Cloning and Functional Expression of a Gene Encoding a P1 Type Nucleoside Transporter from Trypanosoma brucei*

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Nucleoside transporters are likely to play a central role in the biochemistry of the parasite Trypanosoma brucei, since these protozoa are unable to synthesize purines de novo and must salvage them from their hosts. Furthermore, nucleoside transporters have been implicated in the uptake of antiparasitic and experimental drugs in these and other parasites. We have cloned the gene for a T. brucei nucleoside transporter, TbNT2, and shown that this permease is related in sequence to mammalian equilibrative nucleoside transporters. Expression of the TbNT2 gene in Xenopus oocytes reveals that the permease transports adenosine, inosine, and guanosine and hence has the substrate specificity of the P1 type nucleoside transporters that have been previously characterized by uptake assays in intact parasites. TbNT2 mRNA is expressed in bloodstream form (mammalian host stage) parasites but not in procyclic form (insect stage) parasites, indicating that the gene is developmentally regulated during the parasite life cycle. Genomic Southern blots suggest that there are multiple genes related in sequence to TbNT2, implying the existence of a family of nucleoside transporter genes in these parasites.

African trypanosomes are protozoan parasites that are widely distributed in Africa and cause sleeping sickness in humans and nagana in domestic cattle (1). These infectious agents present a major public health problem that is complicated by the paucity of effective drugs available for treatment. Thus, many of the currently employed drugs are expensive, toxic, and sometimes ineffectual and suffer from the increasing occurrence of drug resistance (2). Biochemical pathways that are distinct between the parasite and its host offer the potential for developing novel therapeutic agents that could selectively interfere with trypanosome metabolism. Some of the most promising pathways for therapeutic exploitation are those for purine salvage, since protozoan parasites lack the de novo pathway for purine biosynthesis and consequently express a unique complement of purine salvage enzymes that enable host purine acquisition (3). The first step in the salvage of purines is their transport across the parasite plasma mem-

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EXPERIMENTAL PROCEDURES

Chemicals—[2,5,8-3H]Adenosine (54.4 Ci mmol⁻¹) was purchased from NEN Life Science Products, [2,8-3H]Guanosine (34 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals Inc., and [8-3H]adenosine [2,5,8-3H] was purchased from American Radiolabeled Chemicals Inc.
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Uptake Assays—Xenopus oocytes injected with TbNT2.2 cRNA or water as control were incubated for 3 days after injection. Prior to assay, oocytes were incubated for 30 min in ND96 buffer at room temperature. Uptake of [3H]adenosine, [3H]inosine, and [3H]guanosine was assayed by incubating oocytes with radiolabeled substrates for the indicated time periods. The oocytes were then quickly washed in ND96 buffer, and the samples were prepared for liquid scintillation counting as described previously (26). For each data point, the pmol of labeled substrate transported were calculated and plotted as a function of incubation time. These data were fit to a straight line by a linear regression analysis with CA-Cricket Graph III software (Computer Associates International, Inc.). To determine [3H]adenosine and [3H]guanosine uptake, both the saturation and inhibition experiments were performed using PLATINUM Taq DNA Polymerase High Fidelity (Life Technologies) following the manufacturer's instructions. For general DNA amplification, the TbNT2.2 ORF was obtained by conceptual translation of the TbNT2.2 sequence (GenBank/EBI Data Bank accession number AF153409), shown in Fig. 1 along with its alignments to Leishmania donovani Nucleoside Transporter LdNT1.1 (15) and LdNT1.1 protein sequence. Three oligonucleotides, O1, O2, and O3 (see “Experimental Procedures”), were designed against the trypanosome EST and used to screen a T. brucei cdNA library. Two positive clones, designated TbNT1 and TbNT2.1, were partially characterized and shown to encode proteins with significant sequence identity (approximately 30%) to LdNT1.1. A third clone, TbNT2.2, was obtained by PCR amplification of trypanosome genomic DNA using oligonucleotide primer O4, representing the first 5 amino acids of the TbNT2.1 ORF, and O5, representing the complement of sequence within the 3′-UTR of TbNT2.2 spliced leader sequence (22) (in italic type), was used as forward primer, and O5 was used as reverse primer. PCR amplification was performed using PLATINUM Taq DNA Polymerase High Fidelity (Life Technologies) following the manufacturer's instructions. reverse transcriptase-PCR products were subcloned into the pGEM-T Vector System (Promega) and characterized by sequencing as described above.

