The Mitochondrial Calcium Uniporter Interacts with Subunit c of the ATP Synthase of Trypanosomes and Humans

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ABSTRACT Mitochondrial Ca\textsuperscript{2+} transport mediated by the uniporter complex (MCUC) plays a key role in the regulation of cell bioenergetics in both trypanosomes and mammals. Here we report that Trypanosoma brucei MCU (TbMCU) subunits interact with subunit c of the mitochondrial ATP synthase (ATPc), as determined by coimmunoprecipitation and split-ubiquitin membrane-based yeast two-hybrid (MYTH) assays. Mutagenesis analysis in combination with MYTH assays suggested that transmembrane helices (TMHs) are determinants of this specific interaction. In situ tagging, followed by immunoprecipitation and immunofluorescence microscopy, revealed that T. brucei ATPc (TbATPc) coimmunoprecipitates with TbMCUC subunits and colocalizes with them to the mitochondria. Blue native PAGE and immunodetection analyses indicated that the TbMCUC is present together with the ATP synthase in a large protein complex with a molecular weight of approximately 900 kDa. Ablation of the \textit{TbMCUC} subunits by RNA interference (RNAi) significantly increased the AMP/ATP ratio, revealing the downregulation of ATP production in the cells. Interestingly, the direct physical MCU-ATPc interaction is conserved in \textit{Trypanosoma cruzi} and human cells. Specific interaction between human MCU (HsMCU) and human ATPc (HsATPc) was confirmed \textit{in vitro} by mutagenesis and MYTH assays and \textit{in vivo} by coimmunoprecipitation. In summary, our study has identified that MCU complex physically interacts with mitochondrial ATP synthase, possibly forming an MCUC-ATP megacomplex that couples ADP and P\textsubscript{i} transport with ATP synthesis, a process that is stimulated by Ca\textsuperscript{2+} in trypanosomes and human cells.

IMPORTANCE The mitochondrial calcium uniporter (MCU) is essential for the regulation of oxidative phosphorylation in mammalian cells, and we have shown that in \textit{Trypanosoma brucei}, the etiologic agent of sleeping sickness, this channel is essential for its survival and infectivity. Here we reveal that \textit{Trypanosoma brucei} MCU subunits interact with subunit c of the mitochondrial ATP synthase (ATPc). Interestingly, the direct physical MCU-ATPc interaction is conserved in \textit{Trypanosoma cruzi} and human cells.

KEYWORDS ATP synthase, c ring, \textit{Trypanosoma}, mitochondrial calcium uniporter

The \textit{Trypanosoma brucei} group of parasites causes nagana in cattle and African trypanosomiasis, or sleeping sickness, in humans. Two of the best-studied life cycle stages of \textit{T. brucei} are the procyclic form (PCF), which is found in the tse tse fly vector, and the bloodstream form (BSF), which is present in the blood of the infected animal host. Although both stages have a single mitochondrion, the PCF mitochondrion has a respiratory chain, while the BSF mitochondrion does not possess a functional respiratory chain or oxidative phosphorylation and relies on the reverse action of the ATP synthase to maintain a mitochondrial membrane potential (1–4) required for protein (5) and Ca\textsuperscript{2+} (2) transport. Both stages have a functional mitochondrial Ca\textsuperscript{2+} uniporter (MCU) (6–8), which is essential for growth and virulence (9).
Trypanosomes have significant differences in the composition and function of the MCU complex (MCUC), compared to mammalian cells: we have found paralogs of trypanosome MCU that we named MCUb, MCUC, and MCUd that are necessary for mitochondrial Ca\(^{2+}\) transport; MCUb and MCUd are present only in trypanosomatids (10, 11). These subunits form, together with MCU and MCUb, hetero-oligomeric complexes in membranes (10), in contrast to the \textit{in vitro} homotetramers of recombinant MCU described for fungi (12–15) and zebra fish (15) and to the homopentamers described for \textit{Caenorhabditis elegans} (16). The MCUb subunit is a Ca\(^{2+}\)-conducting subunit and does not have a dominant negative effect on the channel like its mammalian ortholog (17). The mitochondrial calcium uptake 1 (MICU1) and MICU2 proteins do not have a gatekeeper function at low Ca\(^{2+}\) concentrations (18), as occurs with the mammalian orthologs (19–25). Finally, the trypanosomatid genomes lack orthologs encoding subunits present in the mammalian MCU complex, like MCU regulator 1 (MCUR1) (26) and essential MCU regulator (EMRE) (27).

The mitochondrial ATP synthase of \textit{T. brucei} is a large multisubunit protein that is composed of two oligomeric components, a peripheral hydrophilic F\(_1\) complex and a base piece/stalk Fo complex, and contains additional subunits with no obvious homology to proteins outside the kinetoplastids (28). As in the mammalian enzyme, the F\(_1\) domain has three catalytic sites, while the hydrophobic F\(_0\) complex is embedded in the inner mitochondrial membrane and contains a proton channel (29). In mammalian mitochondria, the ATP synthase is attached to both the phosphate carrier (PiC) and the adenine nucleotide translocator (ANT), forming the so-called ATP synthasome (29, 30). A similar association of the ATP synthase with the adenine nucleotide carrier was also reported for \textit{Leishmania mexicana} mitochondria (31) but not for \textit{T. brucei} PCF mitochondria (32). The synthasome catalyzes the synthesis of ATP coupled to the mitochondrial entry of P\(_i\) by the phosphate carrier and the exchange of ADP for ATP by the adenine nucleotide translocator.

