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***Crithidia fasciculata* adenosine transporter 1 (CfAT1), a novel high-affinity equilibrative nucleoside transporter specific for adenosine**

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Abstract

Most eukaryotic organisms including protozoans like *Crithidia*, *Leishmania*, and *Plasmodium* encode a repertoire of equilibrative nucleoside transporters (ENTs). Using genomic sequencing data from *Crithidia fasciculata*, we discovered that this organism contains multiple ENT genes of highly similar sequence to the previously cloned and characterized adenosine transporter CfNT1: CfAT1 and CfNT3, and an allele of CfAT1, named CfAT1.2. Characterization of CfAT1 shows that it is an adenosine-only transporter, 87% identical to CfNT1 in protein sequence, with a 50-fold lower K_m for adenosine. Site directed mutation of a key residue in transmembrane domain 4 (TM4) in both CfNT1 and CfAT1 shows that lysine at this position results in a high affinity phenotype, while threonine decreases adenosine affinity in both transporters. These results show that *C. fasciculata* has at least two adenosine transporters, and that as in other protozoan ENTs, a lysine residue in TM4 plays a key role in ligand affinity.

Keywords

adenosine; *Crithidia fasciculata*; equilibrative nucleoside transporter; ligand affinity; purine metabolism

Equilibrative nucleoside transporters (ENTs) play important roles in purine/pyrimidine metabolism in a wide variety of eukaryotic species [1–3]. In protozoan parasites, the endogenous role of ENTs in purine nucleoside and/or nucleobase uptake from the host environment is essential due to a lack of purine *de novo* synthesis in such organisms [4]. ENTs also play a role in the uptake and/or cellular retention of nucleoside analog drugs used in the treatment of a variety of cancers and viral infections [3,5], opening the possibility that genetic variation in the protein sequence or expression level of ENTs may be clinically relevant to resistance to these drugs [2,6]. While the ENT family is of significant biological interest, like many membrane protein families little structural information exists to aid in the elucidation of its ligand recognition and translocation mechanisms.

Studies of chimeras between mammalian transporters as well as site-directed mutagenesis of mammalian and protozoan ENTs have traced ligand discrimination and affinity determinants

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to amino acids in the N-terminal half of the molecule, and most notably TMs 4 and 5 [3,7,8]. To uncover if other regions of transporter structure might influence ligand specificity and/or affinity, we undertook to compare ENTs from *Crithidia fasciculata* with highly similar protein sequences but distinguishable biochemical characteristics. Previous work on adenosine transporter CfNT1 and inosine-guanosine transporter CfNT2 in *C. fasciculata* suggested the existence of multiple adenosine-transporting ENTs in this organism [9]. In addition, an adenosine uptake activity that was partially inhibited by cytidine was measured in whole *Crithidia* cells [9,10], but CfNT1-dependent adenosine transport was not inhibited in this way [9], suggesting biochemical differences might exist between CfNT1 and the unidentified adenosine transporter(s).

The DNA sequence of the *CfNT1* open reading frame (GenBank ID 10764225) was used as the query sequence in a BLASTn search against preliminary genomic sequencing reads and scaffolds from the *Crithidia fasciculata* strain Cf-C1 provided by Stephen Beverley and The Genome Center (Washington University School of Medicine). Predicted 5' and 3' untranslated regions of CfNT1-like genes from this analysis were used to design 5' and 3' PCR primers for amplification of *C. fasciculata* genomic DNA. Two putative ENT sequences were obtained and named *C. fasciculata* Adenosine Transporter 1 (*CfAT1*) and *C. fasciculata* Nucleoside Transporter 3 (*CfNT3*) (Supplemental Methods). Preliminary assembled scaffolds later obtained from the Beverley/Genome Center team were exact matches for *CfAT1* and *CfNT3* except for a gap in the center of the *CfAT1* sequence, suggesting that both are legitimate gene sequences.

5' RACE was used to clone *CfAT1.2*, an additional sequence highly similar to *CfAT1* and an exact match to a scaffold from the sequencing project team (Supplemental Methods). *CfAT1* and *CfAT1.2* are 99.6% identical and 100% homologous at the protein level, differing only at two positions near the C-terminus (Met vs. Ile, Val vs. Ile; data not shown), and are 87% identical and 93% similar to CfNT1. Comparison of the coding and non-coding regions of *CfAT1* with *CfAT1.2* suggests that the two sequences are alleles located at the same gene locus in this diploid organism, rather than gene duplicates. In addition to the very low number of non-synonymous DNA changes observed in the coding region, identity between the 5' and 3' untranslated regions (UTRs) is very high, whereas there is no appreciable homology between the UTRs of *CfNT1*, *CfAT1* and *CfNT3* (data not shown).

