Dual Functions of α-Ketoglutarate Dehydrogenase E2 in the Krebs Cycle and Mitochondrial DNA Inheritance in *Trypanosoma brucei*

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The dihydrolipoyl succinyltransferase (E2) of the multisubunit α-ketoglutarate dehydrogenase complex (α-KD) is an essential Krebs cycle enzyme commonly found in the matrices of mitochondria. African trypanosomes developmentally regulate mitochondrial carbohydrate metabolism and lack a functional Krebs cycle in the bloodstream of mammals. We found that despite the absence of a functional α-KD, bloodstream form (BF) trypanosomes express α-KDE2, which localized to the mitochondrial matrix and inner membrane. Furthermore, α-KDE2 fractionated with the mitochondrial genome, the kinetoplast DNA (kDNA), in a complex with the flagellum. A role for α-KDE2 in kDNA maintenance was revealed in α-KDE2 RNA interference (RNAi) knockdowns. Following RNAi induction, bloodstream trypanosomes showed pronounced growth reduction and often failed to equally distribute kDNA to daughter cells, resulting in accumulation of cells devoid of kDNA (dyskinetoplastic) or containing two kinetoplasts. Dyskinetoplastic trypanosomes lacked mitochondrial membrane potential and contained mitochondria of substantially reduced volume. These results indicate that α-KDE2 is bifunctional, both as a metabolic enzyme and as a mitochondrial inheritance factor necessary for the distribution of kDNA networks to daughter cells at cytokinesis.

The α-keto acid dehydrogenases are multienzyme assemblies that catalyze the decarboxylation of their respective α-keto acids, which subsequently produce acyl-coenzyme A (CoA) and NADH (1). This family of high-molecular-mass (greater than 1 MDa) complexes consists of pyruvate dehydrogenase (PDH), branched-chain α-keto acid dehydrogenase (BCKAD), and α-ketoglutarate dehydrogenase (α-KD). Each complex is composed of an α-keto acid dehydrogenase subunit (E1), an acyltransferase subunit (E2), and a dihydrolipoamide dehydrogenase subunit (E3). Multiple copies of E2 form the cores of these multimeric complexes, and the remaining components (E1 and E3) surround this structure (1). In each case, the highly conserved E2 has a similar quaternary structure with a covalently attached lipoic acid prosthetic group that swings from one active site to the next (2, 3).

Although E2 has a vital role in α-KD metabolism, the enzyme has also been shown to associate with prokaryotic genomes and the mitochondrial DNA (mtDNA) of multiple eukaryotic organisms (4–9). mtDNA is organized into stable protein-DNA units called nucleoids attached to the mitochondrial inner membrane and is involved in genome replication, segregation, and maintenance (10, 11). Proteins isolated from mtDNA nucleoids from higher eukaryotes revealed the association of the E2 enzymes from the PDH and BCKAD complexes (6). Additionally, α-KD E2 (α-KDE2) associates with the mtDNA nucleoid of *Saccharomyces cerevisiae* and is required for mtDNA maintenance (4, 5).

The protozoan parasite *Trypanosoma brucei* possesses a distinctive mitochondrial genome termed the kinetoplast DNA (kDNA). The kDNA is a large network structure composed of thousands of catenated DNA minicircles (~1 kb) and approximately 50 maxicircles (~20 kb). Maxicircles contain the coding information for components of the oxidoreductase complexes, ATP synthase, and rRNAs, while minicircles encode small guide RNAs (gRNAs) necessary for posttranscriptional editing of maxicircle-encoded mRNAs (12).

Mitochondrial metabolism and ATP production are developmentally regulated in *T. brucei*. In mammals, the bloodstream developmental forms of trypanosomes (BF) utilize high rates of glycolysis to produce ATP and pyruvate but lack functional Krebs cycle enzymes and mitochondrial respiratory complexes needed for oxidative phosphorylation (13). Thus, all ATP is produced by glycolytic substrate level phosphorylation. In the midgut of the insect vector, the tsetse fly, trypanosomes rapidly differentiate into the procyclic developmental form (PF) and develop a fully functional mitochondrion capable of converting proline to succinate and transferring reducing equivalents to NADH via the Krebs cycle enzymes (14).

Despite the lack of conventional oxidative phosphorylation in the BF, the kDNA must be faithfully replicated and segregated, since it encodes enzymes needed in the PF and other developmental stages in the tsetse fly (15–18). A number of structural proteins and replication enzymes necessary for kDNA replication and segregation have been identified (19, 20), and the mechanism of kDNA replication has been described in detail (21). During the mitochondrial S phase, minicircles are released from the kDNA network by a type II topoisomerase and relocate to the kinetoflagellar zone (KFZ), a region between one face of the kDNA disk and the basal bodies of the flagellum (22). Next, free minicircles bind the universal binding protein (UBP) at discrete foci in the KFZ where replication initiates, giving rise to replicative intermediates containing theta structures (23, 24). Replicating minicircles migrate to antipodal sites on the elongated kDNA network where single-strand gaps from lagging-strand replication are filled and...
the newly replicated minicircles, containing a single gap at the origin of replication, are reattached to the network (11, 25–27). RNA interference (RNAi) knockdown or conditional knockouts of several of the key replicative enzymes results in a rapid loss of minicircles (27–29). Complete kDNA replication results in a network with twice the number of minicircles and maxicircles that undergoes lateral elongation and finally segregation to daughter cells, ensuring equal inheritance of minicircles and maxicircles.