Calcium ion (Ca\(^{2+}\)) is a key element in the pathway responsible for the activation of mitochondrial oxidative phosphorylation (33). In mammalian cells, intramitochondrial Ca\(^{2+}\) stimulates a pyruvate dehydrogenase phosphatase that activates the pyruvate dehydrogenase or allosterically activates 2-oxoglutarate and isocitrate dehydrogenases, resulting in increased ATP production (34–38). We found that trypanosomatid pyruvate dehydrogenase phosphatases are directly stimulated by Ca\(^{2+}\) (39). Ca\(^{2+}\) also increases the specific activity of the F\(_{0}\)F\(_1\) ATP synthase in mammalian mitochondria (40), but as yet there is no clear mechanism to how Ca\(^{2+}\) activates the ATP synthase. The interaction of the MCU complex with the ATP synthasome would thus have physiological significance since this is a biological machine that couples ADP and P\(_i\) exchange with ATP production, a process that is stimulated by Ca\(^{2+}\). Here we report the direct physical interaction of the MCU complex with subunit c of the \textit{T. brucei}, \textit{Trypanosoma cruzi}, and human ATP synthases (TbATPc, TcATPc, and HsATPc, respectively), which is important for the bioenergetics of the cells.

**RESULTS**

**Proteomic analysis of tandem-affinity-purified TbMCU complex.** Tandem affinity purification (TAP) is a widely used method for the isolation of protein complexes under native conditions. We adapted the method developed by Panigrahi et al. (41) and modified by Jensen et al. (plasmid pLew79-MH-TAP) (42) using an inducible system to overexpress the \textit{T. brucei} MCU (TbMCU) in the \textit{T. brucei} PCF 29-13 cell line. The TbMCU subunit was fused to a TAP tag consisting of a c-MYC-His tag (MH) and a protein A domain separated from a calmodulin-binding peptide (CBP) by a tobacco etch virus (TEV) protease cleavage site (Fig. 1A). The sequential purification over IgG-Sepharose and calmodulin resin under native conditions (Fig. 1B to D) resulted in the mass spectrometry identification of 130 proteins (see Data Set S1A in the supplemental material), from two independent experiments. Of the proteins identified, the ones with the highest scores were TbMCU (Tb427tmp.47.0014) and 19 subunits (\(\beta\), \(\alpha\), \(\delta\), OSCP, \(\gamma\), p18, Tb1, Tb2, and 11 ATP synthase-associated proteins [28]) of the ATP synthase.
Interestingly, we also detected TbMCUb and TbMCUc, which we know interact with TbMCU (10), as well as voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and phosphate carrier (PiC). In this regard, the FoF1 ATP synthase, ANT, and PiC form what is known as ATP synthasome (30). Similar mass spectral results were obtained by immunoprecipitation (IP) of *T. brucei* cells overexpressing TbMCU-hemagglutinin (HA) using anti-HA–agarose beads (Fig. S1 and Data Set S1B). Conspicuously absent were subunits a (encoded by the mitochondria) and c of the ATP synthase, although this could be expected giving their very hydrophobic nature and the need of special techniques for their chromatographic isolation and mass spectral detection (43).

**Physical interaction of *T. brucei* ATP synthase subunit c with TbMCU.** As a “reverse” approach to identify the association of TbMCU with a potential ATP synthasome, we generated *in situ* HA-tagged *T. brucei* ATPβ (TbATPβ), TbATPp18 (a subunit that binds to each of the TbATPα subunits [44, 45]), TbANT, and TbPiC PCF cell lines. These proteins colocalized with MitoTracker (MT) to mitochondria of *T. brucei* PCF (Fig. S2A). Interestingly, TbMCU was immunoprecipitated by TbATPβ-HA or TbATPp18-HA, but not by TbANT-HA or TbPiC-HA, using anti-HA antibodies (Fig. S2B and C), suggesting that TbMCU is closely associated with the ATP synthase and probably is loosely associated with other components of the ATP synthasome.

In order to validate the interaction of TbMCU with the ATP synthase, we used split-ubiquitin membrane-based yeast two-hybrid (MYTH) assays (46) to determine the
direct physical interactions between TbMCU and ATP synthase subunits in yeast. We followed the method that we used previously to determine the interaction among TbMCU, TbMCUb, TbMCUc, and TbMCUd (10). The split-ubiquitin system allows detection of in vivo interaction between membrane proteins that have their N and/or C terminus located in the cytosol. The membrane topology predicted by Protter of five ATP synthase membrane subunits or associated proteins showed that these membrane proteins could be localized to the yeast plasma membrane with either the N or C termini facing the cytosol (Fig. S3). In the MYTH assays, TbMCU (the bait, without the mitochondrial targeting signal [MTS]) is fused to the C-terminal half of ubiquitin (Cub) and the artificial transcription factor LexA-VP16 (TF), as described previously (10). Each of the 10 ATP synthase subunits selected (a [synthesized with yeast optimized codons], p18, Tb1, Tb2, c, Tb1, Tb2, c, H9251, H9252, and 3 associated proteins) (the prey, without MTS) was fused to the mutated half of ubiquitin (NubG), and the interaction of the protein partners was monitored by the release of the TF, which translocates to the nucleus, where it binds to LexA operators situated upstream of reporter genes (HIS3, ADE2, and lacZ) via its Lex DNA binding domain. The reporter genes enable the yeast to grow on defined media lacking histidine or/and adenine, while lacZ encodes the enzyme β-galactosidase (β-Gal), resulting in growth of yeast in selective medium and color development in β-Gal assays (Fig. 2B). TbMCU with the yeast invertase (SUC) signal sequence instead of MTS was expressed in pBT3-SUC as bait and targeted correctly to the yeast plasma membrane (10) (Fig. 3E). The 10 ATP synthase subunits were expressed in pPR3N or pPR3C as preys and targeted correctly to the yeast plasma membrane or cytosol as predicted (Fig. 2C). The yeast reporter strain expressing the bait TbMCU alone did not grow on the selective synthetic dropout (SD) plates (SD medium with a triple dropout [SD-3DO], SD-4DO, and SD-4DO plus X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]), indicating that the bait was not self-activated (10). Surprisingly, the strain expressing TbMCU as bait and only ATP synthase subunit c as prey enabled

