4-2010

Sequence and biochemical characterization of equilibrative nucleoside transporters from Crithidia fasciculata: seeking ligand binding residues

Cassandra S. Arendt
Pacific University

Stephen Beverley
Washington University in St Louis

Buddy Ullman
Oregon Health & Science University

Sequence and biochemical characterization of equilibrative nucleoside transporters from Crithidia fasciculata: seeking ligand binding residues

Abstract

Eukaryotic cells acquire purines via *de novo* synthesis or salvage mechanisms. Unlike human cells, protozoan parasites from the Apicomplexan and Trypanosomatid lineages such as *Plasmodium, Toxoplasma, Leishmania, Trypanosoma* and *Crithidia* species lack *de novo* purine synthesis enzymes, and therefore rely on salvage of pre-formed purine bases for growth and proliferation, making purine salvage an attractive drug target. A key step in purine salvage is transport of purine nucleobases and nucleosides into the cell, and each species encodes a unique repertoire of transporter proteins from the equilibrative nucleoside transporter (ENT) family, each with its own ligand preference. No detailed structural information for ENT proteins yet exists, but residues within four of the eleven transmembrane domains (TMs) appear to be important for ligand selectivity. Recently we have described the pivotal role of a lysine residue within TM4 of the *Crithidia fasciculata* purine nucleoside transporter CfNT2 in ligand discrimination. Here we describe the cloning of additional ENT genes from *C. fasciculata* using partial genomic sequencing and molecular techniques, and biochemical characterization of the encoded proteins. The role of sequence variations among the *C. fasiculata* ENTs in determining ligand preference may shed additional light on residues and regions of ENTs that contribute to ligand binding.

Disciplines

Pharmacy and Pharmaceutical Sciences

Comments

Poster presented at the Experimental Biology 2010 meeting.

Sequence and biochemical characterization of equilibrative nucleoside transporters from *Crithidia fasciculata*: seeking ligand binding residues

Cassandra S. Arendt[1], Stephen Beverley[2] and Buddy Ullman[3]

[1]Pacific University School of Pharmacy, Hillsboro, OR. Email: csarendt@pacificu.edu

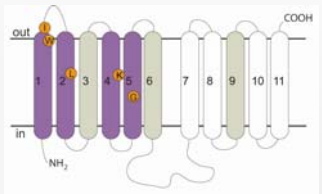
[2]Department of Molecular Microbiology, Washington University, St. Louis, MO

[3]Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, OR

Program Number 699.1

INTRODUCTION

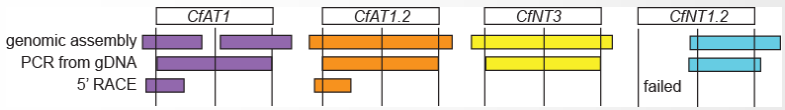
Eukaryotic cells acquire purines via *de novo* synthesis or salvage mechanisms. Unlike human cells, protozoan parasites from the Apicomplexan and Trypanosomatid lineages such as *Plasmodium*, *Toxoplasma*, *Leishmania*, *Trypanosoma* and *Crithidia* species lack *de novo* purine synthesis enzymes, and therefore rely on salvage of pre-formed purine bases for growth and proliferation, making purine salvage an attractive drug target in these organisms. A key step in purine salvage is transport of purine nucleobases and nucleosides into the cell, and each species encodes a unique repertoire of transporter proteins from the equilibrative nucleoside transporter (ENT) family, each with its own ligand preference. ENT proteins have 11 transmembrane domains (TMs; see the figure), and residues that affect ligand specificity in a number of different ENTs have been identified in TMs 1, 2, 4 and 5 (see [1]).



We are interested in identifying additional residues that impact ligand affinity and specificity in these transporters in order to further define the ligand binding site. Previous work has shown that adenosine transport in *Crithidia fasciculata* (a close relative of the *Leishmania spp.*) is carried out by CfNT1 as well as at least one other adenosine transporter [2]. We therefore cloned genes encoding additional adenosine-transporting ENTs in *C. fasciculata* to determine if they would be suitable for construction of chimeras to identify additional regions of the transporter sequence that influence ligand specificity. Good candidates would be highly similar in protein sequence but biochemically distinguishable. Here we describe the cloning of *CfNT1*-like genes from *C. fasciculata* using genomic sequencing and molecular techniques, and preliminary biochemical characterization of one novel adenosine transporter, CfAT1. The role of sequence variations between CfAT1 and CfNT1 in determining ligand preference may shed additional light on residues and regions of ENTs that contribute to ligand binding.

