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The assembly of F_1F_O-ATP synthase is disrupted upon interference of RNA editing in *Trypanosoma brucei*

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ABSTRACT

Throughout eukaryotes, the gene encoding subunit 6 (ATP6) of the F_1F_O-ATP synthase (complex V) is maintained in mitochondrial (mt) genomes, presumably because of its high hydrophobicity due to its incorporation into the membrane-bound F_O moiety. In *Trypanosoma* species, a mt transcript that undergoes extensive processing by RNA editing has a very low sequence similarity to ATP6 from other organisms. The notion that the putative ATP6 subunit is assembled into the F_O sub-complex is ostensibly challenged by the existence of naturally occurring dyskinetoplastic (Dk) and akinetoplastid (Ak) trypanosomes, which are viable despite lacking the mtDNA required for its expression. Taking advantage of the different phenotypes between RNA interference knock-down cell lines in which the expression of proteins involved in mtRNA metabolism and editing can be silenced, we provide support for the view that ATP6 is encoded in the mt genome of *Trypanosoma* species and that it is incorporated into complex V. The reduction of the F_1F_O oligomer of complex V coincides with the accumulation of the F_1 moiety in ATP6-lacking cells, which also appear to lack the F_O ATP9 multimeric ring. The oligomycin sensitivity of ATPase activity of complex V in ATP6-lacking cells is reduced, reflecting the insensitivity of the Dk and Ak cells to this drug. In addition, the F_1 moiety of complex V appears to exist as a dimer in steady state conditions and contains the ATP4 subunit traditionally assigned to the F_O sub-complex.

1. Introduction

Trypanosoma brucei is a pathogen with a broad impact on the inhabitants of sub-Saharan Africa, sub-species of which are the causative agents of human sleeping sickness and ruminant nagana. The haemoflagellate switches between the tsetse fly vector and mammalian host, where it exists in the procyclic and bloodstream stages (PS and BS, respectively), respectively, and has to adapt to the different environments of the insect midgut and mammalian bloodstream. During its life cycle the parasite undergoes substantial physiological and morphological changes (Matthews, 2005), the transformation of its single mitochondrion belonging to the most dramatic of those (Vickerman, 1985). The mitochondrion in the BS is considerably reduced and appears to lack cristae (Schneider, 2001; Hannaert et al., 2003). This morphological difference, in addition to the absence of cytochrome-containing respiratory complexes III and IV, reflects the exclusivity of glycolysis in the energy metabolism of *T. brucei* in the glucose-rich bloodstream of the mammalian host. In contrast, the PS contains a fully developed mitochondrion, appearing as a reticulated structure meandering throughout the cytoplasm, with the participation of complexes III and IV in the generation of ATP via oxidative phosphorylation (Besteiro et al., 2005).

Although the BS has a reduced mitochondrion, it is hardly a dormant organelle, as it still requires the expression of the mitochondrial (mt) (kinetoplast) DNA (kDNA), consisting of thousands of interlocked minicircles and dozens of maxicircles (Shlomai, 2004; Liu et al., 2005). The latter molecules are the equivalents of classical mtDNA, containing genes required for the organelle's biogenesis, most of which encode subunits of the respiratory complexes. The expression of the majority of these genes is not as straightforward as in other eukaryotes however, as their maturation requires extensive RNA editing in the form of post-transcriptional insertion and/or deletion of uridines (U) in pre-ordained positions within the mRNA sequence. A diverse population of minicircles encode small guide (g) RNAs, which direct the enzymatic machinery encompassed by the editosome protein complex to properly edit the mRNAs (Lukeš et al., 2005; Stuart et al., 2005).

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Trypanosomes maintain the mt membrane potential ($\Delta\Psi$m) required for the import of essential proteins (Nolan and Voorheis, 1992; Bertrand and Hajduk, 2000) and Ca^{2+} (Vercesi et al., 1992). Typically, eukaryotes rely on respiratory complexes I, III and IV to pump protons outside of the mt matrix to generate $\Delta\Psi$m, and the latter two complexes indeed have such a role in the PS (Horváth et al., 2005). However, noteworthy experiments have shown that in the BS it is the F_OF_1-ATP synthase (complex V) that adopts the role of sustaining $\Delta\Psi$m (Vercesi et al., 1992; Schnaufer et al., 2005; Brown et al., 2006).

In a typical mitochondrion, complex V acts as a dynamo, driven by the flux of protons down the electrochemical gradient inherent in $\Delta\Psi$m, which is coupled to the production of ATP. Its mechanism of action can be explained by exploring the remarkably conserved structure of complex V. An extensive compositional study of the F_OF_1-ATP synthase in *T. brucei* (Zíková et al., 2009) has demonstrated the presence of most of the subunits comprising the core structure of the complex in bacteria, archea and eukaryotes (Boyer, 1997; Velours and Arselin, 2000), as well as several proteins that are unique to kinetoplastids. Complex V is composed of two parts: the hydrophobic F_O moiety embeds the complex into the mt inner membrane, while the F_1 sub-complex extends into the mt matrix. The F_O part is composed of one ATP6 subunit (subunit *a* in the mammalian nomenclature), one or two ATP4 (*b*) subunits and a ring of 10–15 ATP9 (*c*) subunits. Interaction between the F_O ATP4 and F_1 oligomycin sensitivity-conferring protein (OSCP) subunits forms the peripheral stalk, which is believed to comprise part of a stator running parallel to the central stalk (Mulkidjanian et al., 2007). The action of complex V is reversed in the BS, in which ATP is consumed in order to pump protons out of the matrix (Schnaufer et al., 2005; Brown et al., 2006).

The essential ATP6 subunit is retained in virtually all mt genomes, presumably due to its extreme hydrophobicity (Funes et al., 2002). In *T. brucei*, a pan-edited mt transcript, requiring information from an estimated 21 gRNAs for the insertion of 448 Us and deletion of 28 Us to render a translatable open reading frame (ORF), has been hypothesised to encode ATP6 (Bhat et al., 1990; Corell et al., 1993). Such an assignment was supported by analysis of the in silico translated product, revealing a similar hydropathy profile and low sequence similarity of the C-terminus to ATP6 orthologues from other species (Bhat et al., 1990). However, conclusive evidence for this speculative designation is missing (Stuart et al., 1997).

