Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes

Citation for published version:

Digital Object Identifier (DOI):
10.1073/pnas.1305404110

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the National Academy of Sciences

Publisher Rights Statement:
APC paid.
Freely available online through the PNAS open access option.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes

Samuel Dean1,2, Matthew K. Gould1, Caroline E. Dewar, and Achim C. Schnauper3

Institute for Immunology and Infection Research and Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom

Edited by Paul T. Englund, Johns Hopkins University, Baltimore, MD, and approved July 14, 2013 (received for review March 20, 2013)

Viability of the tsetse fly-transmitted African trypanosome Trypanosoma brucei depends on maintenance and expression of its kinetoplast (kDNA), the mitochondrial genome of this parasite and a putative target for veterinary and human antitrypanosomatid drugs. However, the closely related animal pathogens *T. evansi* and *T. equiperdum* are transmitted independently of tsetse flies and survive without a functional kinetoplast for reasons that have remained unclear. Here, we provide definitive evidence that single amino acid changes in the nuclearily encoded F1F0-ATPase subunit γ can compensate for complete physical loss of kDNA in these parasites. Our results provide insight into the molecular mechanism of compensation for kDNA loss by showing F0-independent generation of the mitochondrial membrane potential with increased dependence on the ATP/ADP carrier. Our findings also suggest that, in the pathogenic bloodstream stage of *T. brucei*, the huge and energetically demanding apparatus required for kDNA maintenance and expression serves the production of a single F1F0-ATPase subunit. These results have important implications for drug discovery and our understanding of the evolution of these parasites.

Author contributions: S.D., M.K.G., and A.C.S. designed research; S.D., M.K.G., C.E.D., and A.C.S. performed research; S.D., M.K.G., C.E.D., and A.C.S. analyzed data; and S.D., M.K.G., and A.C.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Frequently available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KF293288).

1S.D. and M.K.G. contributed equally to this work.

2Present address: Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom.

3To whom correspondence should be addressed. E-mail: achim.schnaufer@ed.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305404110/-/DCSupplemental.

viability | surra | dyskinetoplastic | RNA editing | mitochondrial DNA

Salivarian trypanosomes are extracellular protozoan parasites that cause important diseases in humans (human African trypanosomiasis) and their livestock (nagana). They predominantly infect the blood but, depending on the (sub)species, also other organs, such as the CNS. Transmission typically occurs through the saliva of blood-sucking insect vectors during feeding. The life cycle of African trypanosomes, such as Trypanosoma congolense, *T. brucei brucei*, *T. b. gambiense*, and *T. b. rhodesiense*, is fully dependent on cyclical development in the tsetse fly vector and highly complex (1). Thus, these parasites are restricted to areas inhabited by the tsetse fly (i.e., sub-Saharan Africa).

However, some pathogenic trypanosome species have adapted to efficient tsetse-independent transmission, abandoning any developmental stages associated with that vector, and therefore, they were able to escape from the African tsetse belt. *T. evansi* is transmitted mechanically by biting flies when the insect’s blood meal on an infected host is interrupted and a second host is bitten with trypanosome-contaminated mouth parts shortly thereafter (2). *T. evansi* infects various mammalian animals, including livestock, and it is the pathogenic trypanosome with the widest geographical distribution. The disease caused by this parasite, therefore, has many different names but is known as surra in large parts of Asia. The second species, *T. equiperdum*, causes a sexually transmitted horse disease called dourine and predominantly infects genital tissues (2). *T. evansi* and *T. equiperdum* are morphologically indistinguishable from each other and *T. b. brucei*, and their status as independent species has been questioned (3, 4).