Genomic PCR Amplification of TbNT2.2—100 ng of genomic DNA from T. brucei EATRO 110 was employed as template for PCR amplification. The oligonucleotide O1 (5′-GCGGTACCACATACTCGCTGTGTA-3′), representing the first 5 amino acids of the TbNT2.2 ORF, including a KpnI restriction site (underlined) and a consensus Kozak sequence (22) (in italic type), was used as forward primer, and O5 was used as reverse primer. PCR amplification was performed using Pfu Turbo Polymerase (Stratagene) following the manufacturer's instructions. Amplified fragments were subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Clones were characterized by restriction mapping and sequencing.

DNA and Duded Amino Acid Sequence Analysis—For general DNA sequence analysis of TbNTs, the MacVector software (Intelligenetics) was used. GAP and PILEUP from the University of Wisconsin Genetics Computer Group (19) were used for pairwise and multiple amino acid sequence alignments. Transmembrane segments were predicted using the TMPRED software (23).

Expression in Xenopus Oocytes—The TbNT2.2 genomic clone was subcloned into the EcoRI site of the Xenopus expression vector pL2-5 (24), linearized and in vitro transcribed with T7 RNA polymerase (Life Technologies) in the presence of CAP analog (Amersham Pharmacia Biotech) as described previously (25). Stage V and VI Xenopus oocytes were injected with 15 fmol of DNA (~15 ng), isolated in ND96 buffer in 3 days at 16 °C as described (26), and used for uptake assays.

1 The abbreviations used are: EST, expressed sequence tag; ORF, open reading frame; cdNA, copy RNA; UTR, untranslated region; PCR, polymerase chain reaction; hENT1 and hENT2, human equilibrative nucleoside transporter 1 and 2, respectively.

Trypanosome Nucleoside Transporter

Guanosine (5 Ci mmol−1) was purchased from Movarek Biochemicals. All other chemicals were of the highest commercial quality available.

**Growth of Parasites and Isolation of Nucleic Acids—**Procyelic forms of T. brucei strain EATRO 110 were grown at 26 °C in SDM-79 medium (16). All other chemicals were of the highest commercial quality available.

**Hybridization, cdNA Library Screening, and Sequencing—**The dESTN99278 EST sequence represents a T. brucei nucleoside transporter identified from a TBLASTN search (19) of the nonredundant database of the GenBank EST database. The T. brucei EST EATRO 110 were cultured at 26 °C in SDM-79 medium (16). Nucleic acids were purified from trypanosomes following established procedures (18). Southern and Northern blot analyses were performed using standard protocols (18).

**Reverse Transcription-PCR Amplification of TbNT2.1—**To obtain the full-length TbNT2.1 cDNA clone, polyadenylated RNA from T. brucei EATRO 110 bloodstream forms was primed with oligo(dT)12 to synthesize cdNA using the ThermoScript TM reverse transcriptase-PCR system (Life Technologies, Inc.). Synthesized cdNA was then used as template for PCR amplification. The T. brucei spledin leader sequence was located at the 5′-end of all trypanosome mRNAs (20, 21) was used as forward primer (5′-AACGCTATTTAGAACAGTTTCTGTACTATATGAG-3′), and the oligonucleotide O5, representing the complement of sequence within the 3′-UTR of TbNT2.2 326 nucleotides downstream from the 3′-end of the ORF (5′-CGCTTTCTCTTGTTCTCTAAA-CTTGTGACTGAG-3′) was used as reverse primer. PCR amplification was performed using PLATINUM Taq DNA Polymerase High Fidelity (Life Technologies) following the manufacturer’s instructions. reverse transcriptase-PCR products were subcloned into the pGEM-T Vector System (Promega) and characterized by sequencing as described above.

**Genomic PCR Amplification of TbNT2.2—**100 ng of genomic DNA from T. brucei EATRO 110 was employed as template for PCR amplification. The oligonucleotide O4 (5′-GGGCTACCATGGCAATCCTGCC-3′), representing the first 5 amino acids of the TbNT2.2 ORF, including a KpnI restriction site (underlined) and a consensus Kozak sequence (22) (in italic type), was used as forward primer, and O5 was used as reverse primer. PCR amplification was performed using Pfu Turbo Polymerase (Stratagene) following the manufacturer's instructions. Amplified fragments were subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Clones were characterized by restriction mapping and sequencing.