While the mechanism of network segregation is poorly understood, the association of the kDNA with a specialized portion of the mitochondrial membrane, immediately adjacent to the basal bodies of the flagellum, is required for kDNA segregation (11, 30). The association of the kDNA with the basal bodies has been defined morphologically as a filamentous network of proteins known as the tripartite attachment complex (TAC) (11). The TAC maintains a physical connection between the kDNA and flagellum and fractionates with a larger flagellum-kDNA complex (FKC) upon cell solubilization with nonionic detergents (11, 31). The TAC extends from the basal bodies of the flagellum to the outer mitochondrial membrane (exclusion zone filaments) and continues across the inner mitochondrial membrane to the KFZ face of the kDNA (unilateral filaments). To date, only two components of the TAC have been identified. (i) p166 was the first protein component identified in the TAC (30). This large nuclear-encoded protein was initially discovered during an RNAi library screen for kDNA replication/segregation defects (30). p166 contains an N-terminal mitochondrial import signal, as well as a short transmembrane domain, and associates with the unilateral filaments of the TAC. The RNAi knockdowns of p166 resulted in altered kDNA structure due to asymmetric segregation of the kDNA, but no effect of kDNA replication was observed (30). (ii) Alternatively edited protein 1 (AEP-1), encoded by an alternatively edited cytochrome oxidase III (COIII) mRNA, is a chimeric protein with a unique N-terminal 60-amino-acid DNA binding domain and five C-terminal transmembrane domains of COIII (32). AEP-1 associates with the unilateral filaments of the TAC, where it serves as a maintenance factor for kDNA (33).

The structural organization of the kDNA network has given rise to unique mechanisms of replication, segregation, and inheritance of this mitochondrial genome. Central to these processes is the FKC, which ensures segregation of the newly replicated kDNA networks and distribution to daughter cells at cytokinesis. Considering the complexity of the FKC, it is likely that both structural and motor proteins, necessary for segregation and inheritance, remain to be defined (11, 21). In this study, we report the expression of α-KDE2 in the oxidative-phosphorylation-deficient BF T. brucei. We show that α-KDE2 is expressed and localizes to the BF mitochondria, despite the lack of α-KD activity. Surprisingly, we found that α-KDE2 localized to antipodal sites on the kDNA network and is a stable component of the FKC. RNAi knockdown of α-KDE2 results in increased numbers of dyskinetoplastic cells with a corresponding increase of cells with two kinetoplasts, consistent with a role for α-KDE2 in the distribution of kDNA at cytokinesis and not in either segregation or replication. Finally, depletion of α-KDE2 results in collapse of the mitochondrial membrane potential in dyskinetoplastic cells and a reduction in total mitochondrial volume. These data demonstrate the importance of α-KDE2 as a bifunctional protein necessary for the maintenance of the kDNA and mitochondria in T. brucei.

**MATERIALS AND METHODS**

**Cell culture.** Procyclic-form T. brucei was grown in Cunninghams’s (SM) medium supplemented with fetal bovine serum (FBS) (Gemini Bioproducts, West Sacramento, CA). Bloodstream form T. brucei was maintained in HMI-9 medium containing FBS and Serum Plus medium supplement (SAFC Biosciences, Lenexa, KS). The α-KDE2 RNAi cell line was cultured in HMI-9 medium that was supplemented with tetracycline-free FBS.

**Construction of α-KDE2-PTP and α-KDE2 RNAi cell lines.** Primers 5′-GGGCCCCAAGATACACTTCCAGAGGCGAC-3′ and 5′-GGCGGCCGCGGTTGTCGATCGCACAGA-3′ against the α-KDE2 (Tb1i.01.3550) open reading frame (ORF) amplified 912 bp of Pf- and BF-667 genomic DNA, which was subsequently cloned into the pC-PTP-NEO expression vector (34). The fragment was digested with Apal and NotI for insertion into the vector. Constructs were linearized with a unique restriction site for transfection into the two T. brucei cell types. For the α-KDE2 RNAi cell line, primers 5′-CCCCGAGGAGGCTACGACATTCAACGAGA-3′ and 5′-CCAGCCTTCTCATGGTGTTGAGGATA-3′ were used to amplify a partial α-KDE2 sequence (425 bp) from BF-9013 genomic DNA that was ligated into the inducible pZIP RNAi vector (35). NotI was used to linearize the construct for transfection. All bloodstream constructs were transfected using the Lonza nucleofector system (Lonza, Walkersville, MD). The procyclic α-KDE2-PTP construct was transfected using the Bio-Rad electroporation system (Bio-Rad, Hercules, CA).