The notion that this pan-edited mRNA encodes the ATP6 subunit, and that it is assembled into the F_O moiety is, however, further complicated by the existence of natural and laboratory-induced dyskinetoplastic (Dk) and akinetoplastic (Ak) trypanosomes, which are viable despite a partial and complete loss of kDNA, respectively (Schnaufer et al., 2002). These cells are locked in the BS because they lack the mitochondrial-encoded subunits of the cytochrome-containing complexes III and IV, and are viable despite the absence of the ATP6 gene (Schnaufer et al., 2002). Yet RNA editing is essential in the BS of *T. brucei* (Schnaufer et al., 2001), suggesting that the product of ATP6 is not entirely dispensable. Furthermore, the consumption of ATP by the F_1 moiety of complex V is required for maintaining $\Delta\Psi$m, which is considered indispensable for the survival of any trypanosome, including the Dk and Ak strains (Schnaufer et al., 2005). One interpretation of these findings is that the protein product of the edited mRNA putatively assigned as ATP6 in the mt genome of *Trypanosoma* spp. is not incorporated into the F_O moiety, perhaps because it does not encode this subunit. Another explanation postulates that in the Ak cells mutations have evolved in the nuclear-encoded subunits of the complex to offset the absence of ATP6, in which the ATPase activity of the F_1 is uncoupled from the incomplete F_O that lacks proton pumping capacity. A single amino acid substitution in the C-terminus of the γ subunit from a laboratory-induced Dk strain was elegantly demonstrated to be such a mutation (Schnaufer et al., 2005), while additional mutations in the same region with a putative compensatory role were identified in several natural Ak strains (Lai et al., 2008). Consistent with such a scenario is the insensitivity of *Trypanosoma brucei evansi* to oligomycin, a specific inhibitor of F_O, to which the BS *T. brucei* remains susceptible (Opperdoes et al., 1976; Schnaufer et al., 2005). Furthermore, the putative ATP6 mRNA is edited in both PS and BS trypanosomes, while other transcripts are preferentially edited only in one of these stages (Stuart et al., 1997; Schnaufer et al., 2002).

Despite significant efforts during the last two decades, proteins translated from edited mRNAs remain elusive. To date, apocytochrome B (cyB) and cytochrome *c* oxidase subunit 2 (cox2) from the model trypanosomatid *Leishmania tarentolae* remain the only directly observed translation products of a moderately edited mRNA, as detected by Edman degradation of conspicuous protein signals (Horváth et al., 2000, 2002), whereas those translated from a pan-edited mRNA have yet to be found (Horváth et al., 2002; Panigrahi et al., 2009). Underscoring the invisible nature of these mt-encoded proteins is that they have not been detected in a proteomics survey of the organelle (Panigrahi et al., 2009). Furthermore, ATP6 was not detected in any of the isolations of complex V from *T. brucei* (Zíková et al., 2009) or the monoxenous flagellate *Crithidia fasciculata* (Speijer et al., 1997). Attempts to generate antibodies against proteins predicted from pan-edited RNAs using synthetic oligopeptides were also ineffective (A. Horváth and J. Lukeš unpublished data; Rob Benne and Dave Speijer, personal communication). Unfortunately, direct recombinant manipulation of kDNA is still not feasible despite valiant efforts (Sbicego et al., 1998; Schnaufer et al., 2000).

We have thus decided to address the translation and incorporation of the predicted ATP6 protein by taking advantage of the different phenotypes resulting from RNA interference (RNAi)-mediated silencing of two nuclear-encoded proteins involved in mtRNA metabolism: mtRNA binding protein 2 (MRP2) and kRNA editing proteins KREPA6 (summarised in Table 1). MRP2 with its partner MRP1 forms a heterotetrameric MRP1/2 complex, which facilitates annealing of gRNAs to their cognate mRNAs (Schumacher et al., 2006). RNAi knock-down (KD) of MRP2 in the PS resulted in the down-regulation of a subset of never-edited and edited mRNAs, with the important exception of ATP6 (Vondrušková et al., 2005), which consequently caused the disruption of complexes III and IV (Table 1) (Zíková et al., 2006). Since KREPA6 is a subunit of the editosome (Stuart et al., 2005), its depletion in the PS results in a general reduction of RNA editing, affecting mRNAs for subunits of complexes III and IV, as well as ATP6 (Table 1) (Tarun et al., 2008). Although the secondary effect of its silencing on the respiratory complexes was not directly investigated, their disruption is considered inevitable, since this outcome is observed

Table 1
Summary of mitochondrial transcripts affected by the RNA interference (RNAi) silencing of mitochondrial RNA binding protein 2 (MRP2) and kinetoplastid RNA editing protein 6 (KREPA6).

RNAi KD	Effect on mitochondrial RNAs						Reference
	I	III	IV	rRNA	RPS12	ATP6	
KREPA6	E	E	E	ND	E	E	Tarun et al. (2008)
MRP2	NE and E	E	NE	0	E	0	Vondrušková et al. (2005)

Mitochondrial RNAs: I = subunit of complex I (NADH dehydrogenase); III = subunit of complex III (cytochrome *c* reductase); IV = subunit of complex IV (cytochrome *c* oxidase); rRNA = mitoribosomal RNA; RPS12 = ribosomal protein subunit 12.
Effect: E = decrease in edited mRNA; NE = decrease in never-edited RNA; 0 = no effect; ND = not determined; KD – knock-down.

in the RNAi KD of the terminal uridylyl transferase required for gRNA maturation, which affected all edited mRNAs in a similar fashion (Aphasizhev et al., 2002; Nebohacova et al., 2004). Here we present several lines of evidence, albeit indirect, for the existence of a genuine ATP6 subunit of an apparently non-canonical complex V in the mitochondrion of PS *T. brucei*.

2. Materials and methods

2.1. Strains, cultivation and isolation of mitochondria

The *T. brucei* MRP2 and KREPA6 RNAi cell lines were described elsewhere (Vondrušková et al., 2005; Tarun et al., 2008). The PCR-generated fragment (forward primer with BamHI restriction site: 5′-TGGGATCCAACACTGCACCATGGATTG-3′; reverse primer with XhoI site: 5′-AAGCTCGAGTGGATGTTCTTTCCCCTC-3′; both restriction sites underlined) of ATP4 (p18) was cloned into the p2T7–177 vector. The ATP4 knock-down cell line was established by transformation of the parental 29-13 cell line with resulting cassette, as described elsewhere (Hashimi et al., 2008). All transgenic cells, as well as the 29-13 cell line, were cultivated in semi-defined media (SDM)-79 medium under conditions described elsewhere (Vondrušková et al., 2005). The *T. brucei* strain 920, *Trypanosoma brucei equiperdum* strain 818 and *T. b. evansi* strain 810 were described previously (Lai et al., 2008). The kinetoplast-mt vesicles from 5×10^8 non-induced and induced PS cells were isolated by hypotonic lysis as described elsewhere (Horváth et al., 2005). Pelleted mt vesicles were stored at −70 °C until required.

2.2. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) reactions were performed as in Hashimi et al. (2008). Primers for amplification of pre-edited and edited mt mRNAs are as described elsewhere (Carnes et al., 2005), as are those for KREPA6 (Tarun et al., 2008). The primer pair for detection of MRP2 cDNA is MRP2-qPCR-Fw (5′-GAAGCTTGGCTG TGTCCTTC-3′) and MRP2-qPCR-Rv (5′-TGCGTCCGAATACGATTACA-3′). Relative RNA abundance between RNAi-induced and non-induced samples were calculated as described previously (Carnes et al., 2005; Hashimi et al., 2008; Lai et al., 2008; Tarun et al., 2008).