| Accession no. | Mascot score | Length (aa) | Mass (kDa) | Description |
|---------------|--------------|-------------|------------|-------------|
| Tb427.03.1380 | 2,838        | 519         | 55.7       | ATP synthase subunit beta |
| Tb427.07.7420 | 1,839        | 584         | 63.5       | ATP synthase subunit alpha |
| Tb427.06.3740 | 1,063        | 657         | 71.4       | Heat shock 70-kDa protein |
| Tb427.10.180  | 1,038        | 305         | 34.4       | ATP synthase subunit gamma |
| Tb427.10.8030 | 920          | 255         | 28.8       | ATP synthase subunit OSCP |
| Tb427.06.4990 | 820          | 182         | 20.1       | ATP synthase subunit delta |
| Tb427tmp.05.1710 | 570       | 188         | 21.3       | ATP synthase subunit p18 |
| Tb427tmp.02.4120 | 534       | 269         | 27.6       | ATP synthase-associated protein |
| Tb427.10.520  | 502          | 396         | 46.8       | ATP synthase subunit Tb1 |
| Tb427tmp.47.0014 | 485       | 307         | 34.8       | MCU |
| Tb427.05.2930 | 472          | 370         | 43.3       | ATP synthase subunit Tb2 |
| Tb427tmp.211.1750 | 372       | 317         | 34.3       | PiC or TbMCP11 |
| Tb427.10.300  | 296          | 254         | 28.4       | MCU |
| Tb427tmp.01.4621 | 255       | 149         | 16.8       | Calmodulin |
| Tb427tmp.03.0475 | 243       | 106         | 12.1       | ATP synthase-associated protein |
| Tb427tmp.47.0022 | 226       | 169         | 20.2       | ATP synthase-associated protein |
| Tb427tmp.02.3610 | 207       | 144         | 16.1       | ATP synthase-associated protein |
| Tb427.10.14820 | 203         | 307         | 34.1       | ANT or TbMCP5 |
| Tb427tmp.02.2510 | 201       | 270         | 29.2       | VDAC |
| Tb427tmp.02.1760 | 176       | 249         | 27.7       | MCU |
| Tb427.04.3450 | 161          | 114         | 13.7       | ATP synthase-associated protein |
| Tb427.10.9830 | 147          | 157         | 17.2       | ATP synthase-associated protein |
| Tb427.03.2880 | 121          | 104         | 12.6       | ATP synthase-associated protein |
| Tb427.03.1690 | 78           | 145         | 17.1       | ATP synthase-associated protein |
| Tb427.03.2180 | 78           | 156         | 17.9       | ATP synthase-associated protein |
| Tb427.07.840  | 60           | 124         | 14.5       | ATP synthase-associated protein |
| Tb427.05.3090 | 57           | 101         | 11.7       | ATP synthase-associated protein |

aMethods used and a complete list of proteins identified are available in the supplemental material (Text S1 and Data Set S1). aa, amino acids. Accession numbers are from TriTrypDB.
bAlso identified by HA-tag IP (Fig. S1C).
growth on the high-stringency selective SD-4DO plates and had high X-Gal activity (Fig. 2B), suggesting that TbMCU interacts strongly with ATP synthase subunit c.

Direct physical MCU-ATPc interaction is conserved in trypanosomes and humans. Like mammals, trypanosomes have 3 isoforms of ATP synthase subunit c. These isoforms differ in their cleavable MTS, whereas their mature proteins are identical in both T. brucei and T. cruzi (Fig. S4A). Since both MCU and ATP synthase subunit c are well conserved in most eukaryotes (47, 48), we investigated whether MCU-ATPc interaction was conserved in T. cruzi and human cells. We also investigated whether the other subunits of the TbMCU complex interacted with ATP synthase subunit c. TbMCUC subunits (TbMCU, TbMCUb, TbMCUc, and TbMC Ud), T. cruzi MCU (TcMCU), and Homo sapiens MCU (HsMCU) were expressed as baits for MYTH assays, while TbATPc (identical to TcATPc) and HsATPc (Fig. S4B) were expressed as preys. Remarkably, each TbMCUC subunit, excluding TbMCUb, strongly interacted with TbATPc, TcMCU interacted with TcATPc, and HsMCU interacted with HsATPc (Fig. 3A and B). The MCU-ATPc interactions were confirmed by reciprocal coimmunoprecipitations (co-IP) (Fig. 3C and D) and
immunofluorescence subcellular colocalization (Fig. 3E and F) using antitag antibodies, anti-VP16 for the bait, and anti-HA for the prey. The lack of TbMCUb-TbATPc interaction was consistent with the absence of coimmunoprecipitation in yeast lysates and also with lack of colocalization of these proteins in the yeast plasma membrane (Pearson coefficient correlation [PCC] of 0.4638), while there was colocalization of TbATPc with TbMCU, TbMCUc, and TbMCUd and of TcATPc and HsATPc with TcMCU and HsMCU, respectively (Fig. 3E and F). However, we cannot rule out that a different topology of TbMCUb could be responsible for this negative result.

