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 (PNAS)

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.
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 nuclearly 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 F₁ independent generation of the mitochondrial membrane potential with increased dependence on the ADP/ATP 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.
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 acriflavine (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 ECSO value for Acr was sevenfold greater than for the *T. b. brucei* 427 WT strain (Fig. S2). Interestingly, the *T. equiperdum* and *T. evansi* 164DK (the source of the L262P mutation) were broadly equivalent (Fig. S3), suggesting that they had undergone a secondary adaption. Like the γL262P-expressing cells, all cell lines that had survived Acr treatment were kDNA+ (Table 1). 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. S34, lane 8). Whether this absence is a general feature of the *T. evansi* and *T. equiperdum* F1γ-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, 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><em>T. b. brucei</em> 164DK (USA, mouse, 1971)</td>
<td>17; 20</td>
</tr>
<tr>
<td>CTT→CCT</td>
<td><em>T. equiperdum</em> BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 3</td>
</tr>
<tr>
<td>A273P</td>
<td><em>T. equiperdum</em> STIB8784 (unknown)</td>
<td>4</td>
</tr>
<tr>
<td>CGG→CG</td>
<td><em>T. equiperdum</em> STIB842 (unknown)</td>
<td>4</td>
</tr>
<tr>
<td>A281del</td>
<td><em>T. equiperdum</em> ATCC30019 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td>TCTGCTATG→TCT—ATG</td>
<td><em>T. equiperdum</em> ATCC30023 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. equiperdum</em> STIB8818 (China, horse, 1979)</td>
<td>4; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> Antat 3/3 (South America, capybara, 1969)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> C13 (Kenya, camel, 1982)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> CPOgz1 (China, water buffalo, 2005)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> E110 (Brazil, capybara, 1985)</td>
<td>This work; 44</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> E9/CO (Columbia, horse, 1973)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> SS143M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> S573M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB8805 (China, water buffalo, 1985)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB8807 (China, water buffalo, 1979)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB8810 (China, water buffalo, 1985)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> Stock Kazakh (Kazakhstan, camel, 1995)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> KETRI2479 (Kenya, camel, 1981)</td>
<td>4; 51</td>
</tr>
</tbody>
</table>

In all strains investigated, the L262P and A273P mutations are homozygous, whereas the A281del and M282L mutations are heterozygous.
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 inducibly expressed either γWT or γL262P in *T. evansi* Antat 3/3 (these parasites are heterozygous 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 induced expression of an ectopic L262P 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 Fγ subunit has permitted loss of kDNA, the DK trypanosomes now depend on continued expression of this mutated subunit to remain viable. Because these specific Fγ 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 knockout 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. evansi* was not viable after ablation of TbTop2mt expression, a protein essential for kDNA replication (24). To validate this observation, we ablated TbTop2mt 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 TbTop2mt 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 F0 part of the ATP synthase involves subunit α, 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 ΔΨ 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 F1 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 F1F0-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 T. brucei as well as certain shows an increased importance for the role T. equiperdum parts of the enzyme and increased dependence for ATP was also suggested as part of the reaction mechanism that allows this particular T. evansi strain to survive but by itself, be insufficient, or it could be of no significance. The strain in question is a Kenyan isolate and representative of the rare type B strains (30) (Table 1).

What is the biochemical mechanism that enables BF trypanosomes to generate a \(\Delta \Psi m\) in the absence of a functional \(F_0\) part? Our study shows that introduction of an \(F_1\) L262P mutation results in oligomycin resistance and, consequently, provides a functional \(F_0\) enzyme. This scenario is reminiscent of \(mgi\) mutations in yeast (Fig. S1 B–D), although in that organism, it is not clear whether uncoupling is directly linked to viability without a mitochondrial genome (31). A reduced \(K_m\) for ATP was also suggested as part of the mechanism for yeast \(mgi\) mutations (32), and the effects of the trypanosome \(\gamma\) mutations on \(F_1\)-ATPase kinetics remain to be investigated. Both mechanisms could result in increased ATPase activity and be necessary to provide sufficient ADP for an efficient electrogenic exchange with ATP by the AAC (17, 26, 33). Our finding of bongkrekic acid hypersensitivity in cells expressing the mutant \(\gamma\) shows an increased importance for the role of the AAC in these cells. There is evidence in yeast and Leishmania for an AAC/F_0-ATPase supercomplex (33, 34), which might explain the apparent preference for the ATPase as the source of ADP in mtDNA cells. Such a supercomplex might generate localized zones of increased \(\Delta \Psi m\) and, in association with the protein import machinery, exploit them for protein import, similar to the supercomplex of Tim21 and respiratory complexes III and IV reported for yeast (35). A number of protists, including many important parasites, lack classical mitochondria and instead, harbor related organelles classified as hydrogenosomes or mitosomes that usually lack an organellar genome. How these organelles generate a \(\Delta \Psi m\) is unclear (36, 37), and trypanosomes promise to be an excellent experimental system to further investigate this important problem in cell biology.

How did T. evansi and T. equiperdum evolve from T. brucei, and can the findings reported here help to address this question?

**Fig. 3.** The L262P mutation allows BF T. b. brucei to survive inhibition of mitochondrial gene expression. (A) Cumulative growth of T. b. brucei after RNAi-mediated knockdown of REL1 (dashed lines). Cells expressed either \(\gamma\)WT (open circles) or \(\gamma\)L262P (closed squares). RNAi was induced with 1 \(\mu\)M Tet; uninduced control cultures are shown as solid lines. (B) Western analysis of REL1 protein expression from whole-cell lysates taken at the 72-h time point in A; \(\alpha\)-tubulin was used as a loading control (Lower).
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 antitrypanosomal 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$_1$F$_0$ 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 these parasites.

Another important conclusion from our study is that ATP synthase subunit α seems to be the sole kDNA product ultimately required for viability of *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 α, in the presence of a compensatory ATP synthase γ-mutation. The same is true for the numerous nucleurally 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 (Trb927.10.180) 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 T7RNAP 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 Hiromi-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 × 10$^5$ 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$_{50}$/μM.

The correlation between loss of a functional kinetoplast and an oligomycin-sensitive F$_1$F$_0$-ATPase on the one hand and loss of fly transmissibility on the other hand was first recognized by Opperdoes et al. (38). The temporal order of the two critical events in the evolutionary history of these parasites—acquisition of a propensity for efficient tsetse-independent transmission (the basis of which is still mysterious) and occurrence of compensatory changes that allowed kDNA loss—has been debated elsewhere (39–42). However, regardless of whether the γ subunit mutation occurred before or after adaptation to nontsetse transmission, it is evident that it was a key event and, thus a valuable marker for tracing that history. Each mutation in Table 1 is specifically correlated with other genetic polymorphisms (42), and it is, therefore,
EDV 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) formaldehde 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 SPWS2C 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.

LV 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 1,300 × 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, 0.2 mM EDTA, 5 mM MgCl₂, and 6 mM ß-glucose. Fluorescence caused by Rh123 uptake was measured using a FACSCalibur flow cytometer with CellQuest Pro software (Becton Dickinson). Baseline fluorescence was established for each sample before acquiring 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 antibodes; Sinclair Cooper for help with micrincle 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.).