**Fractionation of mitochondrial proteins.** Cultured PF T. brucei (TRUE667) and BF T. brucei (TRU667) cells, isolated from infected Sprague-Dawley rats, were hypotonically lysed, and mitochondria were purified by a previously described method (36). Subcellular fractionation was performed as discussed previously with minor modifications (32, 37). Matrix proteins were purified by incubating mitochondria in 0.5% (vol/vol) Triton X-100, 20 mM HEPES-NaOH (pH 7.6) with 1× Complete protease inhibitor cocktail (Roche Indianapolis, IN) for 45 min on ice. Insoluble material was separated from the matrix fraction by centrifugation at 12,000 × g for 10 min at 4°C. The membrane fraction was collected after purification of the matrix proteins by incubation of the insoluble fraction in 2% (wt/vol) n-dodecyl-β-D-maltoside (Sigma, St. Louis, MO), 50 mM NaCl, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, and 1× Complete EDTA-free protease inhibitor cocktail, pH 7, at 4°C for 1 h on ice. An insoluble fraction was collected by centrifugation at 13,000 × g for 20 min at 4°C, and the soluble fraction was saved for subsequent analysis. The total cell, cytosolic, total mitochondrial, matrix, and membrane fractions of PF and BF T. brucei were denatured in a reducing SDS loading buffer, applied equivalently (2×10⁶ cells) to gels, and resolved by SDS-PAGE. The gels were either stained with Coomassie blue or analyzed by Western blotting.

**BrdU analysis.** The bromodeoxyuridine (BrdU) assay was performed as previously described (38, 39) with few modifications. BF cells (2 × 10⁵) were incubated with 5 μM 5-bromo-2-deoxyuridine (Sigma) and 50 μM 2-deoxyctydine (Sigma) at 37°C for 30 and 480 min. The cells were washed in medium, dried on a slide, and fixed in methanol (−20°C) for 30
min. After fixation, the cells were rehydrated in phosphate-buffered saline (PBS) and pretreated with 2.0 M HCl for an additional 30 min. The slides were neutralized in PBS (three 5-min washes) and prepared for immunofluorescence assay. For FKC localization, slides were fixed with methanol for 10 min and treated with ice-cold 2% paraformaldehyde on the slide for 5 min. Next, the slides were heated at 90°C in 10 mM sodium citrate (pH 6.0) for 10 min. The slides were removed from the bath, allowed to cool to room temperature (27°C), and washed with PBS for immunofluorescence assay.

**Immunofluorescence microscopy.** Log-phase cultured α-KDE2-PTP BF and PF cells were incubated with MitoTracker (Life Technologies, Grand Island, NY), washed, equilibrated with medium for 30 min, and attached to poly-L-lysine slides for 30 additional minutes. The cells were fixed with 0.5% paraformaldehyde for 1 min, washed in PBS, and permeabilized with ice-cold 0.05% Triton X-100 in PBS for 5 min at 4°C. The slides were washed and blocked using 20% fetal bovine serum in PBS. Cells were incubated with protein C polyclonal antibodies (1:200) diluted in blocking buffer and remained in primary antibody for 1 h. The slides were washed, and the cells were incubated with appropriate secondary antibody (1:500) for 30 min. For FKCs, intact and DNaase-treated structures were dried on a slide and fixed with methanol (−20°C) for 10 min. The slides were washed and blocked using 20% fetal bovine serum in PBS. The slides were blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min, and the following primary antibodies were diluted in the blocking buffer and added: monoclonal rat YLI1/2 (1:1,000) and polyclonal rabbit protein C (1:200). The slides were washed in blocking buffer, and an appropriate secondary antibody (1:500) was used, BF α-KDE2 RNAi cells were washed in medium, dried on the slide, and fixed in methanol for 10 min. BrdU-treated FKCs were blocked in 0.5% BSA in PBS for 30 min and incubated with polyclonal rabbit primary C (1:200) and monoclonal mouse BrdU (1:100) primary antibodies diluted in blocking buffer for 90 min. The slides were washed in blocking buffer and incubated in appropriate secondary antibody (polyclonal, 1:500; monoclonal, 1:100) for 1 h. After the secondary antibody, all slides were rinsed in PBS and coated with 4′,6′-diamidino-2-phenylindole (DAPI) containing the anti-fade reagent ProlongGold (Life Technologies). Images were acquired using a Zeiss Axio Observer inverted microscope equipped with an AxioCam HSm and evaluated with AxioVision v4.6 software (Zeiss).

**Activity of the α-KD.** Purified intact PF and BF mitochondria were solubilized in buffer containing 2% (wt/vol) n-dodecyl-β-D-maltoside, 50 mM NaCl, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, and 1× Complete EDTA-free protease inhibitor cocktail on ice for 1 h and centrifuged at 13,000 × g for 20 min. Soluble mitochondrial homogenate (40 μg) was incubated in 1 ml of α-KD assay buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM CaCl2, 0.05 mM EDTA, 0.5 mM thiamine pyrophosphate, 1 mM MgCl2, 3 mM α-ketoglutarate, 3 mM NAD+, and 0.75 mg/ml of coenzyme A at 25°C). The activity of α-KD was monitored spectrophotometrically by the production of NADH per minute at 340 nm.

