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Validation of spermidine synthase as a drug target in African trypanosomes

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Abstract

The trypanocidal activity of the ODC (ornithine decarboxylase) inhibitor DFMO (difluoromethylornithine) has validated polyamine biosynthesis as a target for chemotherapy. As DFMO is one of only two drugs used to treat patients with late-stage African trypanosomiasis, the requirement for additional drug targets is paramount. Here, we report the biochemical properties of TbSpSyn (*Trypanosoma brucei* spermidine synthase), the enzyme immediately downstream of ODC in this pathway. Recombinant TbSpSyn was purified and shown to catalyse the formation of spermidine from putrescine and dcSAM (decarboxylated *S*-adenosylmethionine). To determine the functional importance of TbSpSyn in BSF (bloodstream form) parasites, we used a tetracycline-inducible RNAi (RNA interference) system. Down-regulation of the corresponding mRNA correlated with a decrease in intracellular spermidine and cessation of growth. This phenotype could be complemented by expressing the SpSyn (spermidine synthase) gene from *Leishmania major* in cells undergoing RNAi, but could not be rescued by addition of spermidine to the medium due to the lack of a spermidine uptake capacity. These results therefore genetically validate TbSpSyn as a target for drug development and indicate that in the absence of a functional biosynthetic pathway, BSF *T. brucei* cannot scavenge sufficient spermidine from their environment to meet growth requirements.

Keywords

biosynthesis; complementation; human African trypanosomiasis; *Leishmania major*; polyamine; RNA interference (RNAi); spermidine synthase; *Trypanosoma brucei*

INTRODUCTION

As a result of co-ordinated surveillance and treatment programmes, the number of people infected annually by the protozoan parasite *Trypanosoma brucei*, the causative agent of HAT (human African trypanosomiasis), has fallen to 70000 [1]. However, in some regions, mortality rates for HAT exceed HIV/AIDS and malaria. Current treatments for the late stage of the disease rely on the drugs melarsoprol and DFMO (difluoromethylornithine). However, their use is problematic. With no realistic prospect of a vaccine, new drugs against 'one of the great neglected diseases' are a priority [2].

One area of parasite biology that has attracted attention in terms of drug development is polyamine biosynthesis [3-6]. Polyamines such as putrescine and spermidine are aliphatic organic compounds containing two or more amino groups. In most eukaryotes, these molecules play pivotal roles in several processes, including protein/nucleic acid synthesis and cell proliferation/differentiation, and have been implicated in the development of certain cancers [7]. Trypanosomes are unusual in that spermidine is also used in the synthesis of trypanothione, a glutathione-spermidine dithiol conjugate that plays a central role in several detoxification processes and supplies reducing equivalents used in nucleic acid synthesis [8,9]. As this thiol is both unique and essential to trypanosomatids, any process involving trypanothione is considered a potential target for drug intervention.

The building blocks for polyamine synthesis are arginine and methionine (Figure 1). In most eukaryotes, arginine is hydrolysed to form ornithine and urea, with ornithine metabolized further to putrescine, reactions catalysed by arginase and ODC (ornithine decarboxylase) respectively. Putrescine then reacts with dcSAM (decarboxylated *S*-adenosylmethionine) to form spermidine, a process mediated by SpSyn (spermidine synthase); the dcSAM is derived from methionine as a result of the activity of SAMdc (*S*-adenosylmethionine decarboxylase) (Figure 1). The trypanocidal activity of various inhibitors that target components of this pathway has been reported [10-14]. DFMO, one of two drugs available to treat late-stage HAT, inhibits ODC activity. The interaction between *T. brucei* ODC and DFMO has been extensively studied and the selective killing activity of the drug has been determined [15,16]. The basis for this toxicity stems from the stability of the parasite ODC (half-life ~18 h) compared with that of the mammalian host ODC (half-life ~20 min) [15,17]. When DFMO binds covalently to the mammalian enzyme, the complex is rapidly degraded and replaced with newly synthesized ODC. In contrast, the DFMO-ODC molecule remains within the parasite and is only slowly replaced. Thus the level of active protein is decreased leading to a cessation of putrescine formation. Eventually, the parasite stops growing and the non-dividing cells are cleared by the host immune system. Although the activities of other *T. brucei* enzymes involved in polyamine synthesis have been detected [12,18], their biochemical roles have yet to be fully analysed. Here, using an inducible RNAi (RNA interference) system, we demonstrate that SpSyn (EC 2.5.1.16), a key component of polyamine synthesis, is essential to infective *T. brucei*, therefore validating it as a potential target for chemotherapeutic intervention.