CfNT3 is much less similar to CfNT1 and CfAT1 (72%) in protein sequence than these two proteins are to each other (Figure 1A). However, alignment of the protein sequences of CfNT1, CfAT1, CfAT1.2 and CfNT3 with other protozoan ENTs shows that all four proteins cluster with LdNT1, an adenosine-uridine transporter [11], and not with LdNT2 and CfNT2 (inosine-guanosine transporters, [9,12]) or with LmaNT3 and LmaNT4 (nucleobase transporters, [13–15]) (Figure 1B), suggesting that all may be adenosine transporters.

Reverse transcriptase PCR (rtPCR) was undertaken to determine if the four identified *C. fasciculata* nucleoside transporter genes, *CfAT1*, *CfNT1*, *CfNT2* and *CfNT3* were expressed at the mRNA level in *Crithidia* cells under purine-rich media conditions. Agarose gel electrophoresis of the products showed successful amplification of a band of the expected size (300–400 bp) for each transcript (Fig. 1C, +RT lanes). Direct sequencing of DNA extracted from each of the visualized bands confirmed the presence of PCR products derived from *CfAT1*, *CfNT1*, *CfNT2* and *CfNT3* mRNAs in each reaction mixture, suggesting that are all present at log phase in *Crithidia* cells. No PCR products were produced when the cDNA reaction did not contain reverse transcriptase, indicating that all bands are mRNA-specific (Fig. 1C, –RT lanes). While mRNA and protein levels typically do not correlate with each other in related protozoans such as *Leishmania*, the presence of *CfAT1*, *CfNT1*,

CfNT2 and *CfNT3* mRNAs in *C. fasciculata* allows the possibility that the encoded proteins contribute to purine nucleoside uptake in this organism.

To characterize the biochemical activity of the newly identified genes, *CfAT1*, *CfAT1.2*, *CfNT1* and *CfNT3* were expressed in a *Leishmania donovani* cell line lacking any endogenous purine nucleoside transport capability (*Δldnt1 Δldnt2*; [16]). *CfAT1* proved to be an adenosine transporter as expected from its 93% amino acid sequence similarity to *CfNT1*, with a K_m value of $0.81 \pm 0.34 \mu\text{M}$ ($n = 3$; Fig. 2A). *CfAT1* [^3H]-adenosine transport capability was inhibited only by adenosine itself and by tubercidin, a toxic adenosine analog (Fig. 2C). The adenosine K_m value and inhibition profile for *CfAT1.2* were very similar to those of *CfAT1* (data not shown). *CfNT1* expressed in *L. donovani* exhibited a significantly higher K_m value than that of *CfAT1* ($59.0 \pm 10 \mu\text{M}$, $n = 3$; Figure 2B). Like *CfAT1*, *CfNT1* showed high specificity for adenosine when expressed in *L. donovani* (Fig. 2D), suggesting that neither *CfNT1* nor *CfAT1* is responsible for the cytidine-inhibitable adenosine uptake observed in whole *C. fasciculata* cells [9,10].

Significant 5' untranslated sequence for *CfNT3* was available from the genomic sequencing project, allowing a good prediction of the correct start codon for heterologous expression of this gene in *L. donovani*. A band corresponding to the expected size of HA-CfNT3 protein was detected by anti-HA immunoblotting of the cellular membrane fraction, suggesting that the protein was expressed in the transfected *L. donovani* cells; however, no uptake of [^3H]-adenosine, inosine, adenine or hypoxanthine above background levels could be detected (data not shown). Punctate spots were visualized within fixed cells by anti-HA immunofluorescence, suggesting that HA-CfNT3 was not localized to the cell surface when over-expressed in *L. donovani* cells (data not shown). Cloning of the *CfNT3* open reading frame into two additional expression vectors expected to give lower levels of protein expression also failed to yield cells with measurable adenosine uptake, precluding biochemical characterization of *CfNT3* within the *L. donovani* expression system.