Mitochondrial DNA (mtDNA) of trypanosomatids is organized as the kinetoplast (kDNA), a gigantic network of concatenated, circular DNA molecules (5). The second key feature distinguishing *T. evansi* and *T. equiperdum* from *T. b. brucei* is that they are dyskinetoplastic [DK; i.e., lacking all (akinetoplastic or δkinetoplastic) parts of their mitochondrial DNA (6)]. *T. brucei* kDNA contains two types of molecules. The ~23-kb maxicircle, present in 20–50 copies, contains a typical set of rRNA and protein-coding genes, most of which encode subunits of respiratory chain complexes (6). Most trypanosomatid mitochondrial mRNAs require a unique form of posttranscriptional editing before they can be translated into functional proteins (7). The second kDNA component is a highly diverse set of thousands of ~1-kb minicircles, which encode guide RNAs required for editing. Maintenance and expression of kDNA require numerous essential enzymes and have been suggested as a target for existing and novel drugs for *T. brucei* and other trypanosomatids (8). Indeed, antitrypanosomatid therapeutics, such as pentamidine and ethidium bromide, have been shown to directly affect kDNA (9, 10). *T. equiperdum* strains typically have retained their maxicircle—in some cases with substantial deletions—but have lost their minicircle diversity (4, 6). *T. evansi* strains do not have a maxicircle and either show minicircle homogeneity or are dyskinetoplastic. Consequently, both species are incapable of functional mitochondrial gene expression.

*T. evansi* and *T. equiperdum* cannot develop in the tsetse fly, probably because ATP production in that environment requires oxidative phosphorylation (11) and, therefore, the capacity to express numerous mitochondrial genes. They can only survive as bloodstream forms (BFs), which produce ATP exclusively through glycolysis; however, they still require a mitochondrion, because it hosts other essential activities (12–14). A key process underpinning mitochondrial function is the maintenance of an electrochemical potential, Δψm, across the inner mitochondrial membrane (15). BF *T. brucei*, which lacks the proton pumping respiratory complexes III and IV, generates Δψm using the mitochondrial F0F1-ATP synthase complex functioning in reverse to pump protons from the matrix into the intermembrane space (16–18). Subunit a of the membrane-embedded F0 part is critical for proton translocation (Fig. S14) and kDNA-encoded, and its pre-mRNA requires substantial RNA editing (19). DK trypanosomes are incapable of expressing subunit a, because they lack either the gene itself or most, if not all, guide RNAs. The puzzling fact that these organisms are viable was hypothesized to involve compensatory mutations in the nuclearly encoded F1 subunit γ (Fig. S1 B–D) that enable F0-independent Δψm generation (4, 17).

We tested this hypothesis by generating BF *T. b. brucei* that express mutated γ subunits and investigating their response to kDNA loss. Our results show that a single amino acid change in subunit γ fully compensates for complete loss of kDNA and provide insight into the molecular mechanism of this compensation. This finding has important consequences for our understanding of...
the mitochondrial function in these organisms, their evolution, and the suitability of kDNA as a drug target.

Results

We first investigated the significance of the F₁γ L262P mutation identified in the T. b. brucei 164DK cell line (17). These cells had lost their kinetoplast after several months of in vivo selection with aclarivaine (Acr), a DNA-intercalating compound (20) (Table 1 lists the mutations investigated, and Table S1 lists the trypanosome cell lines and strains used in this study). We first determined whether this mutation confers Acr resistance in a standard 3-d drug sensitivity assay (21). We combined introduction of a single ectopic subunit γ allele bearing the L262P mutation (γL262P) into T. b. brucei 427 with a single (sKO) or double KO (dKO) of the endogenous γ gene, resulting in cell lines sKO + γL262P and dKO + γL262P. Cell lines expressing an ectopic WT allele (γWT) were generated as controls. We then compared the Acr sensitivity of these cell lines with parental T. b. brucei 427, T. evansi Antat 3/3 (which has the A281del mutation) (Table 1), and T. b. brucei 164DK (the source of the L262P mutation).

For T. b. brucei 164DK, the average EC₅₀ value for Acr was sevenfold greater than for the T. b. brucei 427 WT strain (Fig. L4). T. evansi Antat 3/3 had an intermediate EC₅₀ value. Strains expressing γL262P showed considerable resistance, similar to the resistance level of the 164DK cell line, in which this mutation had originally been identified. Although the EC₅₀ for sKO cells was slightly lower than for dKO cells, this result was not statistically significant (P value = 0.062; unpaired two-tailed Student t test). In contrast, cells expressing the ectopic γWT retained the 427 parental sensitivity. These results show that the L262P mutation is sufficient to confer a level of Acr resistance that is similar to the one observed for T. b. brucei 164DK.