**TMHs are determinants of the interactions between TbMCU and TbATPc.** To identify specific interacting domains or motifs of TbMCU that mediate interaction with TbATPc, TbMCU mutants with truncations or substitutions (Fig. 4A) were generated and expressed as baits for MYTH assays, as described previously (10). Deletion of the N- or C-terminal regions of TbMCU (TbMCUΔ1, TbMCUΔ2, and TbMCUΔ3) and mutations of the conserved residues in transmembrane helix 2 (TMH2) (Y235A, F236A, T241E, and Y248A) (TbMCUΔ5) or substitution of TMH2 with the artificial transmembrane “WALP” helix (TbMCUΔ7) did not affect interaction with the TbATPc (Fig. 4C and D), suggesting that the regions flanking the TMHs of TbMCU and the TMH2 are not...
involved in the protein-protein interaction. In contrast, TbMCU mutations of the conserved residues in TMH1 (Q213A, V216P, I217P, and F222A or V216P and I217P), named TbMCUΔ9 and TbMCUΔ10, or replacement of TMH1 with the artificial transmembrane WALP helix (TbMCUΔ6) significantly reduced the interaction with TbATPc (Fig. 4C and D), indicating that the conserved residues V216 and I217 of TMH1 of TbMCU are essential for TbMCU interaction with TbATPc.

Since TbATPc is embedded in the inner mitochondrial membrane and has short mitochondrial intermembrane N- and C-terminal regions, we tested the interactions between its TMHs with TbMCU. To define specific interacting residues or motifs of TbATPc that mediate this interaction, 4 substitution TbATPc mutants (Fig. 4B) were

FIG 4  Determination of specific interactions between the transmembrane domains of TbMCU and TbATPc by mutagenesis and MYTH analyses. (A and B) The scheme depicts the wild-type (WT) and truncated and/or substitution mutant constructs of TbMCU (A) and TbATPc (B). Coil, coiled-coil domain; TM1 and TM2 (black or gray rectangles), transmembrane domains 1 and 2; M, the conserved WDXXEPXTY motif; WALP, artificial TM sequence GWLALALALALALALWLA. Substitutions of the conserved residues of TMH1 or TMH2 of TbMCU or TbATPc are indicated (multiple substitutions were generated, because single substitutions did not significantly alter protein-protein interaction). (C) Growth assay of the yeast NMY51 strain expressing the bait (TbMCU WT and Δ1 to Δ3, Δ5, Δ7, Δ9, or Δ10 mutant) together with the prey (TbATPc WT or Δ1 to Δ4 mutant) on SD selection agar plates as described for Fig. 3A. (D) Quantitative β-Gal activity assay of strain NMY51 coexpressing the bait-prey pairs as described for panel C determined their interaction strength. Each column represents the mean ± standard deviation (n = 3; 6 colonies for each independent experiment). (E) Expression level of each bait or prey as determined by immunoblot analysis using antitag antibodies, VP16 for the bait and HA for the prey, and hexokinase antibodies used as a loading control. TF, transcription factor (LexA-VP16) cleavage (as indicated). First lane, molecular weight markers. (F and G) Fluorescence microscopy images validated proper yeast plasma membrane localization of expressed new baits TbMCUΔ9 and -Δ10 (F) and preys TbATPcΔ1 to -Δ4 (G). Left images are DIC. Scale bars = 5 μm.
generated and expressed as preys for MYTH assays. TbATPc mutations of the conserved residues in TMH2 (F98L, F107L, and F113L, designated TbATPcΔ3, and mutations F98L, E102A, F107L, and F113L, designated TbATPcΔ4), did not affect or slightly affected interaction with the TbMCU (Fig. 4C and D), suggesting that TMH2 of TbATPc is not involved in the protein-protein interactions. In contrast, TbATPc mutations of the conserved residues in the TMH1 (G64A/L, G66A/L, G68/A/L, and G70A/L), named TbATPcΔ1 and TbATPcΔ2, significantly reduced interaction with TbMCU (Fig. 4C and D), indicating that the highly conserved glycine (GXGXGXG) motif in the TMH1 (Fig. S4B) is important for TbATPc interaction with TbMCU.

Expression of the bait-prey pairs in yeast lysates was confirmed by Western blot analyses using anti-VP16 and anti-HA antibodies to detect the baits and preys, respectively (Fig. 4E). The substitutions in the newly generated TbMCU bait mutants (TbMCUΔ9 and TbMCUΔ10) and the TbATPc prey mutants (TbATPcΔ1, TbATPcΔ2, TbATPcΔ3, and TbATPcΔ4) did not alter their plasma membrane localization in yeast, as detected by immunofluorescence microscopy (Fig. 4F and G). Collectively, the mutagenesis and MYTH assays suggested that TMH1s were determinants of the specific interactions between TbMCU and TbATPc.