2.3. Measurement of ATPase activity and inhibition experiments

One milligram of mt proteins was resuspended in 1 ml of the TC buffer (0.2 M KCl; 10 mM Tris–HCl, pH 8.2; 2 mM $MgCl_2$). The reaction was started at room temperature by adding ATP to a the final concentration of 5 mM and after 5 min was stopped by mixing 95 µl aliquots with 5 µl of 3 M $CHCl_3$—COOH, incubated for 30 min on ice, and spun (16,000*g* for 10 min at 4 °C). Ninety microlitres of the supernatant were added to 1 ml of the Sumner reagent (8.8% [w/v] $FeSO_4$; 375 mM H_2SO_4; 6.6% [w/v] $(NH_4)Mo_7O_{24}$). After 15 min incubation at room temperature, absorbance of free P_i was measured at 610 nm. Parallel experiments were performed with untreated samples and those in the presence of 10 µg/ml oligomycin or 1 mM azide. Inhibition experiments were reproduced in 10 independent measurements from four RNAi inductions with parallel non-induced controls. Statistical significance of observed differences in inhibition data were determined by Student's *t*-test. $P < 0.05$ was considered significant.

2.4. In-gel activity staining and two-dimensional gel electrophoresis

For activity staining, 100 µg of mitochondrial lysate in 0.5 M aminocaproic acid and 2% dodecylmaltoside was loaded per lane and analysed on a 4–15% gradient blue native polyacrylamide (BN) PAGE gel as described elsewhere (Horváth et al., 2005). Immediately after the run, the gel was transferred into either ATPase reaction buffer (35 mM Tris; 270 mM glycine; 19 mM $MgSO_4$; 0,3% [w/v] $Pb(NO_3)_2$; 11 mM ATP) for overnight incubation, or into cytochrome oxidase buffer (50 mM sodium phosphate, pH 7.4; 1 mg/ml 3,3′-diaminobenzidine; 24 U/ml catalase; 1 mg/ml cytochrome *c*; 75 mg/ml sucrose) for 3 h staining, both by slow agitation. The ATPase activity appears as white and cytochrome *c* oxidase activity as brown precipitate. The resulting bands were quantified with the scanning densitometry ImageQuant program (Molecular Dynamics). The BN-PAGE gels for the first dimension of two-dimensional gel electrophoresis were prepared in the aforementioned fashion. The second dimension was resolved on 10% Tricine–SDS–PAGE gels as in Horváth et al. (2005) and Zíková et al. (2006).

2.5. Western blot analysis

After electrophoresis, the BN-PAGE gel was blotted on a nitrocellulose membrane and probed with polyclonal rabbit antibodies raised against complex V subunits (designation in parenthesis) of *C. fasciculata* (F_1 moiety) (Speijer et al., 1997), *L. tarentolae* (ATP4) (Bringaud et al., 1995) and *T. brucei* (ATP9) (Brown et al., 2006) were used at 1:1,000, 1:3,000 and 1:500 dilutions, respectively. Secondary α-rabbit antibodies (1:2,000) (Sevapharma) coupled to horseradish peroxidase were visualised according to the manufacturer's protocol using the ECL kit (Pierce).

2.6. Isolation of F_1 moiety of complex V by chloroform extraction

In a protocol adapted from Linnett et al. (1979), hypotonically-isolated mt vesicles from 4×10^8 cells were resuspended in 300 µl STE buffer (0.25 M sucrose; 20 mM Tris·HCl, pH 7.9; 2 mM EDTA), and sonicated 4 × 10 s with 30 s intervals. The sonicate was centrifuged at 100,000*g* for 30 min at 4 °C, and the pellet was then resuspended in 300 µl STE buffer. A 150 µl vol. of chloroform was added; the two phases were emulsified by vortexing for 10 s and spun in a microcentrifuge at maximum speed for 5 min at room temperature. The aqueous phase was transferred into a new centrifuge tube and ultracentrifuged at 100,000*g* for 30 min at 25 °C. The proteins present in the supernatant were concentrated to 0.4 µg/µl using Microcon YM-30 centrifugation filters (Millipore), in which the solvent buffer was replaced with the resuspension solution (0.5 M aminocaproic acid; 0.25% dodecylmaltoside). This sample was divided and stored overnight at either 0 °C or room temperature to assay for the cold lability of the isolated F_1 moiety. Approximately 5 µg of extracted proteins were loaded per lane of the BN-PAGE gel.

3. Results

3.1. Differential effect of KREPA6 and MRP2 RNAi silencing on ATP6 editing

Inhibition of cell growth was re-evaluated in the PS cells, in which the KREPA6 or MRP2 transcripts were down-regulated by RNAi (Figs. 1A and C). In agreement with previous reports (Vondrušková et al., 2005; Tarun et al., 2008), tetracycline-induced targeting of these transcripts by double-stranded RNA resulted in slower growth. Based on these results, subsequent experiments were performed using cells grown for 6 days in the presence or absence of tetracycline. This time-point was chosen since our study concerns a secondary phenotype affecting the respiratory complexes, a strategy already employed in similar studies (Aphasizhev et al., 2002; Nebohacova et al., 2004), taking advantage of the consequential results of the direct effect of these RNAi KDs on mtRNAs.

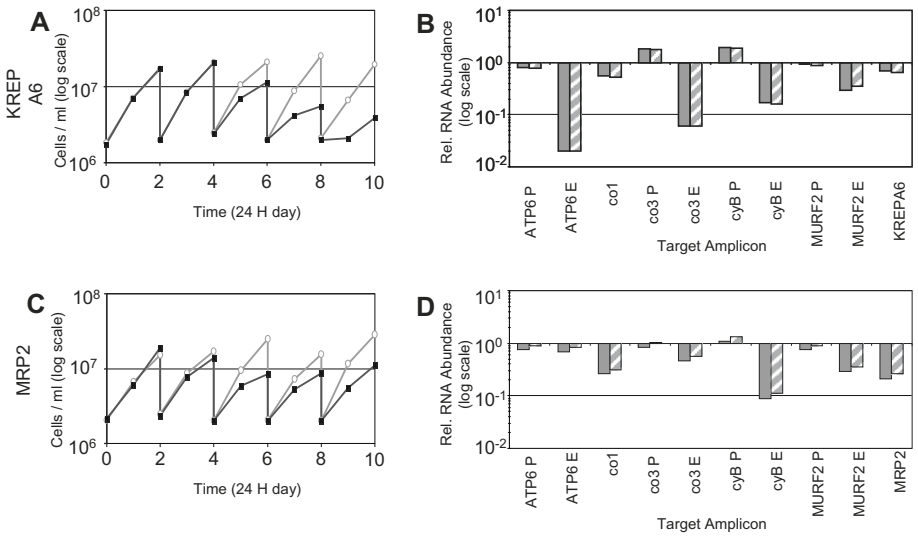

Fig. 1. Effect of RNA interference (RNAi) of mitochondrial RNA binding protein 2 (MRP2) and kinetoplastid RNA editing protein 6 (KREPA6) on procyclic stage (PS) *Trypanosoma brucei* cell growth and editing of ATP6 mRNA. The growth curves for non-induced (grey open circles) and induced KREPA6 (A) and MRP2 (C) RNAi knock-downs (black filled squares) are shown for a period of 10 days. The *y* axis is depicted on a log scale and represents the cell densities of the cultures measured every 24 h, as specified on the *x* axis. Real-time quantitative PCR analysis of a subset of mitochondrial-encoded RNAs, including pre-edited and edited ATP6 mRNAs, as well as the levels of the particular nuclear-encoded and RNAi-targeted mRNA (KREPA6 – B; MRP2 – D) was performed in triplicate on cDNAs generated from cells grown for 6 days in the presence or absence of tetracycline. For each target amplicon, the relative change in RNA abundance due to induction of RNAi-silencing of the particular transcript was determined by using cytosolic transcripts of β-tubulin (striped bar) and 18S rRNA (grey bar) as internal references, since their transcription was not affected by treatment. The following pre-edited (P) and edited (E) mRNAs were assayed: ATPase subunit 6 (ATP6), cytochrome oxidase subunits 1 (co1) and 3 (co3), cytochrome reductase subunit b (cyB) and maxicircle unknown reading frame 2 (MURF2). The average and median SD of the measured triplicate cycle threshold (C_t) values are 0.13 and 0.10, respectively.