EXPERIMENTAL

Parasites

BSFs (bloodstream forms) of the SMB (*T. brucei* single marker cell line) that constitutively express T7 RNA polymerase and the tetracycline repressor protein [19] were grown at 37 °C under a 5% CO₂ atmosphere in modified Iscove's medium [20] containing 2 µg · ml⁻¹ G418. Transformed SMB cells were maintained in this growth medium, supplemented with 2.5 µg · ml⁻¹ hygromycin. Tetracycline-free fetal calf serum (10%, v/v) (Autogen Bioclear) was used in the growth medium. RNA was extracted from parasites by using the RNeasy[®] mini kit (Qiagen).

Biochemical properties

TbSpSyn (*Trypanosoma brucei* spermidine synthase) was amplified from parasite genomic DNA (see Table 1 for primers). The fragment was digested with BamHI + HindIII and cloned into the corresponding sites of the expression vector pTrcHis-C (Invitrogen). In this system, the expressed protein contains an N-terminal His tag and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen). The insert was sequenced using a dye terminator cycle kit (Applied Biosystems) and an ABI Prism 3730 sequencer. *Escherichia*

coli BL21+ transformed with pTrcHis-TbSpSyn were grown in NZCYM broth (Sigma) containing ampicillin, and protein expression was induced by the addition of IPTG (isopropyl β -D-thiogalactoside). Recombinant His-tagged proteins were affinity-purified on an Ni-NTA (Ni²⁺-nitrilotriacetate) matrix column under native conditions as recommended by the manufacturer (Qiagen). The cell lysis, column wash and elution steps were all carried out in the presence of protease inhibitors (Roche). Fractions were analysed by SDS/PAGE and protein concentrations were determined by a BCA (bicinchoninic acid) protein assay system (Pierce).

TbSpSyn activity was followed by monitoring spermidine formation in the presence of putrescine and dcSAM (modified from [21]). A standard 1 ml reaction contained 100 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol, 1 mM putrescine, 10 μ M dcSAM and 100 μ g of TbSpSyn. Reactions were incubated at 37 °C for 30 min and then terminated by placing the test tubes at 95 °C for 5 min. Polyamines were derivatized with dansyl chloride and then analysed by HPLC.

RNAi

An 860 bp DNA fragment corresponding to an internal sequence from *TbSpSyn* was amplified from genomic DNA (see Table 1 for primers), digested with BamHI + XhoI and cloned into the corresponding sites of the vector p2T7^{Ti} [22]. In this vector, the inserted DNA is flanked by two opposing T7 promoters, with each promoter under the control of a tetracycline operator. Constructs were linearized with NotI and electroporated into BSF parasites and transformants were cloned as described [23]. Induction of RNAi was initiated by adding tetracycline (1 μ g · ml⁻¹) to the culture.

Complementation vector

The complete ORF (open reading frame) of *LmSpSyn* (*Leishmania major* SpSyn) (CAC44919) was amplified from *L. major* genomic DNA (see Table 1 for primers), and the resultant 903 bp fragment was digested with SbfI + AscI and then cloned into the corresponding sites of the *T. brucei* constitutive expression vector pTUB-EX [24]. Constructs were digested with NotI + XhoI and electroporated into the RNAi-TbSpSyn cell line and clones were selected. Comparison between *TbSpSyn* and *LmSpSyn* revealed that the two genes were 65% identical and had no conserved stretches > 20 bp. Therefore RNAi targeted at the *TbSpSyn* mRNA should not affect the *Leishmania* transcript.