Next, we investigated if F₁γ mutations identified in DK trypanosomes (Table 1) enable long-term survival of parasites in the presence of 20 nM Acr, a concentration well above the 3-d EC₅₀ for WT cells. Trypanosomes expressing only T. equiperdum were very similar to those observed for the 164DK cell line, in which this mutation was originally identified (Fig. 1B and Fig. S2B); in contrast, the γA273P-expressing cells continued to grow like γL262P-expressing cells (Fig. 1B). The result for γA281del-expressing cells (the mutation present in the vast majority of field isolates; see Table 1) was more complex. Although some clones died as quickly as the negative controls, other clones persisted, and a small number of live cells were still observed after 7 d (Fig. 1B and Fig. S2B, asterisks). After transfer to Acr-free medium, these cells recovered, and when reexposed to Acr, no lag in growth was observed (Fig. 1E), suggesting that they had undergone a secondary adaption. Like the γL262P-expressing cells, all cell lines that had survived Acr treatment were kDNA⁺. Expression of functional F₁γ-ATPase was broadly equivalent (Fig. S3), ruling out variations in expression level as an explanation for the differing Acr sensitivities. The antibody that we used detects the 15 kDa N-terminal β-barrel domain of the α subunit, which in trypanosomatids, is proteolytically cleaved off from the rest of the polypeptide but remains associated with the F₁ complex (22). Interestingly, the β-barrel domain seems to have been lost or altered in T. evansi Antat 3/3, because here, the 15 kDa band was absent (Fig. S3A, lane 8). Whether this absence is a general feature of the T. evansi and T. equiperdum F₁-ATPase and if it is of functional significance remains to be investigated. Loss of the domain is not a direct consequence of kDNA loss, because the dKO + γL262P cells retained the domain after Acr treatment (Fig. S3A, compare lane 3 with lane 4). Growth rates for sKO or dKO cells expressing a mutated γ subunit in the absence of Acr were very similar to WT (Fig. S2 A and B), suggesting that, under standard culture conditions and within the time frame observed, the mutated subunits were fully functional. The γA281del-expressers seemed to not be adapted as well to life without kDNA as cells expressing γL262P or γA273P, which was indicated by the slightly reduced growth rate in Acr-free medium (Fig. S4).

Table 1. ATPase subunit γ sequence variations tested in this study

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Source (origin, host, year of isolation)</th>
<th>Source for mutation; isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L262P</td>
<td>T. b. brucei 164DK (USA, mouse, 1971)</td>
<td>17; 20</td>
</tr>
<tr>
<td>CTT→CCT</td>
<td>T. equiperdum BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 3</td>
</tr>
<tr>
<td>A273P</td>
<td>T. equiperdum BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 3</td>
</tr>
<tr>
<td>M282L</td>
<td>T. equiperdum ATCC30019 (France, horse, 1903)</td>
<td>4; 3</td>
</tr>
<tr>
<td>A281del</td>
<td>T. equiperdum ATCC30023 (France, horse, 1903)</td>
<td>4; 3</td>
</tr>
<tr>
<td>TCTGCTATG→TCT—ATG</td>
<td>T. equiperdum ATCC30019 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td>T. evansi Antat 3/3 (South America, capybara, 1969)</td>
<td>This work; 51</td>
<td></td>
</tr>
<tr>
<td>T. evansi C13 (Kenya, camel, 1982)</td>
<td>This work; 51</td>
<td></td>
</tr>
<tr>
<td>T. evansi CPOgz1 (China, water buffalo, 2005)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi E110 (Brazil, capybara, 1985)</td>
<td>This work; 44</td>
<td></td>
</tr>
<tr>
<td>T. evansi E9/CO (Columbia, horse, 1973)</td>
<td>This work; 51</td>
<td></td>
</tr>
<tr>
<td>T. evansi SS143M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi SS143M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi STIB805 (China, water buffalo, 1985)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi STIB807 (China, water buffalo, 1979)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi STIB810 (China, water buffalo, 1985)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi Stock Kazakh (Kazakhstan, camel, 1995)</td>
<td>This work; 4</td>
<td></td>
</tr>
<tr>
<td>T. evansi KETR12479 (Kenya, camel, 1981)</td>
<td>This work; 4</td>
<td></td>
</tr>
</tbody>
</table>

In all strains investigated, the L262P and A273P mutations are homozygous, whereas the A281del and M282L mutations are heterozygous.

