Adenosine Kinase of *Trypanosoma brucei* and Its Role in Susceptibility to Adenosine Antimetabolites^{∇†}

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Trypanosoma brucei cannot synthesize purines de novo and relies on purine salvage from its hosts to build nucleic acids. With adenosine being a preferred purine source of bloodstream-form trypanosomes, adenosine kinase (AK; EC 2.7.1.20) is likely to be a key player in purine salvage. Adenosine kinase is also of high pharmacological interest, since for many adenosine antimetabolites, phosphorylation is a prerequisite for activity. Here, we cloned and functionally characterized adenosine kinase from *T. brucei* (TbAK). TbAK is a tandem gene, expressed in both procyclic- and bloodstream-form trypanosomes, whose product localized to the cytosol of the parasites. The RNA interference-mediated silencing of TbAK suggested that the gene is nonessential under standard growth conditions. Inhibition or downregulation of TbAK rendered the trypanosomes resistant to cordycepin (3'-deoxyadenosine), demonstrating a role for TbAK in the activation of adenosine antimetabolites. The expression of TbAK in *Saccharomyces cerevisiae* complemented a null mutation in the adenosine kinase gene *ado1*. The concomitant expression of TbAK with the *T. brucei* adenosine transporter gene TbAT1 allowed *S. cerevisiae* *ado1 ade2* double mutants to grow on adenosine as the sole purine source and, at the same time, sensitized them to adenosine antimetabolites. The coexpression of TbAK and TbAT1 in *S. cerevisiae* *ado1 ade2* double mutants proved to be a convenient tool for testing nucleoside analogues for uptake and activation by *T. brucei* adenosine salvage enzymes.

Trypanosoma brucei species comprise the etiological agents of human sleeping sickness and livestock trypanosomes. The WHO estimates that over 23 countries are affected in sub-Saharan Africa, with a total burden of 1,598,000 disability-adjusted life years (<http://www.who.int/tdr/diseases/trypan>). Vaccination is hampered by the antigenic variation of the trypanosomes. Current chemotherapy relies on drugs that are either too expensive (e.g., eflornithine) or too toxic (e.g., melarsoprol) for mass use. The situation is somewhat mitigated by the donation of eflornithine from Sanofi-Aventis to the WHO and by the newly developed drug pafuramidine (50), which is active only against first-stage sleeping sickness but orally applicable and presently under a phase III clinical trial. Still, there is a need for new drugs to treat sleeping sickness. Purine salvage offers attractive opportunities for chemotherapeutic intervention. Like all obligate parasitic protozoa, African trypanosomes cannot synthesize purines de novo and rely completely on salvage from their hosts to build nucleic acids (26). Their purine salvage machinery is remarkably versatile, as *T. brucei* incorporates any of the physiological purine bases or nucleosides and interconverts the corresponding nucleotides (19). This means that no single salvage enzyme is essential and that trypanosomal purine salvage, although vital to the parasite, may not hold suitable drug targets. However, the salvage

pathways may be exploited for drug targeting, i.e., the specific delivery or activation of subversive substrates via enzymes that are absent or different in the mammalian host.

The concept was illustrated for adenosine kinase of *Toxoplasma gondii* (TgAK), which, in contrast to its mammalian counterparts, phosphorylates nitrobenzylmercaptapurine riboside (NBMPR) (6-nitrobenzylthioinosine), rendering it and related compounds specifically toxic to the parasite (10, 17, 29, 56). TgAK null mutants were resistant to NBMPR (10, 52). The loss of adenosine kinase also caused drug resistance in *Leishmania donovani*, to formycin A and cordycepin (24). Cordycepin (3'-deoxyadenosine) from *Cordyceps militaris* is nature's forestalling of the Sanger sequencing method (47). It is a prodrug, with the presumed mechanism of action—chain termination upon incorporation into RNA—demanding prior phosphorylation. Since this is the case for most nucleoside antimetabolites, particular pharmacological importance is allocated to nucleoside kinases from parasites. Adenosine kinase has been dissected to the molecular level in *T. gondii* and *Leishmania donovani* (9, 10, 12, 13, 22, 23, 48), cloned from *Babesia canis* (5) and *Cryptosporidium parvum* (20), and characterized in cell extracts of *Trypanosoma cruzi* (28) and *Eimeria* spp. (36) but has not yet been described for African trypanosomes.