Detection of the growth inhibition phenotype

BSF trypanosomes, transformed with the RNAi construct, were seeded at 1×10^5 cells · ml⁻¹ and incubated at 37 °C in the presence of tetracycline (1 μ g · ml⁻¹). Every 24 h, parasite growth was monitored microscopically and the culture was diluted back to 1×10^5 cells · ml⁻¹. Control cultures incubated in the absence of tetracycline were grown in parallel. Under these conditions, untreated cell lines grew with a doubling time of approx. 7-8 h.

Analysis of polyamines

BSF trypanosomes (5×10^7) were lysed in 10% (v/v) trichloroacetic acid and sonicated and the extract was clarified. Polyamines in the lysate were then derivatized as follows: supernatant (100 μ l) was incubated with 200 μ l saturated sodium carbonate and 400 μ l of dansyl chloride (10 mg · ml⁻¹ in acetone) (Sigma) at 60°C for 1 h in the dark. Excess dansyl chloride was removed by addition of 100 μ l of L-proline (100 mg · ml⁻¹) and incubated at 60°C for a further 30 min. Derivatized polyamines were then extracted into toluene and the organic phase was dried under argon. Pellets were resuspended in acetonitrile and analysed by HPLC.

Spermidine transport studies

[^{14}C]Spermidine trihydrochloride was supplied by GE Healthcare U.K. BSF *T. brucei* or *Trypanosoma cruzi* epimastigotes, in the exponential phase of growth, were pelleted, washed three times with assay buffer (90 mM Tris/HCl, pH 7.5, 3.1 mM KCl, 96.9 mM NaCl, 5 mM MgCl_2 , 2 mM Na_2HPO_4 and 2 mM glycerol) and then resuspended at 2×10^8 cells $\cdot \text{ml}^{-1}$. To initiate uptake, radiolabelled spermidine (0.5 μCi) was added to 50 μl of parasite suspension at a final concentration of 45 μM and the reaction was incubated at 22°C for an appropriate time. The assay was stopped by the addition of 200 μl of 4% (w/v) paraformaldehyde in PBS. The cells were pelleted, washed three times with 1 $\times$ PBS and then lysed in 250 μl of 2% (w/v) SDS in a scintillation vial. A 3 ml portion of scintillation fluid (Ecoscint A; National Diagnostics) was added to vials, which were incubated overnight. The incorporated radioactivity was then measured using a Beckman liquid-scintillation spectrometer. As a control, paraformaldehyde was added to trypanosomes before adding the radiolabelled spermidine. Each time point was performed in triplicate.

HPLC analyses

All HPLC equipment and software were from Dionex. Separations were carried out utilizing a Genesis AQ 4 μm column (150 mm $\times$ 4.6 mm; GraceVydac) by eluting with 20 mM ammonium formate (pH 2.7)/acetonitrile (15:85) passing through a fluorescence detector (Dionex model RF 2000), with a λ_{ex} of 340 nm and a λ_{em} of 510 nm, at a flow rate of 1.0 ml $\cdot \text{min}^{-1}$. Peak identity was confirmed by measuring the retention time and comparison with commercially available spermidine or putrescine. A calibration curve of spermidine or putrescine was generated with Chromeleon (Dionex software) by using known amounts of the standard (0-1000 μM) in acetonitrile.

RESULTS

Isolation of a *TbSpSyn* gene

A DNA sequence (GeneDB accession no. XM_822031) in the *T. brucei* database was identified as containing an ORF related to spermine/spermidine synthases (Pfam PF01564). Based on this, we amplified an 894 bp DNA fragment with potential to encode a 33 kDa protein (designated *TbSpSyn*). Pairwise alignments revealed that *TbSpSyn* had 39-43% sequence identity to SpSyns of mammalian, plant and fungal origin (Figure 2). The similarity extended across the entire length of the trypanosomal protein and included the PAPT (putrescine aminopropyltransferase) family signature motif [(V/A/I)-(V/A/L)-(L/I/V)-(L/I/V)-G-G-G-X-(G/C)-X-X-(L/I/V/A)-X-E] (Prosite PS01330).