**DNA and Duded Amino Acid Sequence Analysis—**For general DNA sequence analysis of TbNTs, the MacVector software (Intelligenetics) was used. GAP and PILEUP from the University of Wisconsin Genetics Computer Group (19) were used for pairwise and multiple amino acid sequence alignments. Transmembrane segments were predicted using the TMPRED software (23).

**Expression in Xenopus Oocytes—**The TbNT2.2 genomic clone was subcloned into the EcoRI site of the Xenopus expression vector pL2-5 (24), linearized and in vitro transcribed with T7 RNA polymerase (Life Technologies) in the presence of CAP analog (Amersham Pharmacia Biotech) as described previously (25). Stage V and VI Xenopus oocytes were injected with 15 fmol of DNA (~15 ng), isolated in ND96 buffer for 3 days at 16 °C as described (26), and used for uptake assays.

**RESULTS**

**Cloning and Sequence of the TbNT2 Gene—**To clone nucleoside transporter genes from T. brucei, we first searched a trypanosome EST database with the sequence from the LdNT1.1 gene, which encodes a nucleoside transporter in the related parasite L. donovani (15) and which was recently cloned in our laboratories. This search identified a single EST (N99278) whose deduced amino acid sequence revealed a significant degree of identity (27% identity over 88 amino acids) to the LdNT1.1 protein sequence. Three oligonucleotides, O1, O2, and O3 (see “Experimental Procedures”), were designed against the trypanosome EST and used to screen a T. brucei cdNA library. Two positive clones, designated TbNT1 and TbNT2.1, were partially characterized and shown to encode proteins with significant sequence identity (approximately 30%) to LdNT1.1. A third clone, TbNT2.2, was obtained by PCR amplification of trypanosome genomic DNA using oligonucleotide primer O4, representing the first 5 amino acids of the TbNT2.1 ORF, and O5, representing the complement of sequence within the 3′-UTR of TbNT2.2. This clone was employed in subsequent expression studies (see below), because the insert began with the initiating methionine codon of the TbNT2 ORF (the first in-frame methionine codon in the full-length cdNA sequence) and did not contain any 5′-UTR sequence that could potentially interfere with expression in the heterologous Xenopus oocyte system. The deduced amino acid sequence of the TbNT2 protein, obtained by conceptual translation of the TbNT2.2 sequence (GenBank/EBI Data Bank accession number AF153409), is shown in Fig. 1 along with its alignments to LdNT1.1 and to two human equilibrative nucleoside transporters, hENT1 (12) and hENT2 (13, 14, 27). TbNT2 exhibits significant sequence identity to all three of these nucleoside transporters (30.4, 22.1, and 24.7% identity, respectively), revealing that TbNT2 is a member of the nucleoside transporter family first defined by hENT1 and suggesting that TbNT2 is likely to be a trypanosome nucleoside transporter. Furthermore, hENT1, hENT2, LdNT1.1, and TbNT2 all possess 11 predicted transmembrane domains, implying that these proteins share a similar topology in the membrane. The topology that has been proposed for hENT1 (12), and which presumably applies for other transporters of this family and for hENT2, has a large hydrophilic loop between putative transmembrane domains 6 and 7 on the cytoplasmic side of the membrane, whereas the loop between transmembrane segments 1 and 2 that contains an N-linked glycosylation site (28) and the COOH-terminal hydrophilic tail are on the extracellular surface (assuming that these trans-

1 The abbreviations used are: EST, expressed sequence tag; ORF, open reading frame; cdNA, copy RNA; UTR, untranslated region; PCR, polymerase chain reaction; hENT1 and hENT2, human equilibrative nucleoside transporter 1 and 2, respectively.
porters are located within the plasma membrane). The TbNT2.1 cDNA clone gave the identical sequence, except for one T to C transition that converted the UUU codon encoding Phe20 in TbNT2.2 into a CUU codon encoding Leu20 in TbNT2.1 (Fig. 1). These results suggest that the two clones may represent either alleles or different copies of the TbNT2 gene. Henceforth, both genes and proteins will be referred to as TbNT2 and TbNT2, respectively, except where we intend to specify the cloned copy of the gene that was employed for a particular experiment.

