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Trypanosoma brucei bloodstream forms express highly specific and separate transporters for adenine and hypoxanthine; evidence for a new protozoan purine transporter family?

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ABSTRACT

The transport of nucleobases and nucleosides in protozoan parasites is known to be performed by Equilibrative Nucleoside Transporter (ENT) family members, including the extensively studied P1 and P2 nucleoside transporters of *T. brucei* bloodstream forms. Studies with P2 knockout parasites suggested the existence of as yet uncharacterised purine transport mechanisms in these cells. Here, we deleted several ENT genes, in addition to P2, including an array comprising three genes encoding for high-affinity broad-selectivity nucleobase transporters − the longest multi-gene *locus* deletion in *T. brucei* to date. It was verified that none of them appreciably contributed to the transport of hypoxanthine in bloodstream forms grown axenically in HMI-9 medium, which was mainly performed by a previously not described hypoxanthine-specific transporter (HXT1) with a K_m of 22 ± 1.7 μM and V_{max} of 0.49 ± 0.06 pmol$(10^7$ cells$)^{-1}$ s^{-1}. The uptake of adenine was also assessed in the knockout cells and was performed by a highly specific adenine transporter (ADET1) with a K_m of 573 ± 62 nM and V_{max} of 0.23 ± 0.06 pmol$(10^7$ cells$)^{-1}$ s^{-1}. Neither HXT1 nor ADET1 displayed any affinity for other natural purines or pyrimidines and could not be completely inhibited by hypoxanthine or adenine analogues. These carriers may be the final pieces in the substantial transporter array trypanosomes can employ to fine-tune the uptake of purines from diverse environments during their life cycles, and may be encoded by genes other than those of the ENT family.

1. Introduction

In higher eukaryotes, transport of nucleobases and nucleosides occurs *via* the concentrative nucleoside transporter (CNT, SLC28) or equilibrative nucleoside transporter (ENT, SLC29) families. Although these families have overlapping substrate specificities, they are structurally and physiologically unrelated: CNTs have 13 transmembrane domains (TMDs) and are sodium- or proton- symporters, which mediate the transport of nucleosides in an unidirectional energy-costly way, whereas ENTs are formed by 11 TMDs and are passive transporters that conduct substrates along the concentration gradient [1–3]. To date, all nucleoside and nucleobase transporters of parasitic protozoa were members of the ENT family, although they are proton symporters, able to concentrate the substrates inside the cell [1,4–6]. Other nucleobase transporter families are NAT, PRT and PUP [7] but, like CNTs, none of these have been found in protozoan genomes.

The best characterized parasites in terms of purine and pyrimidine transport are *Trypanosoma brucei*, the causative agent of Human African Trypanosomiasis (HAT; sleeping sickness), and *Leishmania* spp., causative agents of leishmaniasis. These parasites are known to express ENT family transporters for the uptake of nucleobases and/or nucleosides, which is an essential function because they are unable to synthesize purines *de novo*, relying on salvage from the host environment [1,8,9]. Due to this dependence on transport, several pharmacological approaches have focused on purine transporters as drug carriers for the treatment of parasitic infections, especially HAT [10–14].

During its life cycle, *T. brucei* differentially expresses several mechanisms for the uptake of purine nucleosides and/or nucleobases. The P1 transport activities were reported to be encoded by a tandem repeat of six genes on chromosome 2 (NT2–NT7); all these carriers were shown to have affinity for inosine and adenosine and some of them also displayed a certain level of affinity for hypoxanthine when expressed in oocytes of *Xenopus laevis* [15]. Two more P1-type transporters, NT9 and NT10, were reported to be specifically expressed in short-stumpy

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bloodstream forms [16] and procyclic forms (stage in the mid-gut of the tsetse fly) [8], respectively, whereas the *Tb*AT1/P2 aminopurine transporter is only expressed in long-slender bloodstream forms [17,18].

The long-slender bloodstream forms further express two purine nucleobase transporters, H2 and H3 [10,19], and the procyclic forms also express two purine nucleobase transporters, H1 and H4 [4,20]; these transporters are believed to be encoded by a tandem repeat of NT8 on chromosome 11 [20,21]. The function of the last three members of the ENT family is not yet clear, although one report suggest that NT11.1, NT11.2 and NT12 transport both nucleobases and the drug pentamidine [22]; to date, our own investigations have not been able to confirm this. As the ENT transporters from *T. brucei* and from *Leishmania* spp. have now been cloned and at least partially characterized, we have previously argued that the observed pyrimidine nucleobase transport activities in these species must be mediated by members of a different, as yet unidentified, transporter family [23,24].

The interest on purine transporters in *T. brucei* increased dramatically when it was first reported that parasites resistant to melarsoprol, a first-line drug for treatment of late stage HAT, lack a purine transport activity, which was found to be *Tb*AT1/P2 [17,25–27]. Moreover, *Tb*AT1/P2 was also found to efficiently transport pentamidine [17,28] and is the main transporter of the veterinary trypanocide diminazene [29–31].

The binding model for P2 shows that its specificity for aminopurines is determined by the importance of hydrogen bonds formed between N1 and C6-NH_2 of the purine ring and the transporter binding site, along with interactions between the transporter and N9 [18]. This affinity for a motif NH_2–C(R_1) = N(R_2) also explains the affinity of P2 for diamidine drugs, such as Pentamidine, Melarsoprol and Diminazene [32,33]. Additionally, two other transporters with high (HAPT1) and low (LAPT1) affinity for pentamidine have been reported to interact with diamidine drugs [34,35]. More recently, the HAPT1 activity was found to be encoded by the gene *Tb*AQP2, an aquaglyceroporin [36,37].