Biochemical properties

To investigate the activity of the putative SpSyn, *TbSpSyn* was cloned into pTrcHis-C and expressed in *E. coli* (see the Experimental section). After induction with IPTG, a 37 kDa band was detected in the soluble fraction of bacterial extracts that could be readily purified by one round of affinity chromatography (Figure 3A). Enzyme activity was assayed by monitoring the production of spermidine from putrescine and dcSAM. After performing each reaction, the total polyamine content was derivatized with dansyl chloride and then analysed by HPLC coupled with a fluorescence spectrophotometer. Standard curves were generated by plotting different [spermidine] against the HPLC-derived area under the peak. From this, the polyamine levels in each assay could be determined. Initial reactions using 1 mM putrescine and 10 μM dcSAM in the presence of recombinant *TbSpSyn* demonstrated that the parasite enzyme could mediate the formation of spermidine. To investigate further these interactions, assays were carried out using various concentrations of putrescine (12.5-1000 μM) at a fixed concentration of dcSAM (10 μM). Double reciprocal plots of 1/

TbSpSyn activity against $1/[\text{putrescine}]$ were linear (Figure 3B) and extrapolation allowed the K_m (app) value for putrescine ($205 \pm 65 \mu\text{M}$) and $V_{\max}$ ($11.9 \pm 1.4 \text{ nmol of spermidine formed} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) to be calculated. To determine whether TbSpSyn activity could be saturated by dcSAM, reactions were carried out using a fixed concentration of putrescine ($1000 \mu\text{M}$) and variable concentrations of dcSAM ($0.025\text{--}10 \mu\text{M}$) (Figure 3C). Again, TbSpSyn demonstrated Michaelis–Menten kinetics with the parasite enzyme having a K_m (app) for dcSAM of $0.09 \pm 0.01 \mu\text{M}$.

Investigating the functional significance of TbSpSyn using RNAi

Using a tetracycline-inducible RNAi system, we set out to determine whether TbSpSyn was required for parasite viability. A DNA fragment (860 bp) was generated using PCR and cloned into p2T7^{Ti} [22], and the RNAi construct was transformed into BSF *T. brucei*. To examine whether down-regulation of *TbSpSyn* affected the growth of BSF *T. brucei*, the cumulative cell density of tetracycline-treated parasites was compared with untreated cultures (Figure 4A). In the absence of tetracycline, the RNAi cell lines grew at the same rate as parental cultures. Addition of tetracycline had a dramatic effect (Figure 4A). Within 72 h, there was a significant decrease in the growth rate, with a reduction in the endogenous *TbSpSyn* mRNA (Figure 4B) and a 60% decline in spermidine levels (Table 2). Over the next 48 h, the cell density remained static. Similar results were observed with two independent clones.

To demonstrate conclusively that the observed growth deficiency was due specifically to a reduction in the *TbSpSyn* transcript, a complementation strategy was used. This involved inserting the *LmSpSyn* gene into the tubulin locus of the *T. brucei* RNAi cell line using the constitutive expression vector pTUBEX [24]. In these experiments, the parasites induced to undergo down-regulation of *TbSpSyn* while expressing *LmSpSyn* grew at a rate comparable with non-induced controls (Figure 4A). RNA hybridization confirmed that the level of endogenous *TbSpSyn* had fallen and that expression of the complementing *LmSpSyn* mRNA was occurring (Figure 4B).