**Northern and Southern analyses.** All radiolabeled probes were prepared using a Prime-It random primer-labeling kit (Stratagene, Santa Clara, CA). For Northern analysis, total RNA was extracted from both developmental stages using TriPure Isolation Reagent (Roche). Total RNA was separated on a 7% formaldehyde-1% agarose gel, blotted to a membrane, and evaluated with radiolabeled probes generated from ORF-equivalents) was analyzed by Northern blot hybridization with probes specific for α-KDE1 (~3.0 kb), α-KDE2 (~1.1 kb), and E3 (~1.4 kb) (Fig. 1A). Hybridization was normalized relative to the rRNA ethidium stain for each lane (Fig. 1A). Consistent with published RNA-Seq results, the mRNAs of α-KDE1, α-KDE2, and E3 were expressed in both BF and PF trypanosomes (45) (Fig. 1A). The developmental regulation of nuclear-encoded mitochondrial proteins in *T. brucei* occurs principally at the level of RNA stability and, to a lesser extent, by differential protein stability (41, 42). High-throughput RNA sequencing (RNA-Seq) provides an accurate evaluation of the life cycle-dependent expression of mitochondrial proteins (43–46). Genomic-wide analysis of mRNA levels indicated that the expression of α-KDE1, α-KDE2, and E3 was not developmentally regulated in BF and PF trypanosomes (45, 46). Unlike α-KDE1 and α-KDE2, the E3 subunit is a component of four distinct multienzyme mitochondrial complexes encoded by a single trypanosome gene (47). Therefore, sequence analysis alone cannot be used to determine whether E3 mRNAs encode subunits of the α-KD or other enzyme complexes (46). To verify the RNA-Seq results, total RNA from procyclic and bloodstream trypanosomes (1 × 107 cell equivalents) was analyzed by Northern blot hybridization with probes specific for α-KDE1 (~3.0 kb), α-KDE2 (~1.1 kb), and E3 (~1.4 kb) (Fig. 1A). Hybridization was normalized relative to the rRNA ethidium stain for each lane (Fig. 1A). Consistent with published RNA-Seq results, the mRNAs of α-KDE1, α-KDE2, and E3 were expressed in both BF and PF trypanosomes (45) (Fig. 1A). The activity of α-KD is absent in BF *T. brucei*. Since previous studies have shown that BF *T. brucei* lacks metabolically active Krebs cycle enzymes, the presence of α-KDE1 and α-KDE2 mRNA expression in both BF and PF trypanosomes was unexpected. However, E3 has previously been shown to be essential in BF trypanosomes (47). We therefore next examined these cells for α-KD activity. This mitochondrial enzyme complex specifically converts α-ketoglutarate to succinyl-CoA and subsequently reduces NADH to NAD+ and CO2. This nicotinamide conversion can be measured spectrophotometrically for α-KD activity when α-ketoglutarate is added as a substrate. At 340 nm, the procyclic mitochondrial lyses, but not BF mitochondrial lyses, showed increasing production of NADH (Fig. 1B). To determine whether the lack of α-KD activity in BF trypanosomes was due to an endogenous inhibitor of α-KD in the BF mitochondrial fraction, equal amounts of BF and PF mitochondrial lyses were mixed and added to the same α-KD assay mixture. Since the total protein concentration during this analysis was kept constant, a 2-fold reduction in the NADH produced (µmol/min) was expected if no enzyme antagonists were present (Fig. 1B). Lack of inhibition by the BF lyses indicates that
α-KDE1, α-KDE2, and E3, while expressed in BF mitochondria, do not assemble into a functional enzyme complex, suggesting alternative functions for these proteins in BF trypanosomes. Evidence for the bifunctionality of α-KDE2 in other organisms led us to examine the function of the protein in BF trypanosomes (10).

**α-KDE2 distribution in the mitochondria of trypanosomes.** Krebs cycle-associated α-KDE2 is present in a soluble complex within the mitochondrial matrix. However, a fraction of E2 transferases (α-KDE2, PDHE2, and BCKADE2) has also been shown to be associated with DNA nucleoids attached to the mitochondrial membranes of other cells (5–7). To evaluate the localization of α-KDE2 in *T. brucei*, a C-terminally epitope-tagged version of the protein (α-KDE2-PTP) was constructed (34, 48). Expression of the BF and PF (α-KDE2-PTP) tagged transcripts from a single allele was confirmed by Northern analysis, which identified 1.5-kb (α-KDE2-PTP mRNA) and 1.1-kb (endogenous α-KDE2 mRNA) bands in both cell types (Fig. 2A). Though inserted into the endogenous α-KDE2 locus, α-KDE2-PTP expression was approximately 2-fold higher than that of wild-type (WT) α-KDE2 and is possibly a result of increased mRNA stability due to the 3′ untranslated region (UTR) sequences flanking the PTP coding sequences (Fig. 2A) (34).

We next examined the intracellular localization of α-KDE2-PTP by immunofluorescence microscopy (Fig. 2B). Using an antibody against the protein C epitope, α-KDE2-PTP distribution was compared with MitoTracker Red staining (Fig. 2B). Within the large and highly branched mitochondria of the PF trypanosomes, the α-KDE2-PTP and MitoTracker staining were superimposed (Fig. 2B, top). Similarly, while the BF mitochondrion was reduced to a single, largely unbranched tubular structure, MitoTracker and α-KDE2-PTP colocalized throughout the organelle (Fig. 2B, bottom), despite the lack of α-KD activity (Fig. 1B and 2B).

