ELSEVIER

Molecular & Biochemical Parasitology 117 (2001) 179–186

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

Disruption of the *Crithidia fasciculata KAP1* gene results in structural rearrangement of the kinetoplast disc

Julius Lukeš [a,b], Jane C. Hines [a], Cory J. Evans [c], Nuraly K. Avliyakulov [a], Vidya P. Prabhu [d], Junghuei Chen [d], Dan S. Ray [a,*]

[a] *Molecular Biology Institute and Department of Microbiology and Molecular Genetics, University of California, Los Angeles, 405 Hilgard Ave., Los Angeles, CA 90095-1570, USA*

[b] *Institute of Parasitology, Czech Academy of Sciences and Faculty of Biology, University of South Bohemia, 37005 Ceské Budejovice, Czech Republic*

[c] *Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, 405 Hilgard Ave., Los Angeles, CA 90095-1570, USA*

[d] *Department of Chemistry & Biochemistry, University of Delaware, Delaware, USA*

Received 28 December 2000; accepted in revised form 21 July 2001

Abstract

The mitochondrial DNA (kinetoplast DNA) in trypanosomatids exists as a highly organized nucleoprotein structure with the DNA consisting of thousands of interlocked circles. Four H1 histone-like proteins (KAP1, 2, 3 and 4) are associated with the kinetoplast DNA in the trypanosomatid *Crithidia fasciculata*. We have disrupted both alleles of the *KAP1* gene in this diploid protozoan and shown that expression of the KAP1 protein is eliminated. The mutant strain is viable but has substantial rearrangement of the kinetoplast structure. Expression of the KAP1 protein from an episome restored expression of the KAP1 protein in the mutant strain and also restored a normal kinetoplast structure. These studies provide evidence that the KAP1 protein is involved in kinetoplast DNA organization in vivo but is nonessential for cell viability.

Keywords: Kinetoplast; Trypanosome; Minicircle; Histone-like; DNA-binding protein

1. Introduction

The Kinetoplastida are a group of protozoans widespread in nature that includes important parasites (trypanosomes, leishmanias, phytomonads) and free-living bodonids. They represent one of the earliest branches of mitochondrial eukaryotes and, probably due to their ancient character, retain several unique features [1]. One of them is their mitochondrial (kinetoplast) DNA (kDNA) which consists of dozens of maxicircles bearing cryptic mitochondrial genes, and thousands of minicircles that code for guide RNA genes involved in RNA editing of mitochondrial transcripts (reviewed in [2,3]). kDNA contains up to 40% of the total cellular DNA and occurs either in a primitive non-catenated form [4] or in the form of a single network consisting of mutually interlocked mini- and maxicircles located in the mitochondrial matrix close to the kinetosome of the flagellum (reviewed in Ref. [5]).

Purified kDNA observed by the cytochrome c spreading technique [6] appears as a two-dimensional oval-shaped sheet of DNA larger in size than the cell from which it was isolated. In the model organism *Crithidia fasciculata*, the kDNA network is condensed into a structure that forms a disc 1 μm in diameter and 0.4 μm thick [7,8], with minicircles interlocked on average with three neighbors each by a single interlock [9]. Replication of the kDNA network involves the decatenation of minicircles by a kinetoplast DNA topoisomerase and their release from the center of the network [10]. Newly replicated minicircles are subsequently rejoined to the network and distributed around its

* Corresponding author. Tel.: +1-310-825-4178; fax: +1-310-206-7286.

E-mail address: danray@ucla.edu (D.S. Ray).

0166-6851/01/$ - see front matter
PII: S0166-6851(01)00348-6

perimeter possibly as a consequence of rotation of the condensed network relative to the sites of reattachment [11]. Nicks and gaps in the newly replicated minicircles remain even after rejoining to the kDNA network at the antipodal sites [12–15]. After all minicircles have been duplicated, and remaining nicks and gaps have been sealed, the double size network is divided into two equivalent daughter networks by unknown mechanisms.