In the original report, mtRNAs were assayed in the MRP2-silenced cells by poisoned primer extension (Vondrušková et al., 2005), showing reduction of edited and never-edited subunits of complexes III and IV, without an apparent effect on the ATP6 mRNA (Table 1). We have verified the differential effect on ATP6 editing in the KREPA6 and MRP2 KDs using a more sensitive method based on qPCR (Fig. 1B and D). Using previously described primers against pre-edited and edited ATP6 mRNAs (Carnes et al., 2005), editing of this transcript is virtually abolished in the KREPA6 KD 6 days after tetracycline induction compared with its non-induced counterpart (Fig. 1B). In contrast, MRP2-silencing only slightly reduces the levels of pre-edited and edited ATP6 mRNAs (Fig. 1D), perhaps reflecting a general role in RNA stability and/or processing, suggested for its eponymous complex (Schumacher et al., 2006; Vondrušková et al., 2005). Nonetheless, the level of the translatable ATP6 transcript is considerably closer to the wild-type level upon the depletion of MRP2 compared with KREPA6.

A subset of the mt-encoded RNAs for subunits of respiratory complexes III and IV, plus the edited maxicircle unidentified reading frame 2 (MURF2), were also assayed by reverse transcription-qPCR to confirm that they are affected as originally reported in the KREPA6 and MRP2 KDs (Vondrušková et al., 2005; Tarun et al., 2008) (summarised in Table 1). As expected, the never-edited transcript of complex IV co1 and the edited RNA of the complex III cyB were down-regulated upon MRP2 silencing (Fig. 1D). Editing of co3 and cyB was also reduced in the cells with down-regulated KREPA6 (Fig. 1B). Moreover, editing of the MURF2 transcript, encoding a protein of unknown function, was equally affected in both KDs (Fig. 1B and D).

3.2. The accumulation of the F_1 moiety and reduction of the F_1F_O oligomer of complex V in ATP6-depleted cells

Mitoplasts from the non-induced and tetracycline-induced KREPA6 and MRP2 KDs were subjected to BN-PAGE/in-gel ATPase activity experiments. Complex V was visualised by running these lysates on the BN gels, followed by staining for the in-gel ATPase activity (Fig. 2A). In all samples except the KREPA6-silenced one, relatively strong upper (~2,700 kDa) and lower (~900 kDa) activity bands are present, separated by a triplet of weaker bands. To investigate the identity of these bands, Western blots of parallel BN-PAGE gels were probed with antibodies immunodecorating subunits of complex V (Fig. 2B and C). The α-F_1 β subunit and α-ATP4 antibodies, used in previous studies of *T. brucei* (Schnaufer et al., 2005; Lai et al., 2008), were originally raised against the F_1 moiety of *C. fasciculata* (Speijer et al., 1997), and the F_O-subunit of *L. tarentolae* (Bringaud et al., 1995; Nelson et al., 2004). Both antibodies positively labelled all of the bands with the same relative intensities as the in-gel activity stain. Polyclonal antibodies raised against the *Saccharomyces cerevisiae* ATP4, 6 and 9 subunits did not cross-react with antigens present in *T. brucei* (data not shown).

Multiple bands are resolved by native gel electrophoresis that correspond to different forms of complex V in yeast, such as oligomers, dimers and monomers of F_1F_O, as well as free F_1 particles (Rak et al., 2007). We attempted to identify the form of complex V corresponding to the upper and lower bands by LC–MS/MS. However, only peptides from the catalytic F_1 moiety were identified in all three samples, plus ATP4, possibly because the technique is not amenable to identification of the hydrophobic peptides comprising F_O (data not shown). The presence of the canonical F_O ATP4 in all bands also complicated this task. We resorted to the assignment of these bands by how their estimated molecular weights match the canonical stoichiometry (Boyer, 1997; Velours and Arselin, 2000; Mulkidjanian et al., 2007) of the currently annotated complex V subunits in the GeneDB *T. brucei* genome database (http://www.genedb.org/genedb/tryp/). The top ~2,700 kDa band has been provisionally assigned as an oligomer of complex V. The identity of the lower ~900 kDa band was more difficult to ascertain, since its migration could correspond to either a monomer of complex V or a dimer of the F_1 sub-complex. We favour the latter interpretation, since the isolated F_1 moiety from *L. tarentolae* has been reported to have a similar ~900 kDa size and was also

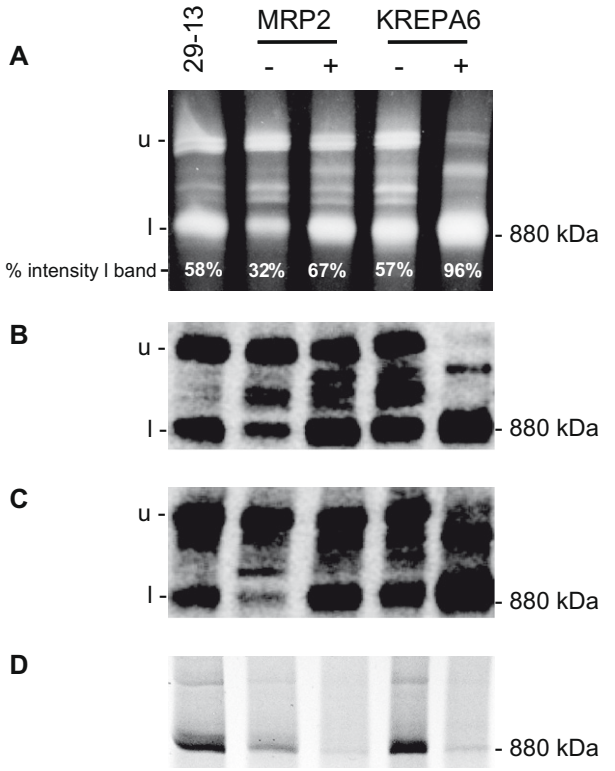

Fig. 2. Decrease in complex V F_1F_O oligomers and accumulation of free F_1 dimers in the ATP6-deficient cells. Mitochondrial complexes from mitochondrial RNA binding protein 2 (MRP2) and kinetoplastid RNA editing protein 6 (KREPA6) knock-down cells, grown for 6 days in the absence (–) or presence (+) of 1 μg/ml tetracycline (tet), respectively, were separated by 4–15% blue native gel electrophoresis and stained for in-gel ATPase activity. Protein (100 μg) was loaded in each lane. 29-13, procyclic *Trypanosoma brucei* strain 29-13. The position of the ferritin dimer molecular weight marker is indicated on the left; u – upper activity bands (~2,700 kDa); l – lower activity bands (~900 kDa). (A) In-gel ATPase activity. The percentage of density of the lower activity bands relative to that of total density of the combined bands is indicated at the bottom. (B) Western blot analysis with antibody against the F_1 β subunit. (C) Western blot analysis with antibody against the F_O subunit ATP4. (D) In-gel cytochrome *c* oxidase (complex IV) activity. The presence of complex IV on blue native gels was detected by incubation with the reaction buffer (see Section 2.4), which yields a dark precipitate upon catalysis by the complex. In both knock-down cell lines, the density of the activity band is reduced by ~70% compared with their non-induced counterparts.