TMH1-TMH1 mediates specific interaction between trypanosomal MCU and ATPc. To investigate whether the TMH1s also mediate specific interactions between other T. brucei MCU subunits (TbMCUc and TbMCUd) and TbATPc and between TcMCU and TcATPc, the TMH1 substitution mutants of TbMCUc (TbMCUcΔ5 and TbMCUcΔ2), TbMCUd (TbMCUdΔ5 and TbMCUdΔ2), and TcMCU (TcMCUΔ1 and TcMCUΔ2) were used (10) or newly generated and expressed as baits for MYTH assays, while the TMH1 substitution mutants of TbATPc (TbATPcΔ1 and TbATPcΔ2, identical to TcATPcΔ1 and TcATPcΔ2, respectively) were expressed as preys (Fig. 5A). The substitutions of newly generated MCU baits (TbMCUcΔ5, TbMCUdΔ5, TcMCUΔ1, and TcMCUΔ2) did not change their plasma membrane localization, as detected by immunofluorescence microscopy (Fig. 5B). Similar to the TbMCU-TbATPc interaction (Fig. 4C and D and Fig. 5D and E), the substitutions of TMH1 of TbMCUc, TbMCUd, or TcMCU or the substitution of TMH1 of TbATPc significantly disrupted their interactions, and the protein-protein interactions were completely blocked when both TMH1s of MCU and ATPc were mutated (Fig. 5D and E). These results suggest that TMH1 of each TbMCUC subunit or TcMCU specifically interacts with TMH1 of the TbATPc via the conserved residues or motifs of the TMH1s. Expression of the bait-prey pairs in yeast lysates was confirmed by Western blot analyses using anti-VP16 and anti-HA antibodies to detect the baits and preys, respectively (Fig. 5C).

TbMCU complex interacts with the ATP synthase of T. brucei. To confirm the TbMCU-TbATPc interaction in T. brucei, we overexpressed HA-tagged TbATPc1, TbATPc2, or TbATPc3 in PCF 29-13 cell lines (Fig. S5). The 3 isoforms of TbATPc colocalized with TbMCU to the mitochondria of PCF, as determined using anti-HA and anti-TbMCU antibodies (Fig. S5A). TbMCU was immunoprecipitated with the 3 TbATPc isoforms using anti-HA antibodies (Fig. S5B). To confirm that the TbMCU complex interacts with TbATPc, we also generated in situ smMYC-tagged or smV5-tagged TbATPc1 in the previously generated triple-smFP-tagged TbMCUCPCF cell line (10) and in the TAP-tagged TbMCU 29-13 cell line generated in this work, respectively. Coimmunoprecipitations (Fig. 6A and B) and colocalizations (Fig. 6D to I) of TbMCUC subunits (TbMCUc, TbMCUd, or TbMCU) with TbATPc revealed that TbMCU complex interacts with TbATPc in T. brucei. Blue native PAGE (BN-PAGE) and immunodetection analyses with anti-CBP and anti-V5 antibodies indicated that the TbMCU complex physically interacts with mitochondrial ATP synthase subunit c in a large protein complex with a molecular weight of approximately 900 kDa (Fig. 6C). This large protein complex possibly associates with TbANT and TbPiC to form a potential “ATP synthaseosome” in T. brucei.

To obtain additional evidence supporting the interaction of the TbMCU complex with the TbATP synthase of T. brucei, we generated in situ smMYC-tagged or smV5-
tagged TbATP\(\beta\) and TbATP\(\gamma\) \textit{T. brucei} PCF cell lines using the triple-smFP-tagged TbMCUC PCF wild-type (WT) cell line (10) and the TAP-tagged TbMCUC PCF29-13 cell line, respectively. The smMYC-tagged or smV5-tagged TbATP\(\beta\)/TbATP\(\gamma\) colocalized with MitoTracker (MT) to mitochondria of PCF (Fig. S6A to D) and coimmunoprecipitated with TbMCUC and TbMCU (Fig. S6E and F), further confirming that the TbMCU complex interacts with the mitochondrial ATP synthase in \textit{T. brucei}.

**Biological significance of the MCU-ATP\(\gamma\) interaction in \textit{T. brucei}**. The MCU-ATP\(\gamma\) interaction provides evidence that the MCU complex forms part of a functional mega-
complex including the ATP synthase, the ANT, and the PIC, which couples Ca\(^{2+}\)/H\(^{+}\) transport with ATP synthesis. We reported previously that downregulation of \(\text{TbMCU}\) increased the AMP/ATP ratio in \(\text{T. brucei}\) PCF grown in glucose-rich medium and that the ratio increased more significantly in the absence of glucose and presence of proline, when mitochondrial metabolism is more active (10). Similarly, ablation of other \(\text{TbMCUC}\) subunits (\(\text{TbMCUC}\) and \(\text{TbMCUD}\)), with exception of \(\text{TbMCUb}\), by RNA interference (RNAi) resulted in an increased cellular AMP/ATP ratio in the absence of glucose and presence of proline, revealing the downregulation of ATP production in the cells (Fig. 7). These results are compatible with the coupling of Ca\(^{2+}\) transport with ATP synthesis.