Expression of the TbNT2 Gene in Xenopus Oocytes—To determine whether TbNT2 was a functional nucleoside transporter and to define its substrate specificity, we expressed the TbNT2 gene in Xenopus oocytes and assayed for uptake of various radiolabeled compounds. The results (Fig. 2) revealed that oocytes injected with TbNT2.2 cRNA transported [3H]adenosine, [3H]inosine, and [3H]guanosine at significantly higher rates than oocytes injected with water, confirming that the TbNT2 protein is a functional nucleoside permease. Furthermore, these results suggest that TbNT2 is a P1 type nucleoside transporter that mediates the uptake of adenosine and inosine.

To further characterize nucleoside transport by TbNT2, we performed substrate saturation curves for adenosine and inosine (Fig. 3) using oocytes expressing this transporter. The results of several independent saturation curves revealed a $K_m$ value for adenosine of $0.99 \pm 0.09 \mu M$ (mean $\pm$ S.D., n = 4) and a $K_m$ value for inosine of $1.18 \pm 0.62 \mu M$ (n = 3). These values are in the same range as the $0.15 \mu M K_m$ value for adenosine previously reported for P1 type transport in intact bloodstream form trypanosomes (6), and they confirm that TbNT2 is a high affinity purine nucleoside transporter. The 6-fold difference in $K_m$ values for adenosine obtained in parasites compared with oocytes could be due to physiological differences between Xenopus oocytes and trypanosomes, such as distinct membrane potentials, or the possibility that kinetic parameters obtained with intact parasites were influenced by metabolic processes.

To further probe the substrate specificity of TbNT2, we performed additional uptake assays using $0.5 \mu M$ [3H]adenosine as substrate and various unlabeled compounds as competitors at a concentration of $50 \mu M$ (Fig. 4). Significant inhibition was apparent for unlabeled adenosine, inosine, guanosine, 8-aminoguanosine, 6-thioguanosine, allopurinol riboside, and thio- purinol riboside, but not for adenine or any of the other nucleosides or nucleobases tested. The drug pentamidine, a high affinity substrate for the P2 transporter ($K_m = 0.84 \mu M$ (7)), did not inhibit uptake of adenosine by TbNT2 at either 1 or 10 $\mu M$ concentration (Fig. 4B), nor did 1 $\mu M$ putrescine, another antagonist of the P2 transporter (29) (data not shown). Together, these experiments confirm that TbNT2 has the substrate specificity of a P1 type nucleoside transporter. Finally, the ability of the protonophores carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone and 2,4-dinitrophenol to partially inhibit uptake of adenosine by TbNT2 (Fig. 4B) suggests that this permease may be a proton symporter, consistent with the observations of de Koning et al. (4) that nucleoside transport in intact procyclic parasites is dependent upon proton motive force.

Gene Organization and RNA Expression in Trypanosomes—The identification of at least one other closely related but nonidentical gene, TbNT1, suggested that trypanosomes might contain a family of TbNT2-like genes. Although TbNT1 has not yet been fully characterized, we have probing Southern blots of
T. brucei genomic DNA (Fig. 5A) to obtain a measure of the complexity of related sequences within the parasite genome. Most of the restriction digests revealed multiple bands that hybridized with a probe representing the 5'-half of the ORF, suggesting the presence of multiple genes of related sequence. In particular, the HindIII digest (Fig. 5A, lane 2) contained at least six major hybridizing bands that were detectable in this experiment and in other similar Southern blots, although the TbNT2 gene does not contain any HindIII sites within its ORF. However, digestions with two restriction enzymes that possess 8-base pair recognition sequences, NotI and SfiI (Fig. 5A, lanes 7 and 9) revealed the presence of a single hybridizing band of >20 kilobases. This result suggests that all of the TbNT2-related genes that hybridize under these conditions are clustered together within the genome. A complete characterization

FIG. 2. Functional expression of TbNT2 gene in Xenopus laevis oocytes. Shown is the time course for uptake of 0.5 μM [3H]adenosine (Ado) (A), 0.5 μM [3H]inosine (Ino) (B), and 0.5 μM [3H]guanosine (Guo) (C) by oocytes injected with TbNT2 cRNA (closed circles) or by oocytes injected with water (open circles) as control. For each time point, uptake (pmol) into at least three oocytes was measured and averaged; error bars represent S.D. of these values.

