the uptake of rCNT2 and rCNT2-A346S was very statistically significant (p < 0.001). A previous study reported that hydrogen bonding was critical to substrate interaction in CNT2 (15). To test whether a decrease in hydrogen bonding capability led to a decrease in 2CdA uptake relative to that of rCNT2, we made three additional mutants in rCNT2: rCNT2-S355A, rCNT2-S440A, and rCNT2-T441A, changing the residue back to the human equivalent, with the expectation being that these variants would lose some ability to transport 2CdA. The mutant rCNT2-S440A dramatically reduced both inosine and 2CdA transport, and the results are not shown. rCNT2-T441A specifically reduced the transport of 2CdA (Fig. 6, p = 0.025), and rCNT2-S355A showed a slight, but not significant decrease in 2CdA transport relative to rCNT2.

DISCUSSION

Human and rat CNT2 are highly similar proteins (~80% amino acid identity), and both proteins share the same substrates among the naturally occurring nucleosides (i.e. purine nucleosides and uridine) (4). Despite the similarity in sequence and specificity for naturally occurring nucleosides, hCNT2 and rCNT2 exhibit a dramatic difference in their ability to transport certain nucleoside analog drugs such as Ara-A and 2CdA (10). In particular, 2CdA (and Ara-A) are excellent substrates of rCNT2 and poor substrates of hCNT2. In this study, we took advantage of the large difference in specificity in the rat and human orthologs of CNT2 for 2CdA to identify structural domains and key residues that contribute to the specificity of CNT2 for the synthetic analog 2CdA.
2CdA Specificity

TABLE 2
Uptake of 2CdA in X. laevis oocytes expressing synthetic mutants of hCNT2

The single mutants that were constructed are listed in the column on the left. The number on the right represents the uptake of 2CdA expressed as a percent of the hCNT2-reference. 100% represents the uptake of 2CdA by hCNT2-reference on the day the experiment was performed. The values represent the mean of three experiments that were conducted for each variant, and the error indicates the S.E. between the three repeats. hCNT2-S345A is bolded because it showed the highest increase in 2CdA uptake of any of the changes. Several other variants likely make smaller contributions.

| Mutants constructed | Uptake of 2CdA (% of hCNT2-reference) |
|---------------------|--------------------------------------|
| hCNT2-reference     | 100                                  |
| hCNT2-S345A         | 594 ± 13.9                           |
| hCNT2-S355A         | 594 ± 13.9                           |
| hCNT2-S355A         | 648 ± 40.5                           |
| hCNT2-R401E         | 135 ± 9.8                            |
| hCNT2-Q451H         | 26.9 ± 7.5                           |
| hCNT2-T441A         | 112 ± 6.9                            |
| hCNT2-E515N         | 126 ± 7.5                            |
| hCNT2-T475A         | 139 ± 8.7                            |
| hCNT2-A440T         | 196 ± 34.2                           |
| hCNT2-T529A         | 82.6 ± 3.8                           |

The C-terminal Region of CNT2 Contains the Determinants of Specificity for 2CdA—Our chimera studies indicate that the determinants of 2CdA specificity are located in the C-terminal region of CNT2. These results are consistent with the determinants of substrate specificity for the naturally occurring nucleosides, which were also found in the C-terminal region of the CNTs (7–9). Our further studies with second generation chimeras suggested that there are determinants of 2CdA specificity in both the third and fourth quarters of the protein. That is, 2CdA uptake was low in both RRRH and HHHR suggesting that neither the third nor the fourth quarter alone of rCNT2 could reconstitute its activity for 2CdA. However, together (i.e. HHRR) the two quarters can reconstitute rCNT2 activity for 2CdA.

One Key Amino Acid Residue Contributes to the Recognition and Translocation of 2CdA—Of the 51 amino acid differences in the C-terminal halves of human and rat CNT2, we constructed 14 single mutants based on criteria outlined under “Results” (10 in hCNT2 and 4 in rCNT2), and information from the rabbit ortholog. One single mutant of hCNT2 showed a large increase in 2CdA uptake over hCNT2-reference: hCNT2-S345A (see Table 2). hCNT2-S345A is predicted to reside in a transmembrane domain near other residues that have previously been shown to be relevant for purine and pyrimidine specificity. When the equivalent residue was mutated in rCNT2, the uptake of 2CdA was reduced dramatically, underscoring the importance of the residue at this position for determining 2CdA specificity. Several other single mutants in hCNT2 appear to contribute toward a minor (less than 2-fold) increase in 2CdA uptake over hCNT2. It is possible that some combination of these minor mutants, along with the residue at position 345 would convert hCNT2 to a rCNT2-like phenotype. Chang et al. (15) used computational methods to identify chemical features of nucleoside analogs required for interaction with concentrative nucleoside transporters. Whereas the affinity for CNT1 was found to be due to multiple factors such as electrostatic interactions and steric properties, the affinity of hCNT2 for its substrates was predicted to be dominated by hydrogen bonding interactions (15). In light of these findings, we made three additional mutations in rCNT2, all of which changed a serine or threonine residue to an alanine. rCNT2-T441A showed a statistically significant decrease in 2CdA uptake as compared with rCNT2, but this decrease much less significant than the decrease seen with rCNT2-A346S, but it still is likely to have a minor contribution toward 2CdA specificity. Many of the mutations that caused an alteration in 2CdA uptake by either hCNT2 or rCNT2 involved either the introduction or elimination of an amino acid residue containing a hydroxyl group; this finding supports the contention of Chang et al. (15) that hydrogen bonding could play a major role in the substrate recognition by CNT2.