T. brucei bloodstream forms incorporate radiolabeled adenosine into the nucleotide pool faster than any other nucleoside (19). Trypanosomal adenosine uptake consists of two components: P1 transports adenosine, inosine, and guanosine, and P2 transports adenosine, adenine, melarsoprol, and diamidines (6, 7, 14). While the P1 transporters are redundant (30, 46), P2 is encoded by a single gene, *T. brucei* AT1 (TbAT1) (33, 35). Homozygous disruption of TbAT1 in *T. brucei* bloodstream forms caused resistance to melarsoprol, diamidines, cordyce-

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pin, and tubercidin (21, 35). Cordycepin was found to be a potent and selective trypanocide in vitro (34, 53) but not in vivo (40). However, when cordycepin was coadministered with an adenosine deaminase inhibitor to prevent it from being converted to 3'-deoxyinosine in plasma (44), it cured *T. brucei*-infected mice even from (supposedly) late-stage trypanosomiasis (45). Tubercidin (7-deazaadenosine) is another natural adenosine analogue of potent antitrypanosomal activity. In vivo, its therapeutic window may be widened by the coadministration of NBMPR to block uptake by host cells (16, 39). Here we clone and characterize TbAK and investigate by functional expression in the yeast *Saccharomyces cerevisiae* its potential for the activation (i.e., phosphorylation) of cordycepin, tubercidin, and other adenosine analogues.

MATERIALS AND METHODS

Trypanosome stocks, in vitro culture, and drug sensitivity tests. Bloodstream-form *T. brucei brucei* BS221 (s427) and its *TbAt1*^{-/-} derivative (35) were cultivated at 37°C and in 5% CO₂ in HMI-9 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Amimed) and 1 mM hypoxanthine. "Purine-free" FCS was obtained after passage through a Sephadex G25 column with an exclusion limit of 5 kDa (PD-10; Amersham Biosciences) and elution with Hanks balanced salt solution buffer (GIBCO). Procytic forms were grown at 27°C in SDM-79 medium supplemented with 5% FCS (4). Alamar Blue drug sensitivity tests were performed as described previously (43). In brief, 10⁴ cells were incubated at serial drug dilutions for 70 h, followed by a 2-h incubation with the redox-sensitive dye Alamar Blue as an indicator of cell viability. The assays were performed at least four times, each in duplicate. Fifty percent inhibitory concentration (IC₅₀) values were calculated by nonlinear fitting to a sigmoidal dose-response curve with Prism4 (GraphPad Software, San Diego, CA). Cordycepin, tubercidin, and ABT-702 were purchased from Sigma-Aldrich.

Southern and Northern blots. For Southern blots, genomic DNA was isolated from procyclic trypanosomes (PC427). Total RNA for Northern blots was isolated from cultured trypanosomes with the hot phenol method. A TbAK probe of 315 bp, amplified by PCR from cloned TbAK with the primers TbAK-i-s (GCA AGCTTGGATCCGGTTTACGTTGACAGTTGACGTTAA) and TbAK-i-as (GCTCTAGACTCGAGTGAACACAACAACACGACCTTTTGT), was labeled with digoxigenin (PCR DIG probe synthesis kit; Roche) for Southern blots and [α -³²P]dCTP (Megaprime DNA labeling system; Amersham) for Northern blots. An actin probe was made in the same manner with primers act-s (CCGA GTCACACAACGT) and act-as (CCACCTGCATAACATTG).

RNA interference (RNAi)-mediated gene silencing. A TbAK stem-loop construct was obtained by cloning the PCR product of primers TbAK-i-s and TbAK-i-as (see above) twice, in opposite directions, into the plasmid pALC14 (1). NY-single-marker (T7RNAP/TetR) bloodstream-form trypanosomes (55) were transfected with 10 μ g of NotI-linearized plasmid by electroporation. Transfectants were selected for by cultivation in 0.5 μ g/ml puromycin and 1 μ g/ml neomycin and cloned by limited dilution. Expression of the stem-loop construct was induced by the addition of 1 μ g/ml tetracycline (Tet).