Subcellular fractionation studies confirmed the mitochondrial localization of α-KDE2 in both BF and PF *T. brucei*. In Western blot studies, total cell lysates from nontransfected BF and PF cells (WT) did not contain proteins that cross-react with the antibody (α-protein A) used to detect α-KDE2-PTP. Total BF and PF protein from α-KDE2-PTP-expressing cells (TC) and cellular fractions enriched in cytosol (CY), total mitochondria (TM), mitochondrial matrix (MA), and mitochondrial membrane (ME) were resolved by SDS-PAGE and analyzed by Western blotting with antibodies to visualize α-KDE2-PTP and the basal bodies of the flagellum, respectively. The nucleus and kinetoplast were stained with DAPI (Fig. 3A). The majority of the α-KDE2-PTP signal was exclusively detected in mitochondrial fractions relative to the cytoplasmic HSP-70 and the mitochondrial matrix protein MTP-70. Examination of the submembranous localization of α-KDE2-PTP revealed that approximately 25% localized with the mitochondrial membrane markers, Rieske iron-sulfur protein (ISP) for PF and the alternative oxidase (TAO) for BF (Fig. 2C and D). These results confirmed the localization of α-KDE2-PTP to the mitochondria of PF and BF *T. brucei* and suggested similar submembranous localization in the two developmental stages. To further evaluate the alternative function(s) of α-KDE2 in trypanosome mitochondria, we focused our studies on BF trypanosomes, where the α-KD enzymatic activity is absent.

**α-KDE2 associates with the FKC.** The kDNA network of trypanosomes is anchored to the base of the flagellum by the TAC. This association with the mitochondrial membrane is reminiscent of the attachment of the mitochondrial nucleoid in diverse organisms. Since α-KDE2 is distributed in both the mitochondrial matrix and membranes, it was not possible to determine whether there was a specific association with the kDNA using intact cells. To release the mitochondrial matrix and membrane-associated α-KDE2-PTP, cells mildly fixed with paraformaldehyde were treated with low concentrations of Triton X-100 (0.25%) to create cell ghosts. This treatment permeabilizes many subcellular organelles, including the mitochondrion, while leaving the DNA-associated cytoskeleton intact (31) (Fig. 3A). The cell ghosts were subsequently examined by immunofluorescence microscopy using anti-protein C and anti-tyrosinated α-tubulin (tyr-α-tubulin) antibodies to visualize α-KDE2-PTP and the basal bodies of the flagellum, respectively. The nucleus and kinetoplast were stained with DAPI (Fig. 3A). The majority of the α-KDE2-PTP signal was depleted in these preparations, leaving a prominent structure in close proximity to the kDNA and basal bodies. Three-dimensional reconstruction (3D-R) images further support the close proximity of α-KDE2-PTP to the kDNA and basal body (Fig. 3A).

Since kDNA replication involves the detachment, duplication,
and reattachment of minicircles, the kDNA network grows laterally until it is twice the size of the kDNA network in nonreplicating cells. The newly replicated kDNA network segregates, and along with a newly formed flagellum, is distributed to daughter cells at cytokinesis. Late in the kDNA replication cycle, α-KDE2-PTP distributes with each lobe of the kDNA network in close proximity to the old and newly formed flagella (Fig. 3B). In this image, a single unfixed cell treated with detergent has two flagella, one nucleus, and a single V-shaped kDNA network that has completed kDNA replication and is just beginning network scission (11). Interestingly, α-KDE2-PTP foci are associated with this bilobed kDNA genome, with a single point located on each lobe.

When cells were treated with detergent and CaCl₂, there was a general depolymerization of the subpellicular microtubules, leaving the kDNA associated with the flagellum via the TAC (Fig. 3C) (11). α-KDE2-PTP was localized to either a single point on the kDNA network in nonreplicating cells. The newly replicated kDNA network segregates, and along with a newly formed flagellum, is distributed to daughter cells at cytokinesis. Late in the kDNA replication cycle, α-KDE2-PTP distributes with each lobe of the kDNA network in close proximity to the old and newly formed flagella (Fig. 3B). In this image, a single unfixed cell treated with detergent has two flagella, one nucleus, and a single V-shaped kDNA network that has completed kDNA replication and is just beginning network scission (11). Interestingly, α-KDE2-PTP foci are associated with this bilobed kDNA genome, with a single point located on each lobe.

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kDNA (Fig. 3C, top) or at two points (Fig. 3C, bottom) in asynchronous populations. The orientation of the 3D-R images further supports the close association of α-KDE2-PTP with the kDNA and also better resolves the two discrete α-KDE2-PTP points (Fig. 3C, bottom). The heterogeneity in α-KDE2-PTP foci was likely due to cell cycle differences, with a singular localization associated with kDNA networks that had recently completed replication and segregation (Fig. 3B and C). These results show that α-KDE2-PTP is closely associated with the FKC and cofractionates with the TAC. α-KDE2-PTP maintains an antipodal distribution throughout kDNA replication. Many proteins that maintain the kDNA in T. brucei are organized around the network in specialized regions (21). Antipodal sites, which are structural projections that flank this genome, house many of the replication proteins and are the regions where newly replicated minicircles reattach to the network. The thymidine analogue BrdU is a synthetic base that is incorporated into the free replicating kDNA minicircles during S phase. Initial reattachment of these minicircles to the network leads to labeling of the antipodal sites (38). To determine whether α-KDE2-PTP localized to the antipodal sites, cells were incubated with BrdU for 30 min, fixed, treated with HCl, and stained with DAPI and antibodies for BrdU. Trypanosomes in early S phase showed two sites of BrdU staining flanking the kDNA disk and revealed an antipodal distribution of minicircles (Fig. 4A). Additionally, FKCs prepared from cells treated with BrdU for 30 min revealed colocalization of these minicircles with α-KDE2-PTP at the antipodal sites (Fig. 4B). When BrdU labeling was extended to 480 min, replicated BrdU-labeled DNA was distributed throughout the kDNA network, but the position of α-KDE2-PTP re-
mained constant. In cells that had completed kDNA replication and segregation, we observed a single α-KDE2-PTP staining focus, as has been described for other proteins localized to the antipodal sites (49).