Dramatically different Acr sensitivities were observed for T. b. brucei expressing F₁γ with mutations identified in field isolates of T. evansi and T. equiperdum from various geographical areas (Table 1). Cells expressing γM282L behaved like WT and were dead by 72 h (Fig. 1B and Fig. S2B); in contrast, the γA273P-expressing cells continued to grow like γL262P-expressing cells (Fig. 1B). The result for γA281del-expressing cells (the mutation present in the vast majority of field isolates; see Table 1) was more complex. Although some clones died as quickly as the negative controls, other clones persisted, and a small number of live cells were still observed after 7 d (Fig. 1B and Fig. S2B, asterisks). After transfer to Acr-free medium, these cells recovered, and when reexposed to Acr, no lag in growth was observed (Fig. 1E), suggesting that they had undergone a secondary adaption. Like the γL262P-expressing cells, all cell lines that had survived Acr treatment were kDNA⁺. Expression of functional F₁γ-ATPase was broadly equivalent (Fig. S3), ruling out variations in expression level as an explanation for the differing Acr sensitivities. The antibody that we used detects the 15 kDa N-terminal β-barrel domain of the α subunit, which in trypanosomatids, is proteolytically cleaved off from the rest of the polypeptide but remains associated with the F₁ complex (22). Interestingly, the β-barrel domain seems to have been lost or altered in T. evansi Antat 3/3, because here, the 15 kDa band was absent (Fig. S3A, lane 8). Whether this absence is a general feature of the T. evansi and T. equiperdum F₁-ATPase and if it is of functional significance remain to be investigated. Loss of the domain is not a direct consequence of kDNA loss, because the dKO + γL262P cells retained the domain after Acr treatment (Fig. S3A, compare lane 3 with lane 4). Growth rates for sKO or dKO cells expressing a mutated γ subunit in the absence of Acr were very similar to WT (Fig. S2 A and B), suggesting that, under standard culture conditions and within the time frame observed, the mutated subunits were fully functional. The γA281del-expressers seemed to not be adapted as well to life without kDNA as cells expressing γL262P or γA273P, which was indicated by the slightly reduced growth rate in Acr-free medium (Fig. S4).
These results suggested that strains of *T. evansi* and *T. equiperdum* with an A281del or A273P mutation depend on these mutations to be viable. To test this suggestion further, we inductively expressed either γWT or γL262P in *T. evansi* Antat 3/3 (these parasites are heterogeneous for the A281del mutation; see Table 1). Expression of γWT caused a strong growth defect after 48 h, whereas expression of γL262P had no effect (Fig. 2A), suggesting that replacement of sufficient endogenous (A281del-mutant) γ subunits in the ATP synthase complex with WT subunits severely impairs the viability of these DK cells. This observation also suggested that the L262P mutation from the laboratory-induced DK strain of *T. b. brucei* and the A281del mutation present in *T. evansi* Antat 3/3 are, at least to some extent, functionally interchangeable. In another experiment, we inductively expressed of an ectopic γL262P allele in *T. b. brucei* 427 cells and then treated the cells with 20 nM Acr. As predicted, these cells were resistant to Acr and rapidly lost their kinetoplast. Subsequent repression of the γL262P allele forced these DK cells to rely on endogenous γWT alone and caused a severe growth defect after 24 h (Fig. 2B). In contrast, the growth rate of cells with maintained expression of γL262P remained constant. These results confirm that, after expression of a mutated F_{γT} subunit has permitted loss of kDNA, the DK trypanosomes now depend on continued expression of this mutated subunit to remain viable. Because specific F_{γ} mutations are able to compensate for kDNA loss, all genes exclusively involved in kDNA biogenesis or expression would be predicted to become dispensable. RNA editing ligase 1 (REL1) is essential in the kinetoplast mRNA editing process and its knockdown lethal (23). To determine whether the L262P mutation can compensate for REL1 loss, the γL262P gene or the γWT control was constitutively expressed in *T. b. brucei* 427 engineered for inducible REL1 RNAi. Contrasting with γWT cells, γL262P-expressing trypanosomes showed no growth effect after RNAi-mediated knockdown of REL1 (Fig. 3). Interestingly, γL262P-expressing *T. brucei* was not viable after ablation of *Top2mt* expression (Fig. S5B), a protein essential for kDNA replication (24). To validate this observation, we ablated *Top2mt* expression in *T. evansi* Antat 3/3 and observed a growth defect after 48 h (Fig. S5A). Secondary effects resulting from faulty kDNA replication (for example, potentially toxic accumulation of kDNA) are unlikely to be responsible, because the Antat 3/3 strain used for this study is kDNA− (Fig. S5B) (17), probably as a result of long-term in vitro culture (25). The most likely explanation for the growth defect in *T. evansi* is, therefore, that *Top2mt* has an additional important function outside of its role in kDNA replication. Nevertheless, together with the experiments investigating chemically induced kDNA loss, these genetic data show that specific point mutations of ATPase γ are fully sufficient to compensate for loss of kDNA or its gene expression.