In situ tagging of TbAK. C-terminal fusion of the TbAK gene product with a hemagglutinin (HA) tag was carried out in situ using the system developed by Oberholzer et al. (38). A linear tagging construct containing the hygromycin resistance gene was created by PCR with the plasmid pMOTag4HA and the primers RevUTR (AAAAAAAATCATCAACCACACCATCGAAATCATATCAACCA CCGTATTTGGCATTAACAACCGACAACGAAAAACAAAAAAGTGGCG GCCGTCTAGAACTAGTGGAT) and FwORF (GCGAAA CTGGCCACTAC ACAGACAAGAGGTTATCCAGCGTGACGGTTGCTCTTCCCGA GAAGCCCAGTTTCTCTCTGGTACCGGGCCCCCCTCGA) and transformed into bloodstream-form *T. brucei*. Transformants were selected in 1.5 μ g/ml hygromycin, cloned by limited dilution, and verified by PCR for an in-frame insertion of the HA tag.

Digitonin extraction and Western blotting. Cells (2.5 $\times$ 10⁷) were washed twice in SBG buffer (22 mM glucose, 0.6 M NaCl, 80 mM NaHPO₄, pH 7.9) and resuspended in SoTE buffer (1.2 M sorbitol, 4 mM EDTA, 40 mM Tris-HCl; pH 8.0) containing digitonin (Sigma-Aldrich) at the concentrations indicated in Fig. 3B. After incubation on ice for 5 min, the lysate was centrifuged twice at 6,000 $\times$ g to pellet all insoluble proteins from the supernatant. The soluble and the insoluble fractions were then dissolved in protein sample buffer containing β -mercaptoethanol, subjected to 12% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis, and blotted onto an Immobilon-P membrane (Millipore). For immunodetection, mouse anti-HA 12CA5 (at 1/1,000; Roche) was used. The secondary antibody was rabbit anti-mouse horseradish peroxidase (at 1/3,500; Dako), which was followed by exposure to a chemiluminescent substrate detection system (Pierce). To detect glycosomal proteins, antialdolase from rabbit (at 1/100,000; a gift from Paul Michels, Université Catholique de Louvain, Bruxelles, Belgium) was used, and as a secondary antibody, swine anti-rabbit horseradish peroxidase (at 1/3,300) was used.

Immunofluorescence. For immunofluorescence, 10⁵ cells were washed with phosphate-buffered saline and fixed onto cover slides with 4% formaldehyde. The cells were permeabilized with 2% Triton X-100. After the cells were blocked with 3% bovine serum albumin, the primary antibody, anti-HA rabbit immunoglobulin G polyclonal antibody sc-805 (Santa Cruz; at 1/100), was added and the slides were incubated for 45 min at room temperature, washed with phosphate-buffered saline, and incubated with the secondary antibody, Alexa Fluor goat anti-rabbit immunoglobulin G (Molecular Probes; at 1/1,000), for 45 min at room temperature. Vectashield containing DAPI (4',6'-diamidino-2-phenylindole) (Vector Laboratories, Inc.) was used for mounting.

Expression of TbAK in yeast and drug tests. TbAK was amplified from genomic DNA of *T. brucei* with the primers Fw-Xba/Bam (GGTCTAGAGGA TCCACTTTCAGTCATCTAAAGGTTTGC) and Rev-Xho/Hind (GGCTCGA GAAGCTTTTGGCATTAACAACCGACA), cloned into pGEMT-Easy (Promega), sequenced, and subcloned into the yeast expression vector pRS413. TbAT1 had been cloned into p416-MET25 (33). The constructs were transformed into the yeast strain Y759 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 ade2 *ado1::LEU2*) (31). SD medium consisted of 2% glucose and 0.67% yeast nitrogen base (Difco), complemented with lysine (30 μ g/ml), leucine (30 μ g/ml), histidine (20 μ g/ml), uracil (20 μ g/ml), and, depending on the experiment, adenine (20 μ g/ml), hypoxanthine (20 μ g/ml), adenosine (30 μ g/ml), or S-adenosylmethionine (SAM) (30 μ g/ml). For halo assays, cells (10⁶ per ml) were mixed with SD medium containing 150 μ M hypoxanthine plus 0.8% agar and poured into plates. Test compounds were spotted (spots were 5 μ l at different concentrations), and the plates were incubated for 24 h at 30°C. 8-Aza-7-deaza-2'-deoxyadenosine, 8-azaadenosine, formycin A, and 7-deazaadenine were purchased from Berry & Associates Inc. (Dexter, MI); isodotubercidin and A134974 were purchased from Sigma-Aldrich.*