α-KDE2-PTP is stably linked to mitochondrial structural elements. The antipodal localization of α-KDE2-PTP led us to ask whether the protein stably interacts within the FKC by direct binding to the kDNA or to TAC elements surrounding the genome. To determine if α-KDE2-PTP directly associates with the kDNA, purified FKC preparations were treated with DNase, and TM, FKC, and DNase-treated FKC (FKC+D) fractions were resolved by SDS-PAGE and analyzed by Western blotting (Fig. 4C). The blots were probed with antibodies against TAO, tyr-α-tubulin, and the protein A domain of α-KDE2-PTP. The membrane protein TAO was present in TM but was lost during FKC purification. Both α-KDE2-PTP and the basal body marker tyr-α-tubulin remained associated with the FKC following DNase treatment (Fig. 4C). Treatment with DNase caused mild dilution of the FKC samples, since a similar reduction in signal intensity was observed in the tyr-α-tubulin and α-KDE2-PTP FKC+D fractions. The association of α-KDE2-PTP with the FKC was further confirmed by immunofluorescence microscopy of DNase-treated FKC stained with DAPI and antibodies against tyr-α-tubulin and the protein C domain of α-KDE2-PTP. The positions of the nucleus (n), kDNA (k), flagellum (f), and basal bodies (bb) are indicated.

**FIG 4** Antipodal localization and stable association of α-KDE2-PTP with the FKC. (A) BF α-KDE2-PTP T. brucei cells were incubated with BrdU for 30 min, fixed, and acid treated. The cells were stained with DAPI and probed with a monoclonal antibody against BrdU to show the distribution of recently replicated minicircle kDNA. A magnified image of newly replicated BrdU-labeled minicircles reattached to the kDNA network is shown in the merged image. (B) FKCs were isolated from cells treated with BrdU for 30 min and 480 min, heat treated, and stained with antibodies against BrdU and α-KDE2-PTP. (C) Proteins from TM, FKC, and FKC+D from bloodstream form α-KDE2-PTP cells were resolved by SDS-PAGE and analyzed by Western blotting. The proteins were probed with antibodies against TAO, tyr-α-tubulin, and the protein A domain of α-KDE2-PTP. (D) Immunofluorescence microscopy of DNase-treated FKC stained with DAPI and antibodies against tyr-α-tubulin and the protein C domain of α-KDE2-PTP. The positions of the nucleus (n), kDNA (k), flagellum (f), and basal bodies (bb) are indicated.
RNAi knockdown of α-KDE2 decreases the growth rate and influences kDNA distribution. The localization of α-KDE2-PTP to the FKC in BF T. brucei suggested the protein had an alternative function in kDNA maintenance. To examine this secondary function for α-KDE2, we developed an RNAi cell line by cloning a partial α-KDE2 nucleotide sequence into the inducible pZJM vector (35). Northern analysis of this induced cell line revealed complete loss of detectable α-KDE2 mRNA 24 h after induction (Fig. 5A). Loss of α-KDE2 mRNA levels was accompanied by a 26-fold reduction in cell numbers by day 4 (Fig. 5B). The effect of RNAi knockdown of α-KDE2 on the morphology of the kDNA network was evaluated by DAPI staining (Fig. 5C and D). At the time of induction, over 85% of the cells contained one kinetoplast and one nucleus (1k1n), with a small fraction of cells containing 0k1n, 2k1n, and 2k2n cells (Fig. 5C). By day 3, the percentage of dyskinetoplastic cells (0k1n) lacking detectable kDNA staining had increased to 27% of the total cells (Fig. 5D, E, and F). A similar increase in the number of cells with two kinetoplasts (2k1n and
Inheritance of the kDNA by daughter cells at cytokinesis and not together, these studies showed that etoplastic trypanosomes and cells containing two kinetoplasts. Overall abundance of kDNA is unaltered by RNAi knockdown of /H9251. Hybridization with a probe specific for a conserved sequence found on all minicircles revealed no change in the abundance of both closed and nicked/gapped conformations. Additionally, the abundance of kDNA network-associate minicircles and maxicircles were also assayed, and no differences were observed (Fig. 6B). These results suggest that the overall abundance of kDNA is unaltered by RNAi knockdown of /H9251, consistent with the coordinate increase of both dyskinetoplastic trypanosomes and cells containing two kinetoplasts. Together, these studies showed that /H9251 is necessary for inheritance of the kDNA by daughter cells following segregation of the kDNA.