Previous work with the *L. donovani* adenosine-uridine transporter *LdNT1* [7] and the *C. fasciculata* inosine/guanosine transporter *CfNT2* [8] showed that a lysine residue within TM4 is important for ligand specificity and affinity. Like *LdNT1* and *CfNT2*, *CfAT1* has a lysine at the orthologous position (Fig. 1A, starred residue), while *CfNT1* and *CfNT3* have a threonine. To determine if this residue influences the difference in adenosine K_m between *CfNT1* and *CfAT1*, we exchanged the identities of the residues at position 153 in these transporters, and expressed the resulting *cfnt1-T153K* and *cfat1-K153T* mutant genes in our *L. donovani* expression system. The *cfnt1-T153K* mutant cells exhibited a dramatically increased apparent affinity for adenosine relative to *CfNT1* ($K_m = 1.7 \pm 0.45 \mu\text{M}$, $n = 3$ vs. $59.0 \pm 10 \mu\text{M}$ for *CfNT1*; Fig. 2F), while the *cfat1-K153T* mutant cells had an approximately 10-fold decrease in adenosine affinity ($K_m = 6.3 \pm 1.7 \mu\text{M}$, $n = 2$ vs. $0.81 \pm 0.34 \mu\text{M}$ for *CfAT1*; Fig. 2E). The reduction in apparent V_{max} for adenosine uptake by cells expressing *cfat1-K153T* vs. *CfAT1* may be due to decreased surface expression of the mutant protein and/or a defect in permeant translocation.

Because the *CfAT1* and *CfNT1* proteins have only 66 amino acid differences (87% identical), we anticipated that chimeric proteins that combine portions of these two proteins might be able to fold properly and transport adenosine. Four chimeras were constructed to investigate whether sequence variations outside of TM4 might also influence the difference in adenosine affinity between *CfNT1* and *CfAT1* (Fig. 1A – arrowheads; and Supplemental Fig. 1). Exchanging *CfNT1* sequence for that of *CfAT1* from the N-terminus to the beginning of TM2 (Chimera N1A; 15 amino acid differences) did not significantly affect adenosine K_m compared with *CfAT1* ($0.5 \pm 0.14 \mu\text{M}$ vs. $0.8 \mu\text{M}$), while extending the portion of *CfNT1* sequence to the end of TM3 to encompass an additional nine amino acid

changes (Chimera N3A) did slightly but reproducibly increase the K_m of the chimeric protein for adenosine ($1.2 \pm 0.16 \mu\text{M}$). Interestingly, addition of only two more amino acid changes (Ala for Gly in the 3–4 loop and Thr for Lys in TM4, see Fig. 1A) resulted in a non-functional or non-expressed transporter – cells stably transfected with the plasmid for Chimera N4A demonstrated no detectably saturable adenosine uptake at concentrations up to $50 \mu\text{M}$. It is not clear which additional mutation is responsible for the lack of function of Chimera N4A. Exchanging the C-terminal portion of CfAT1 following the large region of identity from TM4 to the middle of the 6–7 loop with that of CfNT1 did not affect affinity for adenosine (Chimera A6N, $0.4 \pm 0.1 \mu\text{M}$); however, the overall amount of [^3H]-adenosine transported at any given concentration by cells expressing this chimera was significantly less compared with CfAT1-expressing cells (data not shown), suggesting an effect on transporter structure, expression and/or translocation capability.

In conclusion, I have shown that *C. fasciculata* has two adenosine transporter genes encoding proteins of varying ligand affinity (*CfNT1*, *CfAT1*), and possibly a third (*CfNT3*). While the chimeras between CfAT1 and CfNT1 suggest that amino acid differences in TM3 may play a small role in their varying ligand affinities, the primary contributor is the same amino acid position in TM4 that has been previously identified as having a large influence on ligand specificity and affinity in CfNT2 [8] and LdNT1 [7]. Future atomic-level structural analysis of a TM4-lysine containing ENT should shed important light on the role of this residue in both ligand binding and the structural integrity of the transporter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Crithidia fasciculata* has multiple adenosine transporters of the ENT family.
- Like CfNT1, CfAT1 and its allele CfAT1.2 are adenosine transporters.
- *CfNT3* may encode a third adenosine-transporting ENT.
- A Lys residue in TM4 plays a key role in ligand affinity in CfAT1.