**Specific interaction between \(\text{HsMCU}\) and \(\text{HsATPc}\) in vitro and in vivo.** To determine whether TMH1s mediate the specific interaction between \(\text{HsMCU}\) and \(\text{HsATPc}\), \(\text{HsMCU}\) and \(\text{HsATPc}\) mutants (designated \(\text{HsMCUD1}/\text{D2}\) and \(\text{HsATPcD1}\)) were generated (Fig. 8A) and expressed as baits and preys, respectively, for MYTH assays. Similar to the interactions between \(\text{TbMCU}\) and \(\text{TbATPc}\) (Fig. 5D and E), replacements of TMH1s of \(\text{HsMCU}\) and \(\text{HsATPc}\) significantly disrupted or completely blocked their interactions (Fig. 8B and C), suggesting that the TMH1s of both \(\text{HsMCU}\) and \(\text{HsATPc}\) are essential for the protein-protein interaction while they did not alter their expression (Fig. 8D) or plasma membrane localization in yeast (Fig. 8E). To confirm the \(\text{HsMCU-HsATPc}\) interaction in human cells, we carried out reciprocal coimmunoprecipitations of \(\text{HsMCU}\) and \(\text{HsATPc}\) using HEK-293T and HeLa cells, with HEK-293T (MCU-KO) as a

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**FIG 6 Coimmunoprecipitation and colocalization of \(\text{TbMCU}\) complex with \(\text{TbATPc}\) using in situ smFP-tagged ATPc \(\text{T. brucei}\) cell lines.** (A and B) Coimmunoprecipitation of \(\text{TbMCU}\) complex (\(\text{TbMCUC}\)) with smMYC- or smV5-tagged \(\text{TbATPc}\). (A) Cell lysates from the quadruple smFP-tagged (triple-smFP-tagged \(\text{TbMCUC}\) plus smMYC-tagged \(\text{TbATPc}\)) cell line were incubated with anti-MYC or anti-HA antibody, immunoprecipitates were resolved by SDS-PAGE, and input lysates and immunoprecipitates were blotted with antibodies against HA, MYC, or \(\text{TbCyt c}\). The bait proteins (left column, labeled as "Lysate" at the top) and the prey proteins (right column, labeled as "IP" at the top) were detected by Western blotting with the specific antibodies (indicated on the right) using \(\text{TbCyt c}\) as a control. (B) Anti-V5, anti-CBP, and anti-\(\text{TbCyt c}\) immunoprecipitations were performed using lysates from the TAP-tagged \(\text{TbMCU}\) plus smV5-tagged \(\text{TbATPc}\) cell line, and input lysates and immunoprecipitates were blotted with antibodies against CBP, V5, and \(\text{TbCyt c}\). The bait and prey proteins were detected and shown as described for panel A. (C) BN-PAGE analyses of crude PCF mitochondrial vesicles from the TAP-tagged \(\text{TbMCU}\) and TAP-tagged \(\text{TbMCUC}\) plus smV5-tagged \(\text{TbATPc}\) cell line. Immunoblot analyses were performed using antibodies against CBP or V5. Antibodies against \(\text{TbCyt c}\) were used as a loading control but detected on an SDS-PAGE gel. Arrowheads indicate four dominant bands at 250 to 900 kDa, representing \(\text{F}_{0}\)-\(\text{F}_{1}\)-\(\text{MCUC}\) (\(\text{F}_{0}\)-\(\text{F}_{1}\)-\(\text{MCUC}\)), \(\text{F}_{<}\)-ring-\(\text{TbMCUC}\) (\(\text{F}_{<}\)-ring-\(\text{MCUC}\)) dimer, \(\text{F}_{<}\)-\(\text{ATP}\) synthase-\(\text{TbMCUC}\) (\(\text{F}_{<}\)-\(\text{MCUC}\)), and \(\text{F}_{<}\)-ring-\(\text{TbMCUC}\) (\(\text{F}_{<}\)-ring-\(\text{MCUC}\)), respectively. Markers are shown on the left, and the antibodies used in immunoblots are shown at the top. The similar patterns of protein complexes (arrowheads) were detected but shifted approximately 160 kDa in size (from the left blot to the middle and right blots) when \(\text{TbATPc}\) was tagged with smV5 (with a molecular weight of 44.2 kDa), indicating that 4 tagged subunits c were probably incorporated into the complexes of the smV5-tagged \(\text{TbATPc}\) cell line (middle and right blots). (D to F) Colocalization of smMYC-tagged \(\text{TbATPc}\) with MitoTracker (MT) (D), with smHA-tagged \(\text{TbMCUC}\) (E), and with smV5-tagged \(\text{TbMCUD}\) (F) (PCCs of 0.8500, 0.7425, and 0.8110, respectively). (G to I) Colocalization of smV5-tagged \(\text{TbATPc}\) with MT (G), with \(\text{TbMCU}\) (H), and with TAP-tagged \(\text{TbMCUD}\) (I) (PCCs of 0.8771, 0.8339, and 0.8390, respectively). DIC, differential interference contrast microscopy. Scale bars, 10 µm. The merged images indicate colocalization (in yellow).
negative control. The cells were lysed (Fig. 8F) and immunoprecipitated under native conditions with anti-HsMCU or anti-HsATPc antibodies. HsATPc was pulled down with HsMCU using anti-HsMCU, and HsMCU was pulled down with HsATPc using anti-HsATPc, but human heat shock protein 70 (HsHsp70) was not immunoprecipitated with any of the antibodies (Fig. 8G). Furthermore, neither HsMCU nor HsATPc was pulled down from the MCU-KO lysate using these antibodies (Fig. 8G). These results confirm that HsMCU specifically interacts with HsATPc both in the yeast MYTH reporter strain and in human cells.