FIG. 3. Substrate saturation curves for [3H]adenosine and [3H]inosine in oocytes injected with TbNT2. For each [3H]adenosine (A) and [3H]inosine concentration (B), at least three oocytes were incubated with the substrate for 50 min, and the individual velocities were averaged; error bars represent S.D. of these values. The insets display the Hanes plots of these data by plotting [S]/v against [S]/v, adenosine or inosine concentration in μM; v, pmol of substrate oocyte−1.

FIG. 4. Inhibition of transport of [3H]adenosine in TbNT2 expressing oocytes by various compounds. Uptake assays were performed for 50 min in the presence of purines, pyrimidines, and their derivatives or analogs (A) and in the presence of structurally unrelated compounds (B). For each measurement, the concentration of adenosine was 0.5 μM, and the concentration of potential inhibitors was 50 μM in A or as indicated in B. Each bar represents the average of at least three independent measurements, and error bars indicate S.D. values. The asterisks indicate values that are significantly different (p < 0.02) from the no inhibitor control as determined by two-tailed Student’s t test. The vertical line represents the average value for the control (no inhibitor). XanR, xanthosine; 8aminoGuo, 8-aminoguanosine; 9deazaIno, 9-deazainosine; 6TGuo, 6-thioguanosine; Ade, adenine; Gua, guanine; Xan, xanthine; Urd, uridine; Thy, thymine; HPP, allopurinol; HPPR, allopurinol riboside; TPPR, thiopurinol riboside; ETOH 1%, 1% ethanol; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone dissolved in 1% ethanol.
predicted transmembrane segment 10. Only one NX(S/T) consensus N-linked glycosylation sequence (30), NVT (residues 27–29), occurs within TbNT2, although its location is within predicted transmembrane domain 1.

Transporter families defined on the basis of sequence similarity often contain both facilitative and active permeases. Thus, the glucose transporter superfamily contains both mammalian facilitative transporters and bacterial and protozoal proton symporters (31). The protonophore sensitivity of TbNT2 expressed in oocytes (Fig. 4B) and of nucleoside transporters studied in procyclic trypanosomes (4) suggests that these protonol permeases may be active transporters, whereas the related mammalian permeases are facilitative transporters (11). Electrophysiological studies on TbNT2 expressed in oocytes should elucidate whether this permease is an electrogenic symporter that can utilize the pronounced proton electrochemical gradient across the trypanosome plasma membrane (32) to concentrate nucleosides within the parasite.

Possible Family of Nucleoside Transporters in T. brucei—P1 type transporters are expressed in both procyclic and bloodstream form trypanosomes (4, 6). In contrast, TbNT2 mRNA is present at detectable levels only in bloodstream form parasites (Fig. 5B). This result implies that there are other P1 type transporters that are expressed either in procyclics or in both the procyclic and bloodstream stages of the life cycle. The fact that multiple TbNT2-like genes are present in the T. brucei genome (Fig. 5A and data not shown) is consistent with this conclusion, although it is also possible that some of these TbNT2-related genes could encode P2 type permeases that transport adenosine and adenine and that are expressed in bloodstream form parasites (6) or transporters for S-adenosylmethionine (33). Nonetheless, different P1 type transporters might be expressed in each life cycle stage to accommodate the potentially distinct nucleoside composition of the mammalian bloodstream and the tsetse fly gut. There is ample precedent for the existence of multiple isoforms of various transporters in both unicellular and multicellular eukaryotes (31). Humans express the hENT1 and hENT2 isoforms as well as a structurally unrelated family of Na⁺-dependent concentrative nucleoside transporters (34, 35). Furthermore, the existence of nucleoside transporters at both the cell surface and in intracellular membranes of mammalian tissue culture cells (36) raises the theoretical possibility that some organisms might express different nucleoside transporter isoforms that are selectively targeted to distinct membranes within the same cell. Indeed, at least one purine salvage enzyme, hypoxanthine-adenine phosphoribosyltransferase, is present within the mammalian cell membrane with the related parasite L. donovani (37), underscoring the possible need for nucleoside or nucleobase transporters on organellar membranes. Ultimately, it will be important to define the number, arrangement, and function of all the genes present within the cluster of related sequences that contains the TbNT2 gene and to define the potentially unique biological roles of each permease.

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