Spermidine uptake by BSF *T. brucei*

Attempts to complement the RNAi-mediated growth defect adding spermidine to the medium, an approach that has proved successful in analysing *Leishmania* polyamine biosynthesis-null mutants [25–28], failed. Interestingly, exogenous spermidine displayed significant trypanocidal activity at concentrations above $10 \mu\text{M}$ (results not shown). This had been previously noted and attributed to polyamine oxidase activity in the heat-inactivated fetal calf serum that is used to supplement the parasite growth medium [29,30]. At concentrations where no significant toxicity was observed ($<5 \mu\text{M}$), addition of spermidine to the medium failed to complement the RNAi-mediated growth defect, even though this level is >15 -fold higher than that found in blood plasma (300 nM) [31]. One possible explanation for this may be that *T. brucei* lack a high-affinity spermidine transporter related to that in *L. major* and *T. cruzi* [32,33]. To determine whether BSF *T. brucei* can scavenge spermidine from its environment, uptake assays were performed using radiolabelled polyamine (see Experimental section). In these experiments, *T. brucei* displayed no capacity to take up spermidine, even when assays were carried out for 1 h (Figure 5). In parallel assays, *T. cruzi* was shown to transport spermidine. Therefore BSF *T. brucei* is incapable of scavenging spermidine and, as such, must rely on *de novo* synthesis to acquire this polyamine.

DISCUSSION

Polyamines are ubiquitous in nature and play crucial roles in several biological processes. All eukaryotes and eubacteria have evolved uptake and/or biosynthetic strategies to acquire them and it has been proposed that inhibitors targeting these systems may be of importance in treating infectious diseases and certain cancers [3-7]. Here, we demonstrate that SpSyn in BSF *T. brucei* represents a valid target for chemotherapy.

To confirm that the genome database annotation was correct, we performed SpSyn assays using purified protein generated by expression in *E. coli*. These experiments demonstrated that the recombinant enzyme had K_m (app) values for putrescine and dcSAM of 205 and 0.09 μM respectively (Figure 3). An earlier study investigating the biochemical properties of TbSpSyn, partially purified from the parasite, noted that this enzyme functioned as a homodimer of 74 kDa, with K_m (app) values for putrescine and dcSAM similar to our data [18]. It was also observed that activity could be markedly reduced by dicyclohexylamine and cyclohexylamine, compounds that act as competitive inhibitors with respect to putrescine. In many respects, these biochemical properties are typical of PAPT's from other sources (see <http://www.brenda.uni-koeln.de>). For example, the K_m for putrescine of HsSpSyn (human SpSyn) is 80 μM [34]. However, TbSpSyn does appear to have a higher affinity for dcSAM compared with the human enzyme, with HsSpSyn exhibiting a K_m for dcSAM (7 μM) 70-fold higher than its trypanosomal counterpart [34]. This difference may represent a property exploitable in terms of inhibitor design.

Different trypanosomatid parasites use various mechanisms to acquire polyamines. *Leishmania*, for example, possess a polyamine biosynthetic pathway, plus putrescine/spermidine transporters [25-28,32], whereas *T. cruzi* contain a partial biosynthetic pathway and rely on uptake to meet their polyamine requirement [35-37]. To determine the importance of SpSyn to BSF *T. brucei* and investigate whether a biosynthetic and/or transporter mechanism is used to acquire spermidine, we used RNAi to down-regulate expression of *TbSpSyn* (Figures 4A and 4B). This experiment clearly demonstrated that TbSpSyn is essential for the growth of BSF *T. brucei* and that expression of the *LmSpSyn* gene in these cells can complement the resulting growth-deficient phenotype (Figure 4A). We were unable to complement the RNAi-mediated growth defect by addition of spermidine to the medium, suggesting that for the *T. brucei* subspecies used here, *T. brucei brucei*, scavenging alone does not fulfil the parasite's requirements for spermidine. One possible explanation may be the lack of a high-affinity putrescine/spermidine transporter related to that reported in *L. major* and *T. cruzi* [32,33]. Indeed, comparison of the syntenic regions of the trypanosomatid genomes failed to identify the gene encoding this carrier protein in the *T. brucei brucei* database. If this protein is absent, then, based on *ODC* gene deletion experiments, putrescine uptake must occur through other carrier(s) [38-40]. The absence of an effective spermidine permease in *T. brucei*, as demonstrated here (Figure 5), may reflect an adaptation to the host bloodstream environment where the plasma concentration of spermidine is low (~300 nM) compared with the levels detected in mammalian cells (mM) where intracellular parasites such as *Leishmania* and *T. cruzi* reside [31,41].