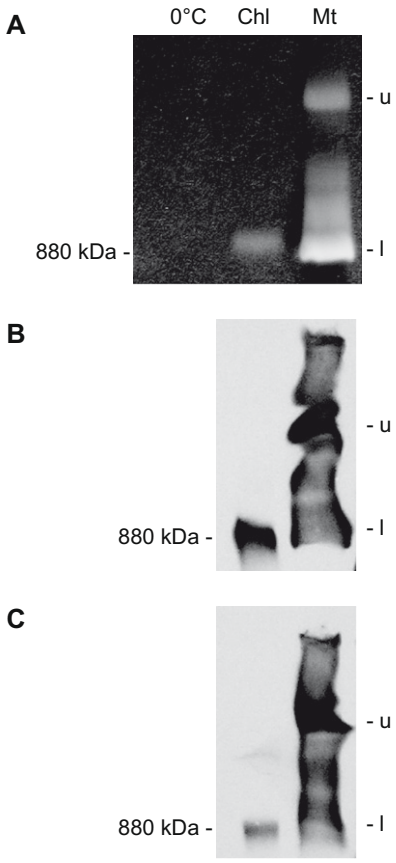

Fig. 3. ATP4 is present in the chloroform-extracted F_1 moiety of procyclic *Trypanosoma brucei*. Protein (100 μg) from lysates of hypotonically-isolated mitochondria and the equivalent of 5 μg of the F_1 moiety after chloroform extraction were resolved on a 4–15% blue native gel. The position of the ferritin dimer (Sigma) used as molecular weight marker is indicated on the left. 0 °C, F_1 moiety isolated by chloroform extraction and incubated overnight at 0 °C; Chl, F_1 moiety isolated by chloroform extraction and incubated overnight at room temperature; Mt, lysates from hypotonically-isolated mitochondria; u – upper activity bands (~2,700 kDa); l – lower activity bands (~900 kDa). After the run, the gel was either stained for in-gel ATPase activity (A), or transferred onto a nitrocellulose membrane and immunodecorated with polyclonal antibodies against either the F_1 β subunit (B) or the F_O subunit ATP4 (C).

proposed to be a dimer (Nelson et al., 2004), which corresponds with our results presented in Fig. 3.

The cells interfered against KREPA6 (in which editing of ATP6 is virtually abolished (Fig. 1B)), exhibit a reduction in oligomerization of complex V and a corresponding accumulation of free F_1 dimers (Fig. 2A). Semi-quantification of the ATPase bands reveal that the lowest doublet represents 96% of the sum density of the upper and lower bands in the lane containing the RNAi-silenced sample, while it comprises 57% and 58% in-gel activity from the untreated and parental cell lines, respectively (Fig. 2A). A similar enrichment of the free F_1 dimers was observed in KREN1 KD cells, in which one of the endonucleases essential for RNA editing has been ablated (data not shown) (Trotter et al., 2005). Such a disruption of complex V is not observed in the MRP2 KDs, in which editing of ATP6 persists, while the amounts of never-edited, pre-edited and edited mRNAs of the other mt-encoded subunits of respiratory complexes are decreased (Vondrušková et al., 2005; Zíková et al., 2006). The in-gel activity of free F_1 represents 32% and 67% of the sum density of the upper and lower bands observed in the non-induced and RNAi-induced samples, respectively (Fig. 2A). It should be noted that the percentage of the free F_1 moiety in the MRP2 KD cells grown in the absence of tetracycline is lower than that observed in the parental strain, while this particle represents a portion of the in-gel ATPase activity that is similar to that observed in the parental cell lines. Such a phenomenon has been previously reported in comparing the in-gel activities of complex IV in the non-induced and RNAi-induced KDs of complex III subunits (Horváth et al., 2005).

Western blot analyses with antibodies against the β and ATP4 subunits of the F_1 and F_O sub-complexes (Fig. 2B and C), respectively, also revealed the predominant accumulation of the F_1 moiety upon RNAi-silencing of KREPA6 compared with that in MRP2 KD as well as the parental cell lines. Furthermore, the immunopositive signals from either antibody are not observed in the upper band of the KREPA6-silenced samples, while they are in the other lanes. Interestingly, a signal corresponding to the uppermost of the three middle bands is observed in the KREPA6-silenced lane of both immunodecorated blots. As expected, the in-gel activity of complex IV was dramatically reduced by approximately 70% when either MRP2 or KREPA6 were down-regulated (Fig. 2D).

3.3. ATP4 is required for the integrity of F_1 moiety

The presence of ATP4, traditionally assigned to the F_O moiety (Boyer, 1997; Velours and Arselin, 2000), in the F_1 sub-complex

prompted us to further investigate this issue. The soluble F_1 moiety was obtained from hypotonically-isolated mt from the 29-13 cells, a method that purifies the sub-complex from the membrane-bound hydrophobic subunits (Linnett et al., 1979). The F_1 moiety was resolved on a BN gel together with the whole mt lysate. Upon staining by the in-gel ATPase activity assay, the lower band from the mt lysate migrates the same distance as the isolated F_1 moiety, whose activity is cold labile (Fig. 3A), a typical characteristic of the isolated F_1 sector. Western blot analyses using antibodies against the F_1 β subunit and F_O subunit ATP4 showed that both are present in the chloroform-extracted F_1 sub-complex (Fig. 3B and C). ATP4 was also reported to be present in the chloroform-extracted F_1 moiety of *C. fasciculata* (Speijer et al., 1997). Migration of the isolated F_O moiety in relation to the ferritin dimer suggests that it exists as a dimer, taking into account the sizes and stoichiometry of the F_1 subunits. This result is in agreement with the size of this particle in *L. tarentolae*, as determined by biochemical isolation (Nelson et al., 2004) or its visualisation by adenylylation with [α-^{32}P] ATP in native gels (Peris et al., 1997).