KDNA abundance is not affected by /H9251 mRNA depletion. Many of the enzymes localized to the kDNA antipodal sites are involved in kDNA replication. RNAi knockdown of these replication proteins leads to kDNA loss (27, 50). In order to evaluate the effect of /H9251 RNAi knockdown on kDNA replication, the abundance of free replicating minicircles was determined in total genomic DNA isolated from induced RNAi cells over a 3-day period. Free minicircles, separated by agarose gel electrophoresis to resolve the covalently closed and nicked/gapped circles, were analyzed by Southern blotting (Fig. 6A). Hybridization with a probe specific for a conserved sequence found on all minicircles revealed no change in the abundance of both closed and nicked/gapped conformations. Additionally, the abundance of kDNA network-associated minicircles and maxicircles were also assayed, and no differences were observed (Fig. 6B). These results suggest that the overall abundance of kDNA is unaltered by RNAi knockdown of /H9251, consistent with the coordinate increase of both dyskinetoplastic trypanosomes and cells containing two kinetoplasts. Together, these studies showed that /H9251 is necessary for inheritance of the kDNA by daughter cells following cytokinesis and not for replication or segregation of the kDNA.

Dyskinetoplastic T. brucei cells have truncated mitochondria with decreased membrane potential. In the BF of T. brucei, the mitochondrial ATP synthase functions as an ATPase, driving the movement of protons across the mitochondrial membrane and establishing a membrane potential (17, 51). A single maxicircle gene encoding the mRNA for subunit A6, numerous minicircle gRNAs for the editing of A6 pre-mRNA, and nuclear-encoded editing enzymes are all necessary for the successful production of the functional ATPase (52). An expected consequence of the generation of dyskinetoplastic trypanosomes by /H9251 RNAi is the loss of A6 expression and the inability of these cells to maintain a mitochondrial membrane potential (Fig. 7). /H9251 RNAi cells were incubated with MitoTracker Red on day 3 of induction, washed, fixed with methanol, and prepared for immunofluorescence analysis with an antibody against the mitochondrial membrane protein TAO and DAPI. All cells containing a DAPI-stainable kinetoplast had overlapping signals for both MitoTracker and TAO. Cells lacking a stainable kDNA network also lacked MitoTracker staining, indicating a loss of membrane potential (Fig. 7A). These dyskinetoplastic cells also showed a dramatic reduction in mitochondrial volume based on TAO staining. A 14-fold reduction in length was observed in extreme cases. Examples of uninduced (−RNAi) and induced (+RNAi) cells with mitochondrial length in micrometers are shown in Fig. 7B. Quantitation revealed that 100% of the kinetoplastids on day 3 contained mitochondria that were more than 5 μm in length (Fig. 7C). Also on day 3, dyskinetoplastic trypanosomes contained mitochondria that were between 2 and 8 μm (77%), and only 23% were 1 μm or less (Fig. 7C). Together, the RNAi knockdown studies showed that /H9251 was necessary for inheritance of the kDNA by daughter cells and that the production of dyskinetoplastic trypanosomes resulted in the rapid loss of mitochondrial membrane potential and reduced mitochondrial volume.

**DISCUSSION**

Whether structural or enzymatic, most proteins are encoded by a single gene and have a single function. However, there are a number of proteins that are multifunctional, with seemingly unrelated activities. In mammals, alternative splicing provides an important mechanism for generation of protein diversity, with literally thousands of splice variants originating from a single gene (53). Trypanosomes lack conventional *cis*-splicing, but alternative *trans*-splicing at the 5’ ends of nuclear-encoded mRNAs and alternative editing of mitochondrial mRNA have recently been recognized as mechanisms for increasing the variety of proteins (32, 33, 40, 45). Expanded functional diversity can also be accomplished without

![FIG 6](http://www.sciencemag.org/content/321/5888/962/F6.large.jpg)

**FIG 6** Effects of α-KDE2 RNAi on minicircle and maxicircle abundance. (A) Total genomic DNA was fractionated by agarose gel electrophoresis and analyzed by Southern blotting over a 3-day period after induction of α-KDE2 RNAi for changes in free replicating minicircle levels. (B) Southern blot analysis of XbaI- and HindIII-digested total genomic DNA for variations in the amounts of kDNA-associated minicircles and maxicircles. Both blots were hybridized with radiolabeled probes for minicircle and maxicircle sequences. Hybridization with a β-tubulin probe was used as a loading control.
differences in protein sequence or posttranslational modification, but rather, by simply having multiple activities associated with the same protein. These “moonlighting proteins” also provide additional complexity to the limited number of proteins encoded by the eukaryotic genome. Seven of the eight Krebs cycle enzymes have alternative functions in eukaryotes. Three of these proteins have been identified as mtDNA maintenance factors in different organisms, suggesting a strong evolutionary trend for metabolic enzymes to be involved in genome preservation (4, 54, 55). Here, we report the moonlighting activity of α-KDE2 and define its function in the inheritance of the mitochondrial genome of African trypanosomes.