The regulation of the intracellular polyamine pools in many eukaryotes is accomplished by a series of complex regulatory and degradation pathways with disruption of these systems promoting unwanted side-effects such as uncontrolled cell proliferation or apoptosis [42,43]. Analysis of polyamine levels in trypanosomes undergoing RNAi-mediated depletion of TbSpSyn revealed that these cells had decreased spermidine content but, unexpectedly, there had been no significant build up of putrescine (Table 2). Overexpression studies of ODC, SpSyn and SAMdc in *Leishmania donovani* also showed that even when enzyme activities were >25-fold higher than wild-type, there was little change in the polyamine content [44].

This strongly implies that trypanosomatids have the ability to control metabolic flux through the polyamine biosynthetic pathway. Considering that the parasite enzymes are stable, compared with their mammalian counterparts, and that regulation of gene expression in trypanosomatids does not generally occur at the transcriptional level [15,45], the processes involved in this regulatory system may be unusual. Recently, a novel mechanism for regulating SAMdc activity in *T. brucei* has been reported [46,47]. An inactive version of SAMdc, known as the prozyme, forms a heterodimeric complex with the functional enzyme, which stimulates activity by > 3 orders of magnitude. It will be of interest to determine whether any other enzyme in the trypanosomatid polyamine biosynthetic pathway displays an unusual regulatory mechanism.

The success of DFMO, one of two drugs used to treat late-stage HAT, has already shown that compounds targeting polyamine synthesis can be used to treat this disease. The present study demonstrates that TbSpSyn, the enzyme immediately downstream of ODC in this pathway, is essential in BSF *T. brucei*. This, coupled with the parasite's inability to scavenge sufficient spermidine from its environment, validates TbSpSyn as a potential target for future drug development.

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Abbreviations used

BSF	bloodstream form
dcSAM	decarboxylated <i>S</i> -adenosylmethionine
DFMO	difluoromethylornithine
HAT	human African trypanosomiasis
SpSyn	spermidine synthase
HsSpSyn	human SpSyn
IPTG	isopropyl β -D-thiogalactoside
LmSpSyn	<i>Leishmania major</i> SpSyn
Ni-NTA	Ni ²⁺ -nitrilotriacetate
ODC	ornithine decarboxylase
ORF	open reading frame
PAPT	putrescine aminopropyltransferase
RNAi	RNA interference
SAMdc	<i>S</i> -adenosylmethionine decarboxylase
SMB	<i>Trypanosoma brucei</i> single marker cell line
TbSpSyn	<i>Trypanosoma brucei</i> spermidine synthase

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Figure 1. Polyamine biosynthetic pathway in trypanosomatids

In one arm of the pathway, putrescine is synthesized from arginine via ornithine by the enzymes arginase (1) and ODC (2), the latter being the target for the trypanocidal agent DFMO. In the other arm of the pathway, methionine is converted into SAM (S-adenosylmethionine) and on to dcSAM by the activities of SAM synthetase (3) and SAMdc (4) respectively. SpSyn (5) then catalyses the formation of spermidine from putrescine and dcSAM. In trypanosomatids, spermidine is required for biosynthesis of the unique thiol trypanothione.

Figure 3. Biochemical properties of TbSpSyn

(A) SDS/10% PAGE gel stained with Coomassie Brilliant Blue. Lane 1, crude extract loaded on to an Ni-NTA column and flow through (lane 2). The column was washed extensively with 10 mM imidazole (lane 3) and the recombinant protein eluted with 200 mM imidazole (lane 4). Markers (M) are given in kilodaltons. The 37 kDa band (indicated) corresponds to recombinant TbSpSyn. (B) Interaction of TbSpSyn with putrescine. TbSpSyn activity was monitored by following the production of spermidine from putrescine (12.5-1000 μM) and dcSAM (10 μM) by using 100 μg of TbSpSyn. (C) Interaction of TbSpSyn with dcSAM. TbSpSyn activity was monitored by following the production of