Several studies have focused on P2 as a carrier for new drugs containing melamine-based units. Interestingly, however, the affinity of the drugs for P2 was not the sole determinant for their trypanocidal effects, and *Tb*AT1/P2 knockout parasites were also able to transport these molecules and keep their IC_{50} values in a low micromolar range [38–40], evidencing the presence of extra transporters with the ability to transport purines and purine-like molecules [1,38,39]. Indeed, unpublished data from the Barrett group suggested the expression of an adenine-sensitive, inosine- and hypoxanthine-insensitive mechanism for (low affinity) adenosine uptake in *Tb*AT1/P2 knockout bloodstream forms [41].

In the light of all these studies, we decided to investigate whether *T. brucei* expresses additional purine nucleobase transporters that have not yet been reported. In order to eliminate some background transport of nucleobases, we knocked out the three genes known to encode high-affinity nucleobase transporters, from the *Tb*AT1/P2-knockout cell line, and used this new lineage as the parental cells for the further knockout (KO) of NT11 and NT12. We verified that none of the deleted transporters are the main purine carriers in cultured bloodstream forms, and describe the existence of two new and highly specific purine transporters, transporting adenine and hypoxanthine. By the process of elimination we conclude that these newly discovered adenine and hypoxanthine transporters are most likely not members of the ENT family and may therefore indicate the existence of a new transporter family in pathogenic protozoa.

2. Materials and methods

2.1. Parasites and culture media

Bloodstream forms of s427 and TbAT1-KO and its derived clonal lines were cultivated in HMI-9 supplemented with 10% of foetal bovine serum (FBS; Gibco) as described [42]. When necessary, 40 μM of adenosine was added to the culture to avoid metabolic limitations.

Alternatively, parasites were cultivated in Creek's Minimum Medium (CMM) [43] with modifications: instead of Gold Serum, we used standard FBS and supplemented the medium with 100 μM each of arginine, leucine, methionine, phenylalanine, tryptophan and tyrosine, and 40 μM of adenosine as purine source. *T. brucei* s427 were kept in serial passage in CMM for at least two weeks prior their use in uptake assays.

2.2. Generation of T. brucei knockout lineages

2.2.1. Knockout of high-affinity nucleobase transporters cluster

The high-affinity nucleobase transporters described independently by Burchmore et al. [20] and Henriques et al. [21] are located in a *tandem* array on chromosome 11 (Tritryp Tb927.11.3610, Tb927.11.3620 and Tb927.11.3630), which enabled us to delete all three genes at the same time, similar to what was done with glucose transporters in *Leishmania mexicana* [44]. In order to achieve this, specific upstream and downstream regions were chosen: due the fact the UTR before Tb927.11.3610 is short (292 base pairs; bp) and very similar to the other two UTRs between the nucleobase transporter sequences, we cloned a fragment (373 bp) consisting of the end of the sequence of the 40S ribosomal protein (Tb927.11.3600) upstream of Tb927.11.3610 and the first 101 bp of the UTR (Fig. 1a), using primers HDK791 and HDK792, which contain restriction sites for PvuII and HindIII, respectively (a complete list of primers is given in Supplementary Table S1). The same logic was used to design the 3′ flank of the cassette, for which 269 bp at the end of the UTR after Tb927.11.3630 and the first 213 bp of Tb927.11.3640 were cloned using primers HDK905 and HDK906, containing the restriction sites for BamHI and SbfI, respectively. The reactions were performed using a high-fidelity DNA polymerase (as described above) and 5 ng of genomic DNA of the Lister s427 wildtype strain. The PCR products were digested overnight with the appropriated enzymes and ligated into a pyrFEKO vector. The final vector was digested with PvuII and SbfI to release the KO cassette, and AclI to cleave the β-lactamase gene and facilitate the visualization of the band of interest. The digestion product was run in 1% agarose gel, and the band of interest purified and transfected into TbAT1-KO [27], generating TbNBT-KO, which lacks all the confirmed *T. brucei* nucleobase transporters.

The TbNBT-KO line was cloned by limiting dilution and confirmed by the absence of amplification of the ORF of the nucleobase transporters with primers HDK901 and HDK902. As an internal control, each reaction contained primers (MB173 and MB174) to amplify a fragment of actin, confirming the presence of DNA in the reaction. After the confirmation of the gene deletion, the resistance genes were removed by transient expression of Cre recombinase [45], enabling the reuse of the markers in the further knockouts.

2.2.2. Knockout of AT-A and AT-E

Using the TbNBT-KO clone as parental cell line, we deleted AT-A (NT11; Tb927.9.15980) and AT-E (NT12; Tb927.3.590), generating two new cell lines: TbNBT/AT-A-KO and TbNBT/AT-E-KO. To do so, UTRs upstream and downstream the target genes were amplified by PCR and cloned into the vector pGL1688, based on pTBT [46] containing either a Hygromycin or Puromycin resistance cassette. The KO cassettes were released from the vector using NotI and XhoI, purified and introduced into TbNBT-KO, yielding single and double knockout strains after one or two rounds of transfection, respectively. Transfectants were cloned and verified by PCR prior to further use.

2.3. Uptake assays

Uptake of [^{3}H]-Hypoxanthine (Perkin-Elmer; 12.8 Ci/mmol) and [^{3}H]-Adenine (Perkin-Elmer; 40.3 Ci/mmol) were performed as