RESULTS

Adenosine kinase from *Trypanosoma brucei*. In order to identify adenosine kinase genes from *T. brucei*, we used the program HMMER (15) to build a hidden Markov model-based profile from 18 known adenosine kinases (EC 2.7.1.20) of all eukaryotic kingdoms and bacteria. The profile (adok.hmm) is available in the supplemental material. Searching the deduced *T. brucei* proteome (version 4 containing 9,152 proteins) with that profile yielded two hits with very trustworthy expectancy values of 10⁻¹⁵⁰. The two proteins, Tb927.6.2300 and Tb927.6.2360, had already been annotated as putative adenosine kinases based on BLAST searches (3). The next-best hit of the hidden Markov model search, Tb11.03.0090, still had an expectancy value of 10⁻⁶ but appeared to be a ribokinase (EC 2.7.1.15) when a BLAST search of Swiss-Prot was conducted, indicating that the *T. brucei* genome contains just the two adenosine kinase genes. The two open reading frames (ORFs) are of equal lengths (1,038 bp) and differ only in seven positions, of which three are synonymous. For the sake of simplicity, we use here the name TbAK for either gene. The two TbAK genes lie on chromosome VI in close proximity. A self-alignment matrix revealed a tandem duplication of 7.8 kb containing TbAK plus four additional ORFs without similarity to known genes (Fig. 1A). Unrelated to adenosine kinase but noteworthy nevertheless was the presence at the TbAK locus of a triplicate sequence of 396 bases (Fig. 1A), within which a 20-mer element (ATGAGTTTTGGAGGGAACAA) turned out to occur also in 15 of the human chromosomes and on

FIG. 1. *Tb4K* locus in *T. brucei*. (A) Self-alignment matrix of 18 kb from chromosome VI (Chrs VI) and predicted genes therein. Gray, uncharacterized ORFs; black, *Tb4K*. About 8 kb are almost perfectly duplicated, and a sequence of 396 bases even occurs three times (asterisks). (B) A Southern blot with a *Tb4K* probe of genomic DNA digested with the enzymes indicated in panel A confirmed the duplication of the locus. (C) A Northern blot of total RNA showed the expression of *Tb4K* in the bloodstream (BS) and procyclic (PC) stages of *T. brucei*.

several chromosomes of chimpanzee and mouse. Randomized versions of the element did not perfectly match any of the GenBank nonredundant DNA sequences. We do not know whether the 20-mer is transcribed, but its repeated occurrence is certainly exceptional. The duplication of the TbAK locus was confirmed by Southern blotting with a TbAK-specific probe (Fig. 1B). Northern blotting results revealed that TbAK is expressed at high levels in both tsetse midgut procyclic- and mammalian bloodstream-form trypanosomes but somewhat higher in the latter (Fig. 1C). This contrasts to what occurs with *Leishmania*, where adenosine kinase is 50 times more active in the macrophage-dwelling amastigote forms than in promastigotes (32). Direct sequencing of reverse transcriptase PCR products indicated that both TbAK genes are transcribed (data not shown).

As shown in Fig. 2, TbAK groups together with known adenosine kinases and is clearly separated from inosine kinases (EC 2.7.1.73) or deoxynucleoside kinases (EC 2.7.1.145). Within the adenosine kinases, the *T. brucei* enzyme forms a significantly distinct branch together with the AK homologues from *Trypanosoma cruzi* and *Leishmania donovani* (LdAK), with which TbAK shares 77% and 74% similarity, respectively. All the charged residues that are indispensable for catalysis in LdAK (Asp₁₆, Arg₆₉, Arg₁₃₁, and Asp₂₉₉) (12, 13) are conserved in TbAK (Asp₁₇, Arg₇₀, Arg₁₃₂, and Asp₂₉₉, respectively). However, with a predicted pI of 5.5, TbAK does not share with LdAK its unusually high pI of 8.8 (11). TbAK also carries the diglycyl motif (Gly₆₂ and Gly₆₃) involved in the domain rotation upon adenosine binding, which allows subse-

FIG. 2. Phylogenetic tree of purine nucleoside kinase amino acid sequences. TbAK clearly groups with the adenosine kinases, and within those kinases, it groups with a subbranch formed by the enzymes from kinetoplastid parasites. Bootstrapping values (in percentages of positive samples per 1,000 rounds) are shown for the major branches.