We also generated an RNAi KD of ATP4 to examine its position within the complex. Silencing of ATP4 resulted in slower growth of the PS cells (Fig. 4A). After 90 h of RNAi-induction, the steady-state level ATP4 protein was virtually eliminated (Fig. 4A, inset), which was the time-point chosen for subsequent experiments. In-gel ATPase activity and Western blot analysis using an antibody against the β subunit showed that the level of the catalytic portion of complex was decreased upon down-regulation of ATP4 (Figs. 4B and C). This result is reminiscent of the apparent destabilization of the β subunit of F_1 upon silencing of its partner, the α subunit, and vice versa (Schnaufer et al., 2005; Brown et al., 2006), which may be an indication that ATP4 also interacts directly with this moiety. This interpretation is consistent with its apparent association with the chloroform-isolated F_1 particle, as well as its interaction with the F_1 OSCP subunit of complex V. Another possibility is that this antibody recognises a genuine F_1 subunit and not the canonical F_O ATP4 subunit. In any case, it is clear that this protein plays a role in maintaining the integrity of the F_1 moiety.

3.4. Formation of the F_O ATP9 multimeric ring is disrupted in the absence of ATP6

Two-dimensional gel electrophoresis was employed to further examine complex V in the KREPA6 KDs, since a polyclonal antibody against ATP9, a subunit forming the multimeric ring of the F_O sub-complex, appears to recognise proteins resolved under denaturing conditions. Native complexes of RNAi cell lines grown in the presence or absence of tetracycline were separated on BN gels in the first dimension followed by SDS–PAGE in the second dimension to break apart individual subunits. As seen in the Western blot in Fig. 5A, the β subunit and ATP4 have been detected in the upper and lower bands (as visualised by in-gel ATPase activity), as well as in the middle bands. However, ATP9 is confined to the region corresponding to the upper bands, and is present to a lesser degree in the middle bands (Fig. 5A). In the KREPA6-silenced samples, ATP4 and F_1 are concentrated in the lowest bands, while ATP9 is undetectable, presumably because in the absence of ATP6, it cannot be assembled into the complex and is degraded (Fig. 5B). This finding further supports the notion that the lower band represents the F_1 moiety.

3.5. Sensitivity to oligomycin is decreased in ATP6-depleted mitoplasts

The Ak trypanosome *T. b. evansi* is insensitive to oligomycin, a specific inhibitor of the F_O subunit (Schnaufer et al., 2005; Opperdoes et al., 1976). We investigated the possibility that the KREPA6-depleted cells may exhibit a decrease in sensitivity to oligomycin

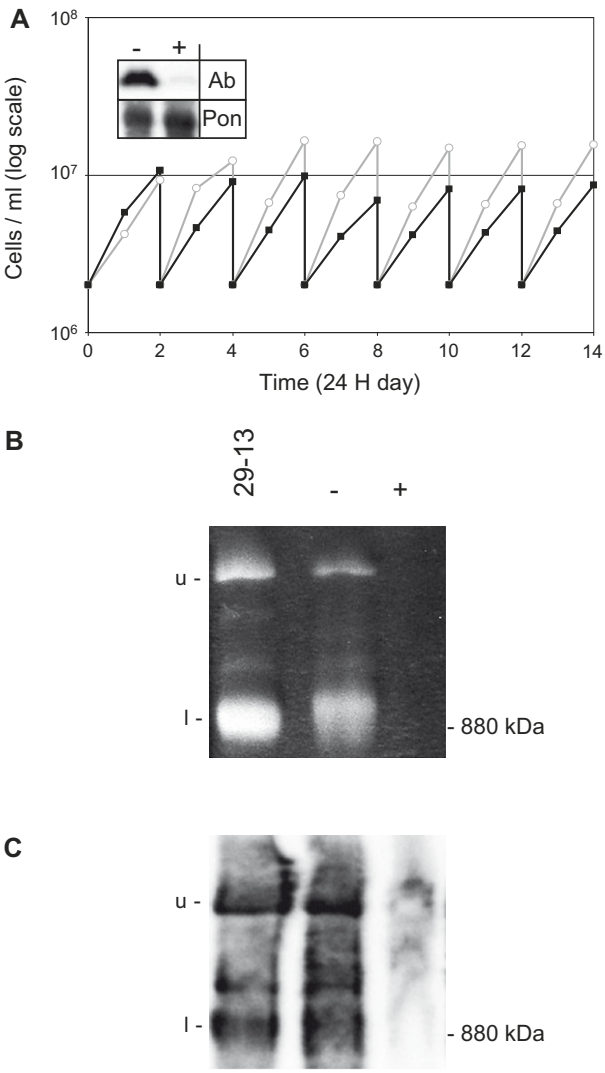

Fig. 4. Down-regulation of ATP4 results in slower growth and disassembly of the F_1 moiety. (A) The growth curves for non-induced (grey open circles) and RNA interference (RNAi)-induced cells (black filled squares) are shown for a period of 14 days and depicted as in Fig. 1A. Inset displays the down-regulation of the ATP4 protein after 90 h of RNAi-induction (+) compared with the non-induced cells (−), as determined by Western blot analysis (Ab). Ponceau staining of the membrane is shown as a loading control (Pon). (B) Complex V from mitochondrial lysates of the *Trypanosoma brucei* 29-13 parental cell line, as well as the ATP4 RNAi knock-down cell line grown for 90 h in the absence (−) and presence (+) of tetracycline, was resolved on a 4–15% blue native gel and visualised by the in-gel ATPase activity assay, as in Fig. 2A. The upper (u, ~2,700 kDa) and lower (l, ~900 kDa) bands, in addition to the position of the ferritin dimer marker (880 kDa), are indicated on the left and right, respectively. Protein (100 μg) was loaded in each lane. (C) Visualisation of F_1 β subunit after the blue native gel was transferred to nitrocellulose and probed with specific antibody. Labelling as in Fig. 2B.

that would be reminiscent of the behaviour of the ATP6-lacking Ak cells. The ATPase activity of hypotonically-isolated mt from the non-induced and RNAi-induced KREPA6 and MRP2 KDs, as well as the parental 29-13 cells, were assayed for the release of P_i in the presence of 10 μg/ml oligomycin and 1 mM azide, which inhibit the F_1 sub-complex (Fig. 6), compared with samples without inhibitors. The observed ~40% reduction of ATPase activity by either inhibitor in mt from the 29-13 cell is similar to what has been previously reported for the BS trypanosomes (Schnaufer et al., 2005). Down-regulation of KREPA6 results in more than 50% lower sensitivity to oligomycin, compared with the non-induced cells, while both cell lines do not differ in their sensitivity to azide (Fig. 6). The observed changes in oligomycin sensitivity are statistically