We have initiated studies aimed at identifying protein components of the kinetoplast and their possible role in the organization and segregation of the kDNA. Several extremely basic proteins have recently been found to be associated with kDNA (kinetoplast-associated proteins-KAP) in *C. fasciculata* [8,16,17]. All are histone H1-like proteins rich in lysine and alanine and can be reversibly cross-linked to the kDNA by formaldehyde treatment in vivo. *KAP1* to *4* genes are single-copy genes that encode proteins with novel 9-amino-acid cleavable presequences. Each of the proteins has been localized to the kDNA disc by fluorescence microscopy and the KAP4 protein was shown by electron microscopy and immuno-gold staining to be present at antipodal sites as well as throughout the kDNA disc [8]. The *KAP2*, *3* and *4* genes were also shown to rescue a chromosome segregation defect in a mutant strain of *E. coli* [8]. In this work, we have disrupted both alleles of the *KAP1* gene, analyzed the altered structure of the kDNA disc of the null strain, and finally rescued the phenotype by reintroducing the gene on a plasmid vector.

2. Materials and methods

2.1. Plasmids

Drug-resistance plasmids were constructed based on the pX vectors [18,19] in which sequences from the 5′ and 3′ flanking sequences of the *Leishmania major* dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) were used for expressing the selectable markers *NEO*, *HYG* and *PHLEO*, encoding resistance to G418, hygromycin and Zeocin (Invitrogen), respectively (Fig. 1). Plasmid pG21 was constructed by cloning a 1.8 kb *Ava*I–*Nru*I fragment containing the *KAP1* gene, plus flanking sequences, into the Promega plasmid pGEM7Zf(+). Plasmid pG21/NEO was constructed by cloning the *NEO* gene with *L. major* 5′ and 3′ *DHFR-TS* flanking regions as a 3.3 kb *Sac*II–*Not*I cassette from plasmid pX.2-KO [20] into *Sac*II and *Not*I sites of the *KAP1* gene. In pG21/HYG, the *HYG* cassette was a 3.4 kb *Xba*I fragment introduced into the *Nhe*I site in the *KAP1* gene. Plasmid p21Ava was constructed by cloning the *Ava*I fragment of pG21 cloned into pGEM7Zf(+). For reintroduction of the *KAP1* gene into disrupted strains, a phleomycin cassette from pXGPHLEO [18] was cloned as a *Sal*I–*Hin*dIII fragment from pXGPHLEO into pUC19, then retrieved as a *Bam*HI fragment and cloned into the *Bam*HI site of p21Ava downstream of the *KAP1* gene creating p21PHLEO.

2.2. Cell growth and DNA transformation

C. fasciculata was grown in BHI medium (Difco) containing hemin (20 μg/ml) and streptomycin (100 μg/ml) at 28 °C. Solid BHI media contained 1% agar (Difco). For disruption of genomic *KAP1* genes, targeting fragments were released from 50 μg of the constructs pG21/NEO and pG21/HYG by *Bsr*GI and *Nru*I digestions, and used to transform cells by electroporation [20]. Single colonies were picked from BHI plates