quent binding of ATP (48), and shares with TgAK the kinase anion hole motif: DTNGAGD in TgAK (48) and DMNGAGD in TbAK (amino acids 293 to 299). Kinetoplastid parasites possess membrane-bound organelles, namely, glycosomes (41), which are specialized for glycolysis but also contain purine salvage enzymes (42). To test whether *T. brucei* adenosine kinase localizes to the glycosome or to the cytosol, an HA tag was added in situ by homologous recombination (38) to the C terminus of TbAK (TbAK does not possess a targeting C-terminal tripeptide (49) that would be masked by the tag). Western blot analysis of HA-tagged TbAK indicated a molecular mass of about 40 kDa, in agreement with the predicted mass of 38 kDa for TbAK. Immunofluorescence microscopy with an Alexa Fluor-coupled secondary antibody gave a granular signal dispersed throughout the cytosol of bloodstream-form trypanosomes (Fig. 3A). The saponin digitonin, a mild detergent that dissolves the plasma membrane at much lower concentrations than are required for internal membranes (51), was used for differential lysis of trypanosomes. Digitonin lysates were centrifuged, and both the pellet (insoluble material) and the supernatant (solubilized) were analyzed on Western blots (Fig. 3B). While TbAK entered the soluble fraction with the use of 0.1 mg digitonin per mg protein, the glycosomal marker aldolase remained with the insoluble material until 0.5 mg digitonin per mg protein was used, demonstrating that TbAK does not reside in the glycosome but in the cytosol. This is in agreement with a recent proteome-wide survey for glycosomal proteins of *T. brucei* (8).

Functional characterization of TbAK in trypanosomes. As an initial test for a possible involvement of TbAK in the trypanocidal action of adenosine analogues, we assessed the effects of the pharmacological inhibition of TbAK on the susceptibility of trypanosomes to adenosine antimetabolites. Drug sensitivity was measured *in vitro* over an exposure time of 72 h,

FIG. 3. Subcellular localization of TbAK in bloodstream-form *T. brucei*. (A) HA-tagged TbAK localized to the cytosol as determined by immunofluorescence microscopy. Bar, 10 μm. (B) Differential lysis with digitonin. P, pellet; S, supernatant; α, anti. Aldolase served as a marker for the glycosome.

using the redox-sensitive dye Alamar Blue as an indicator of cell viability (43). ABT-702 {4-amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2,3-*d*]pyrimidine}, a specific inhibitor of adenosine kinase (27), had an IC_{50} against *T. brucei* bloodstream forms of 3.4 ± 1.1 μM. When applied at the nontoxic concentration of 320 nM, ABT-702 significantly reduced the sensitivity of trypanosomes to cordycepin (3'-deoxyadenosine), raising the IC_{50} from 52 nM to 308 nM ($P < 0.001$, analysis of variance and Tukey's post hoc test) (Fig. 4A). This supports the notion that TbAK activates cordycepin, albeit formal proof that ABT-702 inhibits TbAK is lacking.

FIG. 4. Sensitivity of bloodstream-form *T. brucei* 221 (*TbAT1*^{+/+}) and its *Tbat1*^{-/-} derivative to cordycepin (Cor) (A) and tubercidin (Tub) (B), in the absence (white) or presence (black) of the adenosine kinase inhibitor ABT702 at 320 nM. ABT702 exacerbated the resistance phenotype of *Tbat1* null trypanosomes to cordycepin but not to tubercidin. Error bars indicate standard deviations, and a, b, c, and d indicate significance groups according to the results of analysis of variance and Tukey's post hoc test.

FIG. 5. RNAi-mediated silencing of TbAK in *T. brucei* bloodstream forms. (A) Northern blot analysis on total RNA of control wild-type cells (ctr) and TbAK RNAi cells in the presence (+) or absence (-) of 1 μg/ml Tet. (B) Cordycepin (Cor) sensitivity of TbAK RNAi trypanosomes with or without Tet (1 μg/ml) and/or ABT702 (320 nM).

Homozygous disruption of the adenosine transporter gene *TbAT1* has been shown to cause cordycepin resistance in *T. brucei* bloodstream forms (21). The subtherapeutic application of ABT-702 further reduced the cordycepin sensitivity of *Tbat1* null trypanosomes, raising the IC_{50} from 189 nM to 497 nM ($P < 0.001$) (Fig. 4A). Regarding the antitrypanosomal activity of tubercidin (7-deazaadenosine), however, ABT-702 had no significant alleviating effects. The IC_{50} values of both tubercidin-susceptible BS221 trypanosomes (104 nM) and tubercidin-resistant *Tbat1* null trypanosomes (1.95 μM) were only slightly raised by the addition of the adenosine kinase inhibitor (to 152 nM and 2.07 μM, respectively) (Fig. 4B).