The Chloride Modification of the Purine Base in 2CdA Is Poorly Recognized by hCNT2—Both human and rat CNT2 are able to transport adenosine, yet there are clear species differences in the ability of the orthologs to transport the synthetic analog 2CdA. Our results indicated that the 2'-deoxy phar-

FIGURE 6. Uptake of 2CdA in X. laevis oocytes expressing synthetic mutants of hCNT2 and rCNT2. Top panel, rCNT2 and rCNT2-A346S were incubated with 0.1 μM radiolabeled compound and 1 μM unlabeled compound. Three trials were performed for each condition in oocytes from different frogs. The mean uptake of three experiments is plotted, and the error represents the S.E. between the three trials. The difference in the 2CdA uptake between rCNT2 and rCNT2-A346S is statistically significant. Bottom panel, two single mutants in rCNT2 were constructed (rCNT2-S355A and rCNT2-T441A), changing the hydrophilic residues in rCNT2 to the hydrophobic equivalent residues in human. cRNA from the rat single mutants was then injected into oocytes, and incubated with either [3H]inosine (white) or [3H]2CdA (black). A representative experiment is shown. * indicates a p value of less than 0.05.
specificity are not the same for 2CdA as for the naturally occurring nucleosides. The large difference in specificity between human and rat CNT2 for many synthetic nucleoside analogs suggests that rat may be a poor animal model in the testing of these compounds for their applicability in human therapy. Beginning to understand the complex relationship between transporter proteins and nucleoside analog drugs could facilitate efforts at rational drug design, and preclinical experimentation in relevant animal models, including transgenic animals. Our chimera studies hint at interactions among residues that contribute to 2CdA specificity in both the third- and the fourth-quarters of the protein. For example, an interaction between amino acid residues in the third- and fourth-quarters of rCNT2 may lead to a difference in the folding of the protein and shape of the substrate recognition macrophore had a relatively innocuous effect on transport. In contrast, the 2-chloroadenosine pharmacophore was poorly taken up by hCNT2, whereas rCNT2 and chimera-HHRR were able to transport it very well. Further studies are needed to determine whether these findings are applicable to other compounds that are halogenated on the purine or pyrimidine base such as 8-chloroadenosine or 5-fluorocytosine (16, 17).

There is also a large species difference between hCNT2 and rCNT2 in their ability to transport arabinoside compounds such as Ara-A and Fludarabine. The only difference between Ara-A and adenosine is the configuration of the hydroxyl groups on the ribose sugar (Table 1), indicating that the hydroxyl configuration in arabinosides is not tolerated by hCNT2. These findings indicate that clinically used arabinosides such as Ara-G will likely not be substrates for hCNT2.

Our efforts at determining the region of CNT2 and the individual amino acids that contribute to 2CdA specificity paint a complicated picture when compared with the residues that were found to dictate purine versus pyrimidine nucleoside specificity. The question of specificity for nucleoside analogs, while important, is likely to be more complex since the proteins that transport the nucleoside analog drugs have not been honed by millions of years of selective pressures to transport clinically used drugs. Despite the differences in complexity between nucleoside analog specificity and naturally occurring specificity, it should be noted that the determinants of both are located in similar regions of the protein. Although the region is the same, the individual amino acids that make up the determinants of substrate domain as compared with hCNT2. Unfortunately, interactions such as these cannot be identified without a crystal structure or three-dimensional model of some kind. Our efforts at generating such a model were stymied by the lack of an available crystal structure template on which to base our model of CNT2. Our studies, in combination with crystal structures, could be powerful tools in the design and development of therapeutic nucleoside analogs.

Acknowledgments—We thank Chesi Ho and Leah Lagpacan for excellent technical assistance and Guangqing Xiao, Karin Gerstin, and Leslie Chinn for helpful discussions.

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