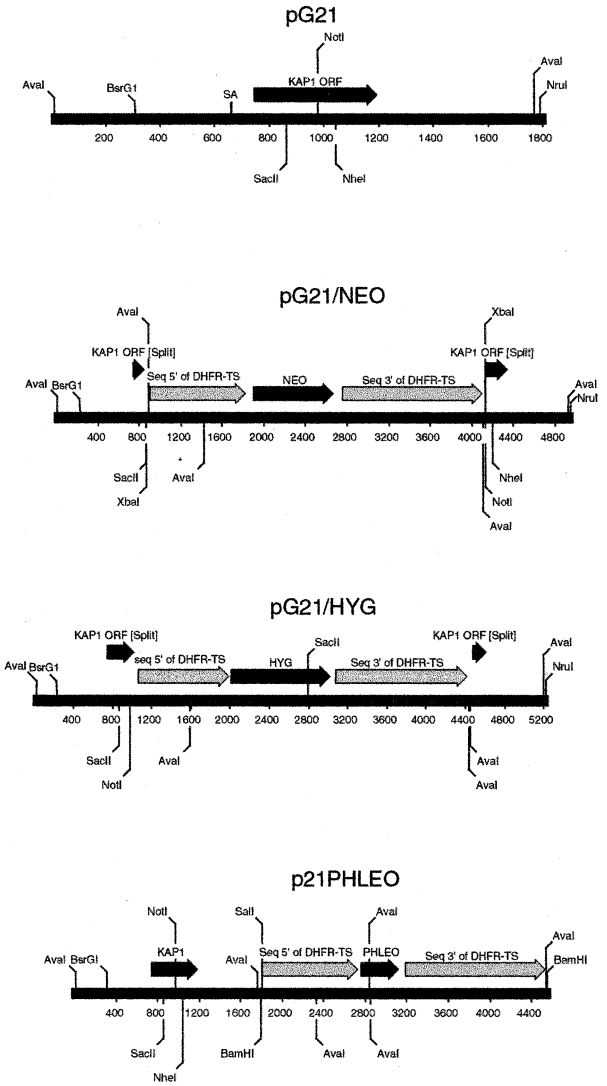

Fig. 1. Physical maps of inserts in plasmids used for disruption of *KAP1* and for episomal expression of the KAP1 protein. NEO, HYG and PHLEO drug-resistance cassettes are derived from the pX plasmids [18,19] and their expression is mediated by 5′ and 3′ flanking sequences from the *Leishmania major DHFR-TS* gene.

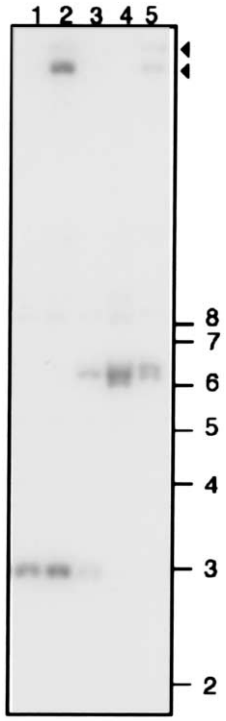

Fig. 2. Southern blots of *Nru*I digests of *C. fasciculata* DNA from wild-type (lane 1), single-disruption (lane 3) and double-disruption (lane 4) strains. Lanes 2 and 5 contain DNA digests from cells transformed with the p21PHLEO plasmid (lane 2, wild type; lane 5, double-disruption strain). A 1.4 kb *Eco*RI fragment derived from a *KAP1* cDNA was used to probe the blot. Molecular-mass markers are indicated in kDa, and the positions of higher forms of the p21PHLEO plasmid are indicated by triangles.

containing 50 μg/ml of G418 or 80 μg/ml of hygromycin and grown subsequently in liquid BHI medium containing 25 μg/ml of G418 or 40 μg/ml of hygromycin.

2.3. DNA isolation and Southern hybridization

Total DNA was isolated from frozen cells as described [21]. Southern blots were performed essentially as described [22] and probed with a 1.4 kb *Eco*RI fragment containing most of the *KAP1* cDNA labeled by random priming using the αdCTP Labeling kit (Ready to Go Beads from Amersham Pharmacia). Each lane on the gel contained 250 ng of DNA except for lane 3 in Fig. 2 in which approximately half of the sample was lost during loading of the gel.

2.4. Western blotting

Cell lysates prepared from *C. fasciculata* cells were analyzed on 12% acrylamide-SDS gels, and blotted to PVDF membranes [20]. Blots were probed with polyclonal antisera (1:10,000) raised against recombinant KAP1 protein in rabbits and affinity purified as described elsewhere [17]. Gels run in parallel were blotted and probed with antisera raised against recombinant KAP3 protein. A secondary goat anti-rabbit IgG antibody (1:15,000) coupled with horse-radish peroxidase was visualized according to the manufacturer's protocols (NEN Scientific).