The proton-translocating function of the membrane-embedded F_{0} part of the ATP synthase involves subunit a, which is thought to be the only ATPase subunit encoded in the kinetoplast (Fig. S1A). To test directly whether a mutated ATPase γ is necessary for generating Δψm in kDNA− cells, we measured Δψm in BF trypanosomes over the course of Acr treatment. For cells expressing only γWT, Δψm decreased after 24 h and was completely abolished after 48 h (Fig. 4A), preceding cell death by at least 24 h. In contrast, Δψm of the γL262P-expressing trypanosomes was not affected by Acr-induced kDNA loss (Fig. 4B). The slight decrease in Δψm during Acr exposure is probably the result of kDNA-independent Acr toxicity, because no difference in Δψm could be observed after removal of Acr from the medium (Fig. S6). The current model for Δψm generation in DK trypanosomes proposes that increased ATP hydrolysis by a mutated F_{1} part facilitates the electrogenic exchange of cytosolic ATP− for mitochondrial ATP− by the ADP/ATP carrier (AAC) (17, 26). We measured sensitivity of our cell lines to oligomycin, an inhibitor of the coupled F_{0}F_{1}-ATPase, and the AAC inhibitor bongkrekic acid. Trypanosomes expressing mutant ATPase γ showed similar levels of oligomycin resistance before and after Acr-induced kDNA loss.
activities, even in kDNA by the AAC (17, and mutations (32), and the effects of cells. In support of the T. evansi mutations on F γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation. The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.

Discussion

The apparently conflicting observations that BF T. brucei depends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum T. equiperdum ΔΨ for γ for ATP was also suggested as part of mitochondria for yeast and the trypanosome O γ for ΔΨ generation.
likely that isolates sharing the same mutation also share the same ancestor. Importantly, the largest group, characterized by the A281del mutation, contains isolates from Africa, Asia, and South America collected during a span of 100 y, and it contains both T. evansi and T. equiperdum isolates. Either these T. evansi isolates are T. evansi isolates that had been misclassified (3) or many isolates of these two species are descendants of the same evolutionary event (43, 44). Based on these considerations, we would propose that, contrary to what was proposed recently (4), the extant strains seem to be the result of a limited rather than large number of independent evolutionary events. It has been suggested that T. evansi/T. equiperdum are analogous to yeast petite mutants (4, 38), and, indeed, the mechanism that allows mtDNA loss in petite-negative yeast and T. brucei is strikingly similar (17). However, it is important to stress that the ability to survive without a mitochondrial genome is only one distinctive feature of these parasites, because the other one, efficient tsetse-independent transmission, has had such dramatic epidemiological consequences.

Maintenance and expression of kDNA has been suggested as a target for existing and novel antitrypanosomatid drugs (8–10). In fact, the Acr compound used for kDNA elimination in the present study was originally developed with the aim of finding a cure for sleeping sickness (45). The fact that single nucleotide changes can make the parasite completely independent of kDNA and its expression suggests that this target needs to be treated with caution, at least for T. brucei. However, a few considerations are important. First, an uncoupled F_0F_1 enzyme will be incapable of proton gradient-driven ATP synthesis, which is thought to be required for survival in the tsetse vector because of the low abundance of glucose in the insect’s midgut (11, 46). The mutation would, consequently be expected to lock the parasite in the mammalian host and prevent spread of resistant parasites. Second, other pathogenic trypanosomatids, including T. congolense, T. cruzi, and Leishmania spp., do not seem to be able to circumvent the need for functional kDNA, which therefore, remains a highly promising target in those parasites.