DISCUSSION

Our studies revealed that three of the T. brucei MCU subunits (TbMCU, TbMCUc, and TbMCUd) physically interact with mitochondrial ATP synthase subunit c (TbATPc) when expressed in yeast membranes. This interaction, which is also observed with the T. cruzi subunits, is mediated by the conserved residues of TMH1s of both MCU and ATPc subunits and was confirmed by their coimmunoprecipitation in yeast lysates and by their colocalization, as detected by immunofluorescence analysis (IFA). The interaction of the TbMCUC with TbATPc was confirmed in trypanosomes in vivo by their coimmunoprecipitation from lysates of the parasite after the in situ tagging of the subunits, by their colocalization, as detected by IFA, and by blue native PAGE and immunodetection analysis. This interaction leads to the pulldown of the ATP synthase complex together with the adenine nucleotide translocator (ANT) and phosphate carrier (PIC) by TbMCU. This pulldown reveals the formation of a potential TbMCUC-ATP synthase “mega-complex” important for the bioenergetics of the cells, as suggested by the increase in the AMP/ATP ratio after downregulation of the TbMCU subunits by RNAi (Fig. 7). We also demonstrated that this interaction is conserved in human cells when HsMCU and HsATPc are expressed in yeast membranes or when they are coimmunoprecipitated from HEK-293 and HeLa cell lysates using specific antibodies.

ATP synthase subunit c is very conserved and it arranges in the so-called c ring, which is an essential component of the F$_{0}$ rotor (reviewed in reference 49). Although the number of c subunits forming the c ring is constant in a given species, it can be in the range 8 to 15, being smaller in eukaryotes than in prokaryotes (49). In eukaryotes,
the inner helix (TMH1) has the highly conserved motif of four glycine residues (GXGXGXG) that we found is necessary for interaction with TMH1 of the MCU subunits of trypanosomes and human cells when expressed in yeast membranes. The outer helix (TMH2) has been proposed to transport H\(^{+}\)/H\(_{1}\) when the c ring rotates counterclockwise (viewed from the matrix) (50). Our MYTH results revealed that TMH1 of each TbMCU, except for TbMCUb, interacts with TMH1 of TbATPc, suggesting that, if this interaction occurs in mitochondria in situ, the TbMCU complex is within the c-ring of the ATP synthase in T. brucei (see Fig. S6G and H in the supplemental material). The c ring is reported to surround an internal phospholipid-containing cavity in bacteria (51), while a recent report on the cryo-electron microscopy (cryo-EM) structure of the porcine ATP synthase (52) identified a helical density in the center of the c8 ring that the authors attributed to subunit 6.8PL. However, they indicated that a structure with better-defined density will be needed to establish the identity of this protein (52). It would be interesting to know whether this helical density corresponds to the MCU.

It has not escaped our notice that the interaction between MCU and the c subunit immediately suggests a potential role for the MCU complex in the formation of the mitochondrial permeability transition pore (mPTP). The mPTP has long been considered a mediator of cell death mechanisms in mammals and as an alternative mechanism to the mitochondrial (intrinsic) pathway of apoptosis (53). The mPTP is a high-
conductance nonselective channel located at contact sites between the inner and outer mitochondrial membranes. Its molecular composition is not yet clear, although several proteins have been shown to be components of this channel, including voltage-dependent anion channels (VDAC), ANT, PiC, cyclophilin D (CypD), and other proteins, such as spastic paraplegia 7 (SPG7) (54) and dimers of the ATP synthase (55). PTP opening can be enhanced by Ca\textsuperscript{2+} overload, oxidative stress, thiol oxidation, pyridine nucleotide oxidation, alkalinization, or low transmembrane potential and is inhibited by cyclosporine, which binds to cyclophilin D (55). Opening of this pore leads to mitochondrial dysfunction and cell death by either apoptosis or necrosis (53). Recent work on the components of the mPTP has implicated the c ring of the ATP synthase (56, 57), a channel inside ATP synthase dimers (55, 58), or the purified ATP synthase itself (59), as forming the pore. Interestingly, vestigial ATP synthases devoid of c subunits maintain mPTP formation features, suggesting that the c ring per se is not the channel (60).

Our results on the interaction of TbMCU with the ATP synthasome suggest that this “megacomplex” couples ADP and P\textsubscript{i} transport with ATP synthesis, a process that is stimulated by Ca\textsuperscript{2+}. Several subunits of ATP synthase appear to be Ca\textsuperscript{2+} regulated. Territo et al. (61, 62) suggested a direct activation of the ATPase by Ca\textsuperscript{2+} with a \(K_{\text{m}}\) of 200 nM, well within the physiological range. Subunit c of Fo ATP synthase from chloroplasts and bacteria was identified as a calcium-binding protein and proposed to be involved in Ca\textsuperscript{2+} gating of the Fo proton (H\textsuperscript{+}) channel (63). The catalytic \(\beta\) subunit of Fo complex from mammalian mitochondrial ATP synthase was also identified as a calcium-binding protein in two studies (64, 65). One proposed the Ca\textsuperscript{2+} binding as a potential mechanism for the Ca\textsuperscript{2+}-dependent regulation of ATP synthesis (64), and the other suggested the binding as a trigger for the mitochondrial permeability transition (65).

Transient knockdown of HsMCU by small interfering RNA (siRNA) resulted in a 3-fold increase of the AMP/ATP ratio in HeLa cells (26). Our work shows that RNAi of TbMCUc or TbMCU\(\text{ld}\) significantly increased the cellular AMP/ATP ratio, similar to what occurs upon knockdown/knockout of other trypanosome MCUC subunits in both \(T.\) brucei (66) and \(T.\) cruzi (11, 67). These results indicate that ATP production is tightly regulated by mitochondrial Ca\textsuperscript{2+} uptake in both trypanosomes and human cells.

In conclusion, coupling of MCU complex with the mitochondrial ATP synthasome is a novel mechanism for Ca\textsuperscript{2+}-dependent regulation of ATP synthesis. Elucidation of the MCUC-ATP synthasome “megacomplex” will significantly advance our understanding of mitochondrial physiology.