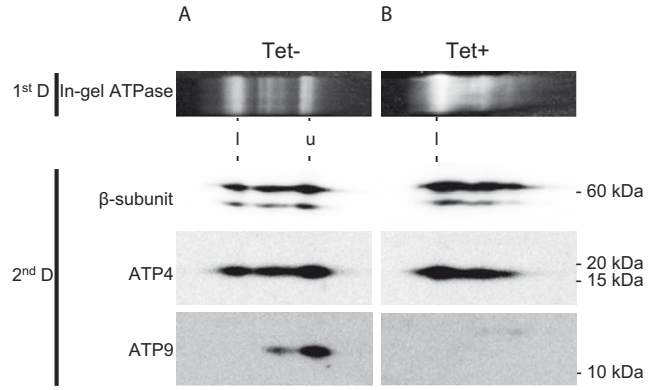

Fig. 5. Incorporation of F_O ATP9 into complex V in the presence and absence of ATP6. Complex V from *Trypanosoma brucei* kinetoplastid RNA editing protein 6 (KREPA6) knock-down cell lines grown for 6 days in the absence (A) and presence (B) of 1 μg/ml tetracycline, were resolved by two-dimensional (2D) gel electrophoresis. Native mitochondrial complexes were separated on a 4–15% blue native gel (1st D), as described in Fig. 1. Complex V was subsequently visualised by in-gel ATPase activity and the upper ~2,700 kDa (u) and lower ~900 kDa (l) bands are indicated just below. The individual subunits of the complexes were separated by 10% Tricine–SDS–PAGE (2nd D), transferred to nitrocellulose and probed with antisera against the F_1 β-subunit, ATP4 and ATP9. These antibodies are indicated on the left. Molecular weight markers are specified on the right.

significant ($P = 0.0288$), while fluctuations in azide inhibition are not ($P = 0.4907$), as determined by the Student's *t*-test. The MRP2 KD cells also exhibit somewhat reduced sensitivity to oligomycin, although they remain more susceptible to the drug than the KREPA6-silenced cells. This relatively minor decrease in sensitivity of the MRP2-silenced cells is not statistically significant (oligomycin $P = 0.1810$; azide $P = 0.5199$), and may be due to the slight decrease of edited ATP6 mRNA, as revealed by qPCR (Fig. 1D).

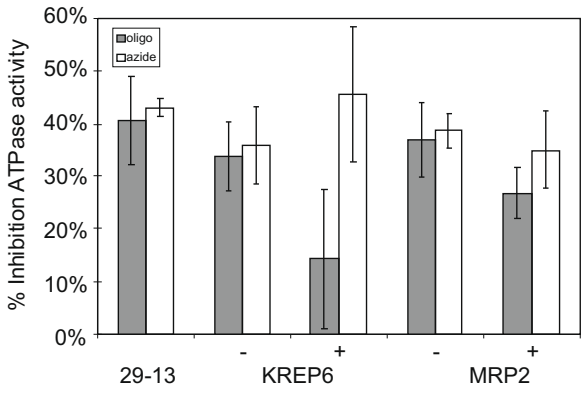

Fig. 6. ATP6-depleted cells exhibit reduced sensitivity to the F_O inhibitor oligomycin. Inhibition of the in vitro ATPase activity of complex V by 10 μg/ml of oligomycin (grey bar), which targets the F_O moiety and 1 mM azide (white bar), interfering with the F_1 moiety, was assayed. Hypotonically-isolated mitochondria from the parental *Trypanosoma brucei* 29-13 cell line and from the mitochondrial RNA binding protein 2 (MRP2) and kinetoplastid RNA editing protein 6 (KREPA6) knock-down (KD) cells, grown for 6 days in the absence (−) and presence (+) of 1 μg/ml tetracycline, were solubilised and incubated with 5 mM ATP. The release of free P_i was measured by absorbance at 610 nm. The vertical axis depicts average percentage of inhibition of ATPase compared with the untreated samples. For both inhibitors, three independent experiments using mitochondria from the 29-13 cells and six each from individual RNA interference-induced and non-induced KREPA6 and MRP2 KDs were performed. Error bars indicate the SD among the 10 individual experiments. The statistical significance of % inhibition of ATPase activity by oligomycin was $P = 0.0288$ and $P = 0.1810$ between induced (+) and non-induced (−) KREPA6 and MRP2 KD cells, respectively, as determined by Student's *t*-test. *P*-values for changes in sensitivity to azide inhibition were $P = 0.4907$ and $P = 0.5199$ for KREPA6 and MRP2 tet + and − samples, respectively.

3.6. Prevalence of the uncoupled F_1 moiety in Dk and Ak trypanosomes

In the induced KREPA6 KD cells, the accumulation of the F_1 moiety is apparent in the absence of the edited ATP6 mRNA (Fig. 2A). Therefore, in the BN-PAGE gels we have assayed the in-gel ATPase activity of lyzed mt purified from the Ak and Dk strains of *T. b. evansi* and *T. b. equiperdum*, respectively, as well as from the PS and BS of *T. brucei* (Fig. 7A). Two prominent bands are apparent in the BS that correspond to the top ~2,700 kDa F_1F_O-oligomer and the bottom ~900 kDa F_1-dimer present in the PS cells. In the Dk and Ak strains only the lower ATPase activity band is apparent, however, with a higher intensity than that found in the BS cells (Fig. 7A). The BS and the Dk trypanosomes show considerably lower in-gel ATPase activity than the PS cells, which is in agreement with previous observations that complex V is down-regulated in the stages infecting mammals (Brown et al., 2001). The native forms of complex V were also visualised by Western blot analysis using an antibody directed against the β subunit, revealing the predominance of the F_1 dimer in the Dk and Ak trypanosomes (Fig. 7B). This finding was confirmed by Western blot analysis of two-dimensional gels resolving complex V, as in Fig. 4, showing that in the Ak *T. b. evansi* strain, the ATP4 subunit is restricted to the lower ATPase activity band.

4. Discussion

Since its seminal discovery (Benne et al., 1986), RNA editing in the mitochondrion of kinetoplastid flagellates has become an intensely studied process (Lukeš et al., 2005; Stuart et al., 2005). There was very little doubt that the edited transcripts are translated into mt proteins. Still, to date only two proteins produced from a moderately edited and never-edited mt mRNA were shown to exist (Horváth et al., 2000, 2002), while all attempts to detect predicted protein products of pan-edited mRNAs failed. An extensive analysis of mt transcripts in *T. brucei* and *L. tarentolae*, however, detected a widespread occurrence of partially edited molecules, with fully edited ones representing only a tiny fraction of the mtRNA population (Decker and Sollner-Webb, 1990; Sturm and Simpson, 1990). In the particular case of pan-edited ATP6, only 10% of analysed molecules could serve as blueprints for the synthesis of the canonical ATP6 subunit of complex V (Ochsenreiter et al., 2008).