RESULTS

1 Genomic sequences of additional *CfNT1*-like genes were obtained by whole genome sequencing and molecular methods.



Genome sequence data were obtained by whole genome shotgun sequencing of *Crithidia fasciculata* clone Cf-C1 (obtained from Larry Simpson, UCLA), using Sanger or several generations of 454 sequencing technologies at the Washington University genome sequencing center. Blast searches of the individual reads generated were performed first with *CfNT1*, which yielded evidence of additional genes. 5' RACE and further iterations of blast searching with these genes against preliminary genomic assemblies ultimately led to the identification of four genes in total, which were named *CfAT1*, *CfAT1.2*, *CfNT3* and *CfNT1.2*. Only the 3' 75% of the *CfNT1.2* gene has been defined to date. Consistent with other genome sequence data suggesting a high level of heterozygosity in *Crithidia fasciculata*, some of these putative genes are allelic (see next panel).

2 DNA sequence analysis indicates that *CfAT1*/*CfAT1.2* and *CfNT1*/*CfNT1.2* are alleles rather than distinct genes.

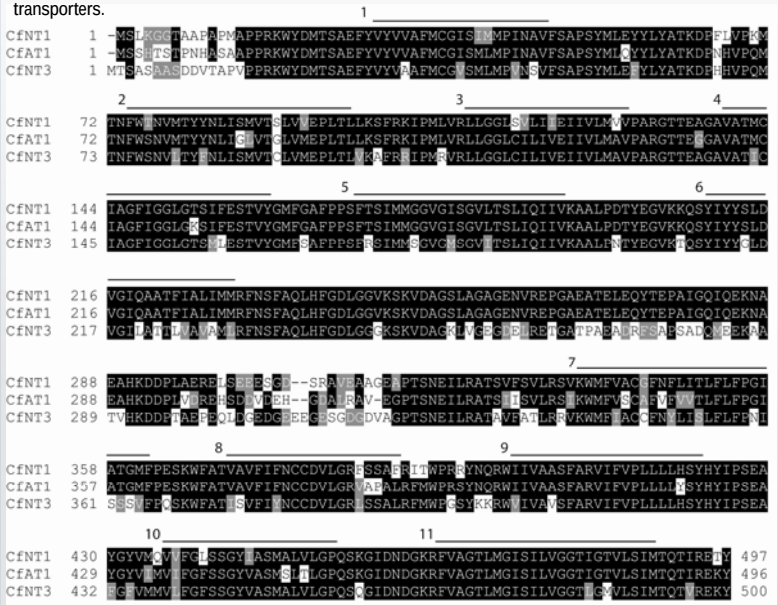
	Synon-ymous	Nonsynon-ymous	5' flank identity	3' flank identity
CfNT1/ CfNT1.2	22/1108	4/1108 (0.36%)	---	90/95 (95%)
CfAT1/ CfAT1.2	36/1488	2/1488 (0.13%)	186/189 (98%)	77/82 (94%)

The low number of nonsynonymous DNA sequence changes and high level of identity in flanking regions support assignment of these sequences as alleles.

3 The novel proteins CfAT1 and CfNT3 are more similar in sequence to adenosine transporters than they are to inosine-guanosine or nucleobase transporters.

A multiprotein sequence alignment and phylogram were generated using Clustal 2.0.12 (www.ebi.ac.uk). CfNT1 and LdNT1 are adenosine transporters from *C. fasciculata* and *Leishmania donovani*, respectively[2,3]. CfNT2 and LdNT2 are inosine-guanosine transporters from the same two organisms [2,4]. LmaNT3 and LmaNT4 are nucleobase transporters from *L. major* [5,6]. CfAT1 and CfNT3 clearly cluster with the other adenosine transporters, suggesting that they are also likely to be adenosine transporters.