The second approach to functionally characterize TbAK in trypanosomes was to knock down its expression by RNAi. A stem-loop construct targeting TbAK was made under the control of a Tet-inducible promoter and transformed into bloodstream-form *T. brucei* that expressed the Tet repressor and T7 RNA polymerase (54, 55). Upon addition of 1 μg/ml of Tet to TbAK RNAi transformant clones, the expression levels of TbAK were strongly reduced but not depleted completely (Fig. 5A). Nevertheless, the addition of Tet reduced the cordycepin sensitivity of TbAK RNAi cells (Fig. 5B). No effect on tubercidin sensitivity was observed (not shown). As usual with these kinds of constructs, there was a certain degree of leakiness, indicated by the slightly reduced expression of TbAK in TbAK RNAi cells (Fig. 5A) and elevated IC_{50} s to cordycepin (Fig. 4A and 5B) in the absence of Tet. Coadministration of Tet and the adenosine kinase inhibitor ABT-702 (at 320 nM) caused a substantial loss of cordycepin susceptibility in TbAK RNAi cells (Fig. 5B). RNAi against TbAK did not affect the growth of trypanosomes in standard cultivation medium containing 1 mM hypoxanthine and 10% FCS. The critical test for TbAK function, the growth of TbAK RNAi cells on adenosine as the sole purine source, was experimentally challenging since the FCS appeared to contain enough purines for the trypanosomes to grow even without a purine supplement. A high background of purines in the medium might also account for the lower toxicity of cordycepin on wild-type trypanosomes, compared to that observed in previous studies (21). In order to obtain a "purine-free" serum, the FCS was passed through a desalting column with a 5-kDa exclusion limit. Using that serum, the parasites proliferated only in the presence of extra purines. However, the loss of all the low-molecular-weight compounds from the serum severely slowed down the growth rate of the

FIG. 6. Functional expression of TbAK in *S. cerevisiae*. Yeast *ade2 ade1* double mutants transformed with TbAK and/or TbAT1, or the respective empty vectors (–), were grown on minimal medium containing as the purine source adenine, SAM, or adenosine. Only concomitant expression of TbAK and TbAT1 allowed growth on adenosine. OD, optical density.

trypanosomes, shifting the population doubling times to around 60 h, compared to 9 h with normal serum. When adenosine (1 mM) was offered as the sole purine source, the addition of Tet initially abolished the growth of TbAK RNAi cells but not of the control cells. After 4 days of adaptation to adenosine though, downregulation of TbAK no longer affected the growth rate of TbAK RNAi cells (data not shown).

Functional characterization of TbAK in yeast. In contrast to trypanosomes, the yeast *Saccharomyces cerevisiae* synthesizes purines de novo but does not take up exogenous adenosine (2). Purine-auxotrophic *S. cerevisiae* strains with null mutations in phosphoribosylaminoimidazole-carboxylase (Ade2p) grow in the presence of adenine or hypoxanthine, which they salvage via phosphoribosyltransferases, but cannot use adenosine as a purine source. They can, however, take up SAM and convert it via SAM-methyltransferase and *S*-adenosylhomocysteine hydrolase to adenosine, which then is incorporated into the nucleotide pool by means of adenosine kinase (Ado1p) (31). Null mutation in Ado1 disrupts this bypass (31). We expressed either TbAK or the adenosine transporter TbAT1, or both genes, in the *ade2 ade1* double mutant yeast strain Y759 (31). Expression of TbAK restored growth on SAM (Fig. 6), proving that TbAK is adenosine kinase. Only simultaneous expression of TbAK and TbAT1 allowed Y759 cells to grow on adenosine (Fig. 6), demonstrating the successful reconstitution of two consecutive steps of trypanosomal adenosine salvage in yeast.