spermidine from putrescine ($1000\ \mu\text{M}$) and dcSAM ($0.05\text{--}10\ \mu\text{M}$) in the presence of $100\ \mu\text{g}$ of TbSpSyn. In (**B**, **C**), reactions were dansylated and spermidine levels were determined by HPLC coupled with a fluorescence detector. Activity is expressed as nmol of spermidine formed $\cdot\ \text{min}^{-1} \cdot\ \text{mg}^{-1}$.

Figure 4. Spermidine biosynthesis is essential for BSF *T. brucei*

(A) The growth of tetracycline-treated (+ tetracycline) and non-treated (- tetracycline) BSF *T. brucei* was followed in the parental (SMB) and RNAi cell lines. The growth defect could be complemented by expressing the *LmSpSyn* gene in the RNAi cell line (RNAi + Comp). The results are expressed as cumulative cell number $\cdot \text{ml}^{-1}$. (B) Down-regulation of gene expression by RNAi. Blots containing 15 μg of *T. brucei* total RNA from tetracycline-induced (96 h) (+) and non-induced (-) BSF cell lines were hybridized with radiolabelled probes as indicated. n.d., Not done. Equal loading was judged by ethidium bromide staining of rRNA.

Figure 5. BSF *T. brucei* lack a spermidine uptake capacity

BSF *T. brucei* or *T. cruzi* epimastigotes (2×10^8 parasites $\cdot$ ml⁻¹ per assay) were incubated with [¹⁴C]spermidine for the times indicated. Cells were prepared and uptake of the radiolabel was measured by scintillation counting (see the Experimental section). Results are the means $\pm$ S.D. for three experiments.

Table 1
Oligonucleotides used in the present study

The sequences in lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning. LmSpSyn, *Leishmania major* SpSyn

	Gene	Primer	Sequence (5'-3')
RNAi	TbSpSyn	TbSpSyn-1	<i>ggatcc</i> TGCTGACGGATGGTTTCGTGA
		TbSpSyn-2	<i>ctcgag</i> TGAGATGGCGGGCAAAGCGTG
Protein expression	TbSpSyn	TbSpSyn-3	<i>ggatcc</i> CTGGACCCGGTCTTCTTGCTGA
		TbSpSyn-4	<i>aagctt</i> TCAAGAATAGTTTGGTTGAG
Complementation	LmSpSyn	LmSpSyn-1	<i>cctgcagg</i> AGCGACTTGTCGATCAGATTT
		LmSpSyn-2	<i>ggcgcgcc</i> TCTCTACTCGTTCAGGTGAGC

Table 2
Polyamine levels in parasites undergoing RNAi

Parasites from three independent wild-type and tetracycline-treated (96 h) RNAi-TbSpSyn cultures were harvested, the polyamines extracted, derivatized with dansyl chloride and then analysed by HPLC, with each sample run in duplicate. The putrescine and spermidine levels were determined using standard curves generated in parallel. All samples were checked by TLC to determine derivative formation. Values are the means $\pm$ S.E.M.

Cell line	<u>[Polyamine] (nmol/10⁸ cells)</u>		
	Putrescine	Spermidine	Putrescine/spermidine ratio
Wild-type			
Culture 1	6.75 $\pm$ 0.15	10.44 $\pm$ 0.15	1.5
Culture 2	10.60 $\pm$ 0.80	17.90 $\pm$ 1.30	1.7
Culture 3	6.25 $\pm$ 0.55	9.45 $\pm$ 0.55	1.5
RNAi cell line			
Culture 1	9.20 $\pm$ 0.60	5.10 $\pm$ 0.30	0.6
Culture 2	12.45 $\pm$ 0.35	7.25 $\pm$ 0.35	0.6
Culture 3	5.25 $\pm$ 0.35	2.85 $\pm$ 0.25	0.5