Although perhaps inefficiently produced, the product of pan-edited ATP6 mRNA appears to be incorporated into the complex V of *Trypanosoma* species, as supported by several lines of evidence presented in this study. The PS cells, in which the 20S editosome subunit KREPA6 was RNAi-silenced, are deficient of ATP6 compared with cells with down-regulated MRP2. Indeed, RNA editing is generally inhibited by silencing proteins directly involved in the process, invariably affecting mt mRNAs encoding subunits of respiratory complexes, whereas complex V escaped such interference in the latter KD. The absence of the ATP6 protein caused by the disruption of RNA editing significantly reduces the level of F_1F_O oligomers of complex V, while an accumulation of the released F_1 moiety, the soluble portion of the ATP synthase, takes place. The KREPA6 KD represents an ATP6-lacking background compared with the MRP2-silenced cells, in which oligomerization occurs at approximately the wild-type level. This situation is also apparent in Dk and Ak trypanosomes, which naturally lack the capacity to express ATP6, since they also exhibit an accumulation of the free F_1 moiety compared with BS *T. brucei* containing the F_1F_O oligomers. Furthermore, we show that ATP9, the subunit responsible for the formation of the multimeric ring of the F_O moiety, is reduced in the absence of ATP6. Finally, the sensitivity of complex V to oligomycin is reduced in cells lacking the capacity

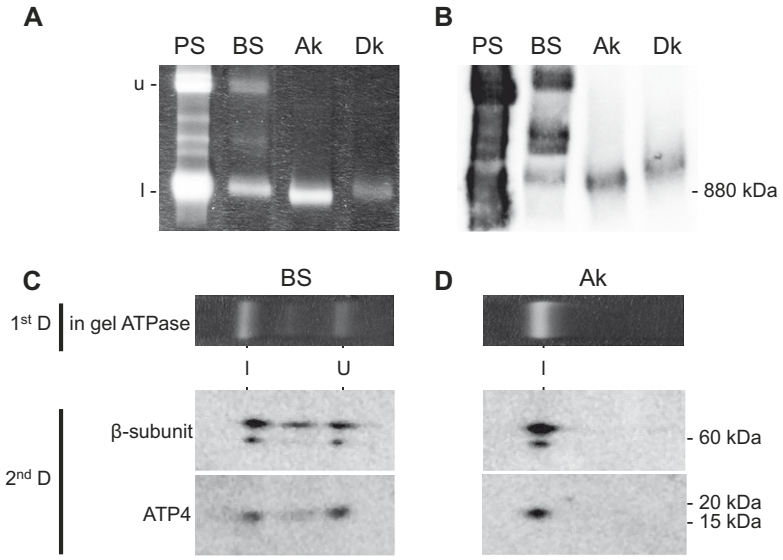

Fig. 7. The prevelance of the F_1 moiety in naturally akinetoplastic *Trypanosoma brucei evansi* and dyskinetoplastic *Trypanosoma brucei equiperdum*. (A) In-gel ATPase activity. Mitochondrial protein complexes were resolved and stained as described in Fig. 1. Protein (100 μg) was loaded in each lane. BS, bloodstream *T. brucei*, strain 920; PS, procyclic *T. brucei* strain 29-13; Ak, akinetoplastic bloodstream *T. b. evansi*, strain 810; Dk, dyskinetoplastic bloodstream *T. b. equiperdum*, strain 818; u – upper activity band (~2,700 kDa); l – lower activity bands (~900 kDa). (B) Western blot analysis with antibody against the F_1 β subunit under the conditions described in Fig. 2. The position of the ferritin dimer molecular weight marker is indicated on the left. (C) Two-dimensional (2D) gel electrophoresis resolution of complex V in the bloodstream *T. brucei* (BS) was performed and labelled as described in detail in Fig. 5. Western blots were probed with antisera against ATP4. (D) Two-dimensional gel electrophoresis resolution of complex V in the akinetoplastic bloodstream *T. b. evansi*, strain 810 (Ak) was performed and labelled as described in detail in Fig. 5. Western blots were probed with antisera against ATP4.

to edit ATP6 mRNAs, which correlates with the situation documented for the Dk trypanosomes naturally lacking ATP6 (Schnaufer et al., 2005).

The deletion of ATP6 in *S. cerevisiae* resulted in the loss of F_1F_O dimers and monomers and the appearance of free F_1, as revealed by in-gel ATPase activity staining (Rak et al., 2007). Our findings in *T. brucei* are consistent with these results. However, ATP9 appears to still be assembled into complex V in the yeast null mutants (Rak et al., 2007). In trypanosomes, the absence of ATP6 appears to destabilise the F_O moiety, as implied by the diminished ATP9 ring, leading to the apparent deficiency of F_1F_O formation. This observation indicates that ATP6 may not be the last subunit incorporated into complex V as has been suggested for the yeast orthologue, as a measure to prevent a premature leakage of protons by the incomplete sub-complex (Tzagoloff et al., 2004; Rak et al., 2007).

Several other differences between kinetoplastid and canonical complex V were underscored by this study. It appears that ATP4, a subunit traditionally assigned to F_O, is firmly associated with F_1 in these flagellates, confirming the same observation made in *C. fasciculata* (Speijer et al., 1997). This situation is possible given its role in the formation of the peripheral stalk by direct interaction with the F_1 OSCP subunit. Alternatively, the designation of this protein associated with the F_1 particle as ATP4 may have been premature. However, it is clear that this protein is associated with the soluble portion of complex V.

This study also suggests that the F_1 subunit exists as a dimer across kinetoplastid flagellates, as originally reported for *L. tarentolae* (Peris et al., 1997; Nelson et al., 2004). While this notion varies from the report of Zíková and colleagues (2009), who show that the *T. brucei* F_1 moiety migrates at the same rate as the ferritin monomer (440 kDa) by native gel electrophoresis, it is consistent with the size of a complex with self-adenylylating activity in both of these trypanosomatid species (Peris et al., 1997), which was later shown to be the F_1 particle (Nelson et al., 2004). This inconsistency may be due to technical aspects of the electrophoresis, such as differences in the range of the acrylamide gradient. Dimerization of this moiety has been reported in other eukaryotes (Dienhart et al., 2002). However, in these organisms it is promoted by an inhibitor protein in response to conditions that lead to diminished $\Delta\Psi m$, such as oxygen deprivation, in order to conserve ATP that would otherwise be spent by reversal of complex V (Dienhart et al., 2002). The chloroform-extracted F_1 moiety of *T. brucei*, whose migration in native gels also suggests a dimer conformation, is apparently not inhibited, since they maintain in-gel ATPase activity. Thus, its apparent dimerization is formed by another mechanism. Homodimers of the ATP4 subunit, assigned to the membrane-bound F_O sub-complex, have been demonstrated to form between two adjacent complexes in yeast (Spannagel et al., 1998). The possibility that this subunit is present in the F_1 sector in trypanosomes may mediate the dimerization of this moiety. Nonetheless, the occurrence of F_1 as a dimer appears to be unique to these organisms.

Although the presented data suggest, to our knowledge for the first time, that a protein product of a pan-edited RNA is indeed translated, and in this case assembled into complex V, due to the limitations of our system, we were unable to directly address the question whether ATP6 is essential for ATP synthesis. A logical experiment would be to test in vitro ATP synthase activity (Allemann and Schneider, 2000) from mt isolated from the analysed KDs. However, this assay requires that the capacity to generate $\Delta\Psi m$ is preserved, which both KDs lack due to the inevitable disruption of proton pumping complexes III and IV. Nevertheless, the notion that ATP6 is indispensable is further supported by reports that RNA editing is essential in the BS *T. brucei*, which uses complex V to sustain $\Delta\Psi m$ (Schnaufer et al., 2001). The maturation of the ATP6 mRNA by this process is redundant in the Dk and Ak trypanosomes because compensatory mutations in some of the F_1 subunits allow the conversion of ATP^{4-} to ADP^{3-} (Schnaufer et al., 2005; Lai et al., 2008). The antipodal exchange of these substrates by the ATP/ADP carrier appears to maintain $\Delta\Psi m$ along the inner mt membrane.

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