The BF of T. brucei lacks α-KD activity yet expresses all three subunits of α-KD. The enzymatic activities of α-KD were expected to fractionate with the mitochondrial matrix; however, α-KDE2 is also associated with mitochondrial membranes (Fig. 2). In yeast, α-KDE2 also associates with the mtDNA nucleoid at the mitochondrial membrane and is necessary for maintenance of the genome (4, 5). Similarly, we found that α-KDE2 in T. brucei associates with the FKC apparatus. The association of α-KDE2 with the FKC is not mediated by direct binding to kDNA, but rather, the protein is physically linked to the FKC in a DNase-resistant manner. Despite apparent similarities in the distribution of α-KDE2 in yeast and trypanosomes, the structure of the kDNA network and its mechanism of replication impose unique requirements. Immunofluorescence microscopy localized α-KDE2 to discrete positions on the kDNA within the FKC. The mechanism of kDNA replication requires a spatial order of the genome, rep-
lization enzymes, and segregation apparatus. The KFZ and the antipodal sites represent defined functional zones surrounding the kDNA and are involved in replication, segregation, and maintenance of the trypanosome mitochondrial genome. Pulse-labeling cells with BrdU allowed the identification of the antipodal sites and showed that α-KDE2 localizes there. Initially, localization of α-KDE2 to the site of minicircle reattachement following replication led us to believe that α-KDE2 might be directly involved in kDNA replication. Moreover, RNAi knockdown of α-KDE2 mRNA resulted in a dramatic increase in cells lacking kDNA staining with DAPI. However, we also observed a corresponding increase in cells with two kDNA networks, and Southern blot hybridization confirmed that the abundance of kDNA was unaltered during RNAi knockdown of α-KDE2 expression despite an increased number of dyskinetoplastic cells. Together, these results indicate that α-KDE2 is not directly involved in kDNA replication or segregation, but rather, is necessary for the distribution of the replicated kDNA networks to daughter cells at cytokinesis. We propose that unequal distribution of the replicated kDNA networks gave rise to a dyskinetoplastic daughter cell and a daughter cell with two kinetoplasts.

The antipodal distribution of α-KDE2 is static throughout the cell cycle, suggesting a structural versus a catalytic function for α-KDE2 at the FKC of T. brucei. In asynchronous populations, α-KDE2 is associated with one or both antipodal sites on the kDNA. We have found α-KDE2 on a bilobed kDNA (prepartitioned), suggesting that the α-KDE2 stably associates as a single site on each lobe of the replicating kDNA network. Complete segregation of this “V-shaped” bilobed network produces daughter kDNA networks with a single podal site containing α-KDE2. This is similar to the results seen in the analysis of the distribution of mitochondrial topoisomerase II in the related organism Crithidia fasciculata (49). Furthermore, while BrdU analysis of kDNA replication revealed an initial colorization of newly replicated minicircles and α-KDE2 at the antipodal sites, continuous uptake of BrdU led to a uniform distribution of the nucleotide analogue throughout the kDNA network, whereas α-KDE2 remained static at the antipodal sites (Fig. 4B).

A possible role for α-KDE2 within the FKC might involve kDNA binding and suggests that α-KDE2 could serve as a bridge to the proteinaceous filaments linking the kDNA network to the mitochondrial membrane. We found that α-KDE2 association with FKC resisted treatment with DNase, but additional studies are needed to determine whether α-KDE2 has DNA binding properties. Within the FKC, the unilateral filaments of the TAC attach to the mitochondrial membranes and extend through the KFZ to a single face of the kDNA disk (11). Only two unilateral filament proteins (p166 and AEP-1) have been reported (30, 33), and possible interactions with α-KDE2 have not been determined. The resistance of α-KDE2 to detergent solubilization and DNase treatment suggests an association with unidentified proteins that make up the structural skeleton in the mitochondria.

Two previously studied TAC proteins, p166 and AEP-1, localize to the kDNA/basal body region of T. brucei and purify with the FKC (30, 33). RNAi knockdown of p166 and expression of a dominant-negative mutant of AEP-1 showed that both are essential for kDNA maintenance and segregation. α-KDE2 RNAi had no effect on kDNA morphology or division but resulted in an increase of dyskinetoplastic cells and a corresponding increase in cells with two kinetoplasts. This suggests that in the absence of α-KDE2, newly replicated kDNA networks fail to partition into the newly formed mitochondrion, leading to unequal kDNA distribution to the daughter cells.

If α-KDE2 is necessary for kDNA inheritance, it is somewhat surprising that the maximum percentage of dyskinetoplastic cells never exceeds approximately 35%. It is well established that BF trypanosomes lack a functional Krebs cycle and oxidative phosphorylation and that ATP is exclusively produced during glycolysis. However, mitochondrial translation is required in BF T. brucei, suggesting an essential function is provided by a mitochondrial gene product (15). It seems likely that the depletion of α-KDE2 by RNAi is lethal to BF trypanosomes due to the moonlighting function of the protein in kDNA inheritance. In BF T. brucei, the formation of a proton gradient across the mitochondrial membrane, necessary for import of nuclear-encoded mitochondrial proteins, is dependent on the expression of a functional F$_{1}$F$_{0}$ ATPase. One essential component of the F$_{1}$F$_{0}$ ATPase is the maxicircle-encoded A6 subunit. Since dyskinetoplastic trypanosomes lack the A6 gene, they cannot maintain a proton gradient and lose the ability to import proteins from the cytoplasm. This occurs rapidly, resulting in loss of membrane potential and reduced mitochondrial volume within 3 days of RNAi induction.

The moonlighting function of α-KDE2 in kDNA distribution to daughter cells further underscores the importance of multifunctional proteins in the maintenance of the mitochondrial genome in eukaryotes. Unexpectedly, we have also demonstrated the essential role of mitochondrion-encoded proteins in the duplication and inheritance of the intact mitochondrion in BF trypanosomes. The complexity of the FKC and kDNA replication cycle has driven the diversification of mitochondrial proteins in trypanosomes, and as additional FKC-associated proteins are identified, it is likely that new moonlighting and alternatively processed proteins will be discovered.

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