Figure 1. Nucleic acid characterization of putative ENTs of *Crithidia fasciculata*

A multiprotein sequence alignment (A) and phylogram (B) were generated using Clustal 2.0.12 (<http://www.ebi.ac.uk>). Alignment was colored using BOXSHADE 3.21 (written by K. Hofmann and M. Baron; public domain; http://www.ch.embnet.org/software/BOX_form.html). Black shading indicates nonhomology amongst at least two of the sequences, while gray shading indicates similar amino acids. Predicted membrane spanning domains for CfNT1 are indicated by the solid lines above the aligned sequences, and the position of residue 153 of CfNT1 and CfAT1 is indicated by an asterisk. Positions of transitions between CfNT1 and CfAT1 sequence in chimeras are labeled with arrowheads. Gene sequence accession numbers and references for all genes: CfNT1 ([9], Genbank ID

10764225), CfAT1 (this work, Genbank ID 404434868), CfNT3 (this work, Genbank ID 404434872), LdNT1.1 ([11], Genbank ID 3450833), CfNT2 ([9], Genbank ID 10764227), LdNT2 ([12], Genbank ID 8272581), LmaNT3 ([15], TriTrypDB ID LmjF.13.1210) and LmaNT4 ([14], TriTrypDB ID LmjF.11.0550).

(C) The presence of mRNA encoding each *C. fasciculata* ENT gene was detected by 5' Rapid Amplification of DNA Ends (5' RACE). DNA-free mRNA was prepared from *Crithidia* parasites maintained in modified M199 medium [17] with 100 μ M xanthine as a purine source using the animal spin protocol of the RNeasy mini kit (Qiagen). For 5' RACE, first strand cDNA synthesis was performed with the SuperScript III First-Strand Synthesis kit (Invitrogen), a gene specific primer (*CfAT1*), oligo dT (*CfNT3*) or random priming (*CfNT1*, *CfNT2*) in parallel samples containing reverse transcriptase (RT+) or mock (RT-). A 5' primer designed to the *C. fasciculata* splice leader [18] and a 3' gene-specific primer (nested 5' of the first-strand primer for *CfAT1*) were used in the subsequent PCR reaction. Products were separated by 2% agarose gel electrophoresis, visualized using ethidium bromide, and verified by sequencing following excision from the gel. The band that corresponds to *CfAT1* in lane 5 is indicated with an asterisk.

Figure 2. Biochemical characterization of CfNT1 and CfAT1 transporters in *Leishmania donovani*

Expression of all nucleoside transporters and their variants in the *L. donovani* *ldnt1* Δ /*ldnt2* line, which lacks all purine nucleoside uptake capability [16], was from the pALTneo-HA vector [19]. *Leishmania* parasites were transfected according to the method of Robinson & Beverley [20] and maintained in modified M199 medium [17] supplemented with 5% heat-inactivated fetal bovine serum (Fisher Scientific, Rockford, IL), 25 μg/ml G418 and 100 μM adenine. Uptake of [³H]-adenosine (34 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) was measured by the oil-stop method and scintillation counting [8] over duplicate 15 sec and 30 sec time courses for *CfAT1*- (A) and *CfNT1*-expressing (B) cells, respectively. The rate of uptake was determined in duplicate by regression analysis at each concentration, and data were fit to the Michaelis-Menten equation in Graphpad Prism 5.01.

K_m values derived from Michaelis-Menten and linear Hanes-Woolf analyses differed by 10% or less for every data set. Data shown are representative of three independent experiments. Uptake of [3 H]-adenosine in parallel samples with and without a 100-fold excess of unlabeled inhibitor (Inh) was measured in duplicate by the oil-stop method at 1 μ M [3 H]-adenosine over 5 sec for *CfAT1*-expressing cells (C) and at 10 μ M [3 H]-adenosine over 30 sec for *CfNT1*-expressing cells (D). Samples containing a 1000-fold excess of unlabeled adenosine were used to determine transporter-independent background uptake (Bk). Corrected uptake levels were used to determine percent inhibition: (uptake with Inh – Bk)/(uptake without Inh – Bk). Data shown are representative of three independent experiments. Point mutants were constructed by site-directed mutagenesis of pALTneo-HA-CfNT1 or pALTneo-HA-CfAT1 by the Quikchange mutagenesis protocol (Stratagene, La Jolla, CA). *L. donovani* Δ ldnt1/ Δ ldnt2 pALTneo-HA-cfat1-K153T (E) and pALTneo-HA-cfnt1-T153K (F) transfectants were assayed for their ability to transport [3 H]-adenosine over a 25 sec time course at a range of concentrations, and data were fit as described above. Data shown are representative of two independent experiments.