The TbAK TbAT1 double transformants provided a convenient means to test nucleoside prodrugs for import and activation by the trypanosomal enzymes. Qualitative halo assays revealed that a number of adenosine analogues depend on TbAK and TbAT1 for activity; cordycepin, tubercidin, 8-aza-adenosine, formycin A, and iodotubercidin were toxic only to TbAK TbAT1 double transformant Y759 cells and not to TbAK or TbAT1 single transformants (data not shown). NBMPPR, 8-aza-7-deaza-2'-deoxyadenosine, 2',3'-dideoxyadenosine, and A134974 (5'-amino-iodotubercidin) were inactive against all of the yeast transformants up to spotting concentrations of 10 mM and so were the purine nucleobase analogues 7-deaza-adenine, aminopurinol, allopurinol, and caffeine. Surprisingly, also melarsen oxide was toxic only to cells expressing TbAK and TbAT1, indicating a role for TbAK in the action of melarsenoprol. However, the IC_{50} of melarsen oxide to bloodstream-form *T. brucei* increased only marginally in the presence of 320 nM ABT-702, from 11 to 13 nM ($P = 0.009$, two-tailed paired *t* test), and no significant effect on melarsen

sensitivity was observed in TbAK RNAi cells upon the addition of Tet (data not shown), indicating that the observed requirement of adenosine kinase for melarsen activity in yeast does not apply to trypanosomes.

DISCUSSION

Adenosine kinase has been identified and found to be of pharmacological importance in a number of protozoan parasites except, somewhat surprisingly, African trypanosomes. Based on AMP production assays with cell extracts, bloodstream-form *T. brucei* was even suggested to lack adenosine kinase (40). On the other hand, adenosine was reported to be the preferred purine nucleoside for salvage by trypanosomes (26). Our findings (Fig. 4, 5, and 6) strongly suggest that TbAK is indeed adenosine kinase and a prerequisite for cordycepin susceptibility in *T. brucei*. With trypanosomes lacking means of transcriptional regulation such as polymerase II promoters, the duplication of the TbAK locus (Fig. 1) may well reflect the importance of adenosine kinase for trypanosomal purine salvage. Nevertheless, TbAK is unlikely to be essential since adenosine can also be converted to AMP by the sequential actions of adenosine nucleosidase (EC 3.2.2.7) and adenine phosphoribosyltransferase (EC 2.4.2.7). This may explain why subtoxic application of the adenosine kinase inhibitor ABT-702 caused resistance to cordycepin but not to tubercidin (Fig. 4). Tubercidin's toxophore resides in the purine ring (lacking N7) and is maintained after incorporation into the nucleotide pool via adenosine nucleosidase and adenine phosphoribosyltransferase, while cordycepin following the same path simply gets converted to adenosine. In yeast, which in contrast to *T. brucei* does not possess adenosine nucleosidase (25, 31), tubercidin activity was TbAK dependent.

Saccharomyces cerevisiae lacks adenosine transporters (2). In order to facilitate the pharmacological characterization of TbAK in the *ade2 ade1* yeast strain Y759 (31), it was coexpressed with TbAT1, enabling the transformants to take up adenosine and analogues thereof. This allowed a simple, qualitative test of potential subversive substrates for import and activation by the two trypanosomal enzymes. Cordycepin, tubercidin, 8-azadeadenosine, formycin A, and iodotubercidin exhibited activity only against TbAK- and TbAT1-expressing cells, demonstrating the pharmacological significance of the two genes. Surprisingly, also melarsen oxide was active only against TbAK and TbAT1 expressors. An involvement of TbAK must be indirect, since melarsen lacks hydroxyl groups that could be phosphorylated. Possibly, adenosine competes with melarsen at the intracellular target site and the overexpression of TbAK increases melarsen sensitivity by reducing the cytosolic adenosine levels (37). However, the phenomenon was not directly translatable to *T. brucei*, where inhibition of TbAK hardly reduced melarsen sensitivity. In trypanosomes, melarsen is complexed by trypanothione to form MelT, which in turn inhibits trypanothione reductase (18). The case of melarsen oxide shows that the yeast system is useful only when the mode of action is conserved between *S. cerevisiae* and *T. brucei*.

In summary, the reconstitution of the first two steps of trypanosomal adenosine salvage in yeast provides a convenient means of testing adenosine antimetabolites for import and activation by *T. brucei*. Parallel inclusion of human adenosine

kinase and/or nucleoside transporters will allow screening for selective antitrypanosomal nucleoside prodrugs in yeast. With nucleoside analogues being widely used in antiviral and anti-tumor therapy, a large number of promising compounds are available for screening, some of which are already registered for use in humans. The specific targeting of subversive substrates toward parasites via their purine salvage pathways is an excellent strategy against *T. brucei* due to its elaborate purine uptake and interconversion machinery.

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