Another important conclusion from our study is that ATP synthase subunit a seems to be the sole kDNA product ultimately required for viability of BF T. brucei. A subunit of the mitochondrial ribosome, RPS12, may be encoded in kDNA (47), and it has been reported that the product of an alternatively edited mRNA, AEP-1, is required for kDNA maintenance (48); however, these proteins would also become dispensable, along with subunit a, in the presence of a compensatory ATP synthase γ-mutation. The same is true for the numerous nuclearly encoded proteins required exclusively for maintenance and expression of kDNA (5, 7, 49). Thus, a single amino acid mutation in BF T. brucei makes a large number of otherwise essential proteins redundant. The compensatory mutations reported in this paper offer an attractive tool for their identification and characterization.

Materials and Methods

Materials, Trypanosome Strains, and Culture Conditions. Details on materials can be found in SI Materials and Methods. All experimental work and culturing were carried out with BF trypanosomes only. ATP synthase γ (Tb927.10.180) genetic manipulations in T. b. brucei were conducted on the Lister 427 strain, except for inducible expression and RNAi experiments, which used the 427 single marker cell line (50). Inducible expression in T. evansi was conducted in a cell line expressing T7RNAS and TETR (17). Cell lines T. evansi Antat 3/3 (51) and T. b. brucei DK 164 (20) were included in growth experiments for comparison. SI Materials and Methods has details on culturing, plasmid construction, and transfection. See Table S2 for oligonucleotides.

Alamar Blue Dose–Response Assay. The Alamar Blue assay was performed essentially as described (21) with minor modifications. Briefly, test compound was doubly diluted in 100 μL Hirumi-modified Iscove’s medium 9/10% (vol/vol) FBS in a 96-well plate; an equal volume of medium containing BF trypanosomes was added to give a final density of 5 x 10^7 cells/mL. The plate was incubated at 37 °C and 5% (vol/vol) CO_2 for 72 h, after which 20 μL 0.5 mM resazurin sodium salt in PBS were added to each well; then, the plate was incubated for another 4 h. Fluorescence was measured in a plate reader with excitation and emission filters of 544 and 590 nm, respectively, EC_{50}.
values were derived from a variable slope (four parameter) nonlinear regression using Prism 5 software (GraphPad).

**DAPI Staining.** Trypanosomes were washed in PBS, settled onto poly-L-lysine coated slides, and fixed in 3% (wt/vol) formaldehyde for 10 min before treating with excess cold methanol for at least 30 min. Slides were then rehydrated in PBS and mounted in glycerol containing 1 μg/mL DAPI before imaging using a Leica SP5WS2 confocal laser microscope (blue diode laser at 405-nm wavelength) with Volocity version 5.2 image analysis software (PerkinElmer).

**PCR and Western Analyses.** Details are given in SI Materials and Methods.

**Δψ Measurements.** Samples of trypanosome cultures, either exposed or unexposed to Acr, were incubated with 260 μM rhodamine 123 (Rh123) for 20 min at 37 °C. Cells were harvested by centrifugation at 2,500×g for 10 min and washed one time with 25 mM Heps, pH 7.6, 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 2 mM EDTA, 5 mM MgCl₂, and 6 mM β-Glucose. Fluorescence caused by Rh123 uptake was measured using a FACSCalibur flow cytometer with CellQuest Pro software (Becton Dickinson). Baseline fluorescence was established for cultures preincubating an aliquot of cells with 10 μM FCCP before adding Rh123; the FCCP concentration was maintained throughout the wash and flow cytometer steps.

**ACKNOWLEDGMENTS.** The authors thank Wendy Gibson, Julius Lukel, and Ken Stuart for sharing parasite DNA or strains; Keith Gull and Ken Stuart for antibodies; Sinclair Cooper for help with minicircle annotation; and Marilyn Parsons and Keith Matthews for helpful comments on the manuscript. This work was funded by Medical Research Council Grant G0600129 (to A.C.S.) and National Institutes of Health Grant R01AI69057 (to A.C.S.).