**MATERIALS AND METHODS**

**TAP-tagged TbMCU cell line.** To construct C-terminally TAP-tagged TbMCU for tandem affinity purification of the TbMCU complex, the full-length cDNA of TbMCU without the stop codon was amplified from \(T.\) brucei genomic DNA by PCR using the primers TbMCU-TAP-F and TbMCU-TAP-R (see Data Set S2A in the supplemental material), digested with BamHI and HindIII, and then cloned in frame into the enzyme-cut pLew79-MH-TAP vector (68) to generate pLew79-MH-TAP (TbMCU) (Data Set S2B).

The recombiant construct pLew79-MH-TAP (TbMCU) was confirmed by sequencing at the DNA Analysis Facility at Yale University (New Haven, CT), NotI linearized, and then purified with Qiagen’s DNA purification kit for cell transfections. After transfection, phleomycin-resistant clones were selected and checked for tetracycline-regulated expression of TAP-tagged TbMCU (Fig. 1B), which is composed of a protein A domain separated from a calmodulin-binding peptide (CBP) sequence by a TEV protease cleavage site (Fig. 1A).

**Purification of epitope-tagged TbMCU complex.** TAP- or HA-tagged TbMCU was expressed by induction with tetracycline (200 ng/ml of culture) for 48 h. The tagged proteins and associated complexes were purified from 600 ml of cells harvested at a density of \(2 \times 10^7\) cells per ml by IP or tandem affinity chromatography (28, 41). First, the harvested cells \((\sim 1.2 \times 10^8\) cells in total\) were washed once in phosphate-buffered saline (PBS) with 6 mM glucose and lysed with 1% Triton X-100 in 18 ml of ice-cold IPP150 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40) with two dissolved tablets of Complete, EDTA-free protease inhibitor cocktail (Roche) on ice for 30 min. Next, the lysate was cleared of debris by centrifugation at 15,000 \(\times g\) for 15 min at 4°C and the supernatant (cleared lysate) containing soluble proteins and tagged-TbMCU complex was further purified by using two complementary methods as described below.

TAP-tagged TbMCU complex was purified by a tandem affinity purification approach as previously described (28, 41), with some modifications (Fig. 1B). The cleared lysate from TAP-tagged TbMCU cell culture was incubated with 300 ml of IgG-Sepharose 6 Fast Flow beads (Pharmacia) overnight with gentle rotation at 4°C. The TAP-tagged proteins bound to IgG-Sepharose were washed three times in a
Poly-Prep chromatography column (Bio-Rad) with 20 ml of IPP150, following equilibration by washing once in 10 ml of TEV cleavage buffer (IPP150, 0.5 mM EDTA, 1 mM dithiothreitol [DTT]). The protein-bead mix was resuspended in 1 ml of TEV buffer containing 10 μl of AcTEV protease (Invitrogen; 10 units/μl) and then incubated at 16°C for 2 h with constant mixing. After collection of the TEV eluate, beads were briefly washed with 0.5 ml of TEV buffer, and the washing was combined with the eluate. The total eluate (1.5 ml) containing tagged proteins and associated complexes was then diluted in 3 volumes of calmodulin binding buffer (IPP150, 10 mM fresh 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl2) and 0.003 volume of 1 M CaCl2 and incubated with 200 μl of calmodulin resin (Stratagene). The mixture was incubated for 1 h with gentle rotation at 4°C and then washed in the same binding buffer. Fractions were eluted with EGTA elution buffer (same as calmodulin binding buffer but containing 2 mM EGTA instead of CaCl2). Eluted fractions were stored in aliquots at –80°C until use.

HA-tagged TbMCU complex was purified by an HA tag IP approach (Fig. S1A) using a Pierce HA tag IP/co-IP kit (Thermo Scientific) according to the manufacturer’s instructions. Briefly, the cleared lysate (supernatant) from an HA-tagged TbMCU cell line (9) was incubated with 200 μl of anti-HA–agarose slurry (350 μl of antibody) overnight with gentle rotation at 4°C. The agarose-bound complex was precipitated by centrifugation at 1,600 g for 5 min at 4°C, resuspended in 850 μl of ice-cold IPP150 with 1× Complete, EDTA-free protease inhibitor cocktail (Roche), and then transferred to a Pierce spin column. The protein-agarose mix was centrifuged at 16,000 g for 10 s, washed once in IPP150, and then washed three times in 0.5 ml of TBS-T (25 mM Tris-HCl, 0.15 M NaCl [pH 7.2], 0.05% Tween 20) by mixing and centrifugation. HA-tagged proteins and associated complexes were eluted in 50 μl of elution buffer (pH 2.8) by centrifugation and immediately neutralized by adding 2.5 μl of Tris (pH 9.5).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.
TEXT S1, DOCX file, 0.1 MB.
FIG S1, PDF file, 0.8 MB.
FIG S2, PDF file, 1.2 MB.
FIG S3, PDF file, 0.6 MB.
FIG S4, PDF file, 1.3 MB.
FIG S5, PDF file, 1.3 MB.
FIG S6, PDF file, 1.1 MB.
FIG S7, PDF file, 0.4 MB.
DATA SET S1, XLS file, 0.1 MB.
DATA SET S2, XLSX file, 0.03 MB.

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G.H. and R.D. designed the experiments. G.H. performed and analyzed the experiments. G.H. and R.D. wrote the paper, and R.D. analyzed the experiments, supervised the work, and secured funding.
We declare no competing interests.

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