Molecular & Biochemical Parasitology 191 (2013) 75–79

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Short communication

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

Cassandra S. Arendt*

Pacific University School of Pharmacy, 222 SE 8th Avenue, Suite 451, Hillsboro, OR 97123, USA

ARTICLE INFO

Article history:
Received 3 February 2013
Received in revised form
26 September 2013
Accepted 29 September 2013
Available online 10 October 2013

Keywords:
Adenosine
Crithidia fasciculata
Equilibrative nucleoside transporter
Ligand affinity
Purine metabolism

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.

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 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 *C. 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

* Current address: 13417 Kinder Pass, Austin, TX 78727, USA.
Tel.: +1 503 807 6030.
E-mail addresses: csarendt@gmail.com, csarendt@yahoo.com

0166-6851/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molbiopara.2013.09.007

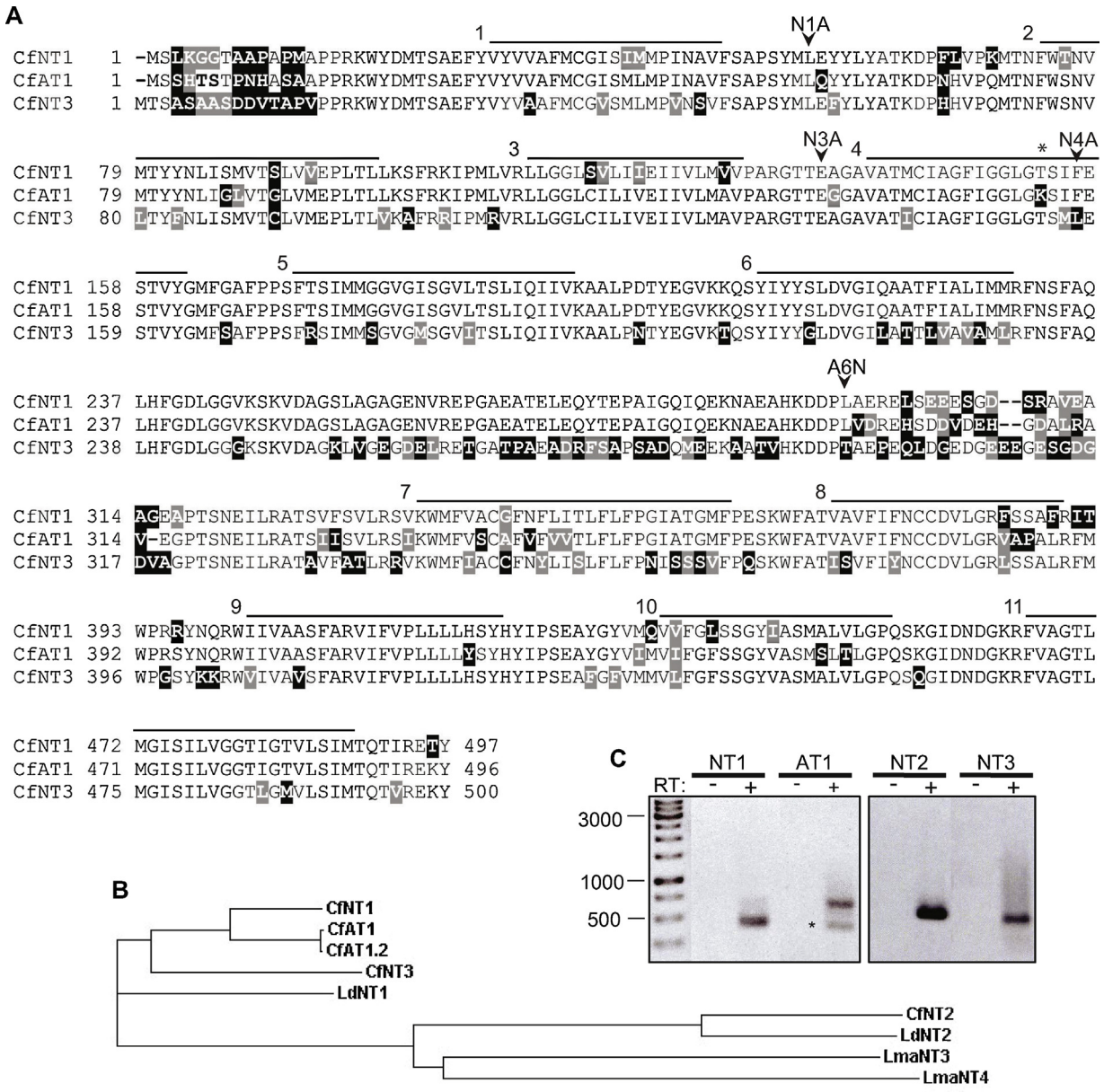

Fig. 1. Nucleic acid characterization of putative ENTs of *Crithidia fasciculata*. A multiprotein sequence alignment (A) and phylogram (B) were generated using Clustal 2.0.12 (http://www.ebi.ac.uk). Alignment was colored using BOXSHADE 3.21 (written by K. Hofmann and M. Baron; public domain; http://www.ch.embnet.org/software/BOX_form.html). 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 μM 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.

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 (Fig. 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