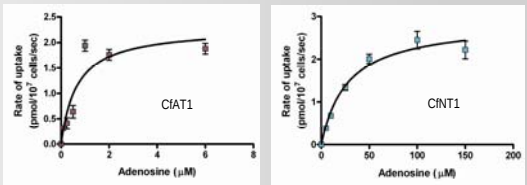
4 Protein sequence alignment of CfNT1, CfAT1 and CfNT3 shows that interesting sequence variations are confined to a few regions of the transporters.



A protein sequence alignment was produced using Clustal 2.0.12. CfAT1 is ~ 87% identical and 93% similar to CfNT1 at the protein level, suggesting that chimeras between these two proteins may be structurally sound. CfNT3 is significantly more divergent in sequence, with only ~73% identity to CfNT1 and CfAT1 at the protein level. CfNT3 deviates most significantly from the other two transporters in TMs 6 through 8. The significance of this portion of the protein to transporter function is unknown.

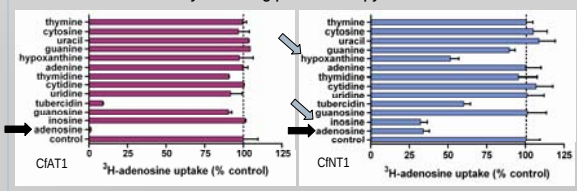
Nonhomologous substitutions between CfNT1 and CfAT1 are concentrated in the N-terminal region and other loops (esp. the large intracellular 6-7 loop). The K to T variation in TM4 may be important for ligand specificity, as the homologous residue in two other transporters has this function (LdNT1 [7] and CfNT2 [1]) as well. Additional changes appear in TMs 7, 8, 9 and 10. While TMs 7, 8 and 10 are thought to lie on ligand translocation channel of ENTs [8], it is not known if residues in these TMs contribute to ligand binding.

5 CfAT1 has a 25-fold higher apparent affinity for adenosine than does CfNT1.



CfAT1 and *CfNT1* were expressed in *L. donovani* cells lacking endogenous purine nucleoside uptake. Uptake of ^{3}H-adenosine into cells was measured by the oilstop method over a 15 sec and 30 sec timecourse for *CfAT1*- (purple) and CfNT1-expressing (blue) cells, respectively, at each concentration. K_m values of ~1 and ~27 μM were calculated from fitting Michaelis-Menten curves in Graphpad Prism 5.01.

6 Unlike CfNT1, CfAT1 does not appear to have any other ligands among the naturally occurring purines and pyrimidines.



CfAT1 was expressed in *L. donovani* lacking purine nucleoside uptake, and *CfNT1* was expressed in *Xenopus* oocytes [2]. Uptake of ^{3}H-adenosine into cells in the presence of a 100-fold excess of unlabeled inhibitor was measured over 5 sec and 45 min for *CfAT1*- (purple) and *CfNT1*-expressing (blue) cells, respectively. Uptake is expressed relative to uninhibited control cells. Tubercidin is a toxic adenosine analog.

SUMMARY

- There are at least three distinct adenosine transporter genes in *Crithidia fasiculata*.
- CfNT1 and CfAT1 differ in protein sequence in several regions of interest, including TMs 2 & 4, which may influence ligand specificity, and the large loop intracellular loop between TMs 6 and 7 whose function is unknown.
- CfNT1 and CfAT1 have distinct biochemical properties, specifically in their apparent affinities for adenosine, and their abilities to be inhibited by inosine and hypoxanthine.
- Chimeras of these two transporters should produce functional proteins, and could allow the identification of functionally significant protein sequence differences and novel ligand specificity determinants.
- Biochemical characterization of the more divergent putative adenosine transporter CfNT3 is ongoing.

REFERENCES

[1] Arendt & Ullman (2010) J Biol Chem 285:6024-6035.
[2] Liu et al (2005) Mol Biochem Parasitol 140:1-12.
[3] Vasudevan et al (1998) PNAS 95:9873-9878.
[4] Carter et al (2000) J Biol Chem 275:20935-20941.
[5] Sanchez et al (2004) Mol Memb Biol 21:11-18.
[6] Ortiz et al (2007) Mol Microbiol 64:1228-1243.
[7] Valdes et al, (2004) Biochemistry 43:6793-6802.
[8] Valdes et al, (2009) J Biol Chem 284:19067-19076.

This work was supported by a Pacific University Faculty Development Grant to CSA, grants AI29646 and AI21903 to SB and grants AI023682 and AI044138 to BU.