2.5. Immunolocalization

Log-phase *C. fasciculata* cells ($\sim 10^7$/ml) were washed in TN buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl), spread on poly-L-lysine-coated slides and fixed in 4% paraformaldehyde/TN for 2 min at RT. Fixation was stopped by washing the slides with 0.1 M glycine/TN for 5 min. Cells were permeabilized in 0.15% Triton X-100, and subsequently washed twice in TN buffer, each step for 5 min at RT. After incubation in blocking solution (20% goat serum/TN) for 30 min at 37 °C in a humid chamber, the blocking solution was supplemented with affinity-purified anti-KAP1 antibody (1:500) [17], and incubation was performed under the same conditions for 45 min. Slides were washed in TNT buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween-20) four times for 5 min at RT and then incubated with blocking solution containing a FITC-conjugated goat anti-rabbit IgG antibody (1:50) for 30 min at 37 °C in a humid chamber, followed by four washes for 5 min each in TNT solution. Finally, the slides were incubated in TNT buffer containing 0.1 μg/ml DAPI for 3 min at RT, rinsed with distilled water and mounted in ProLong Antifade (Molecular Probes) prior to examination by fluorescence microscopy.

2.6. Immunogold labeling and transmission electron microscopy

C. fasciculata cells were fixed and embedded for immunogold labeling at room temperature as described [4]. Thin sections were picked on formvar membrane-coated copper grids and placed in a moist chamber for the following incubations: 15 min in the preincubation solution (10% fetal bovine serum; 0.1% bovine serum albumin [BSA]; 0.02M glycine in 0.1 × phosphate buffered saline [PBS]); 1 h in 1:50 anti-KAP1 antibody in 1% BSA in 1 × PBS; five times 5 min wash in 0.1% BSA; 1 h secondary antibody-protein A coupled with 10 nm colloidal gold; five times 5 min wash in 0.1% BSA; 30 min postfixation in 2% glutaraldehyde. The grids were poststained with 5% uranyl acetate and lead citrate, and examined in a JEOL JEM 1010 electron microscope. For standard transmission electron microscopy, cells were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer at 4 °C for 1 h, embedded in Epon-Araldite resin and processed as described [23].

3. Results

3.1. Disruption of the KAP1 gene

We have used targeted gene disruption to investigate the phenotype of cells in which one or both alleles of the *KAP1* gene were disrupted. Previous studies had shown that *KAP1* is a single-copy gene [17]. In the first disruption plasmid, pG21/NEO, a NEO cassette replaces the central region of the *KAP1* gene (Fig. 1). A G418-resistant transformant was isolated and analyzed by Southern blotting. In blots in which total DNA digested with *Nru*I was hybridized with the *KAP1* cDNA probe, the disrupted allele was identified as a 6.1 kb hybridizing band (Fig. 2, lane 3), while in the wild-type DNA digested with the same enzyme, both alleles migrated at 2.9 kb (Fig. 2, lane 1). The single disruption strain was subjected to another round of electroporation with a targeting fragment containing the *KAP1* gene disrupted by a HYG cassette. DNA isolated from clones resistant to hygromycin was again digested with *Nru*I, and in this case, two hybridizing bands of 6.1 kb and 6.3 kb were observed (Fig. 2, lane 4), consistent with the insertion of the NEO and HYG cassettes into each of the *KAP1* alleles. The wild-type band of 2.9 kb was absent in the double disrupted strain.

The cell doubling time of the double disruption strain in BHI medium lacking drugs was approximately twice that of the wild-type strain. However, the slower growth rate of the double disruption strain may be unrelated to the gene disruption since both the single disruption strain and a double disruption strain rescued by episomal expression of KAP1 also had longer doubling times.

3.2. Rescue of KAP1 null mutant

To further analyze the function of the KAP1 protein in vivo, the *KAP1* gene was reintroduced as an extrachromosomal element into the wild type and double disruption mutant strains. In this plasmid construct a PHLEO cassette conferring resistance to Zeocin was cloned into plasmid pG21 downstream of an intact *KAP1* gene to create the complementing plasmid p21PHLEO (Fig. 1). Double-disrupted and wild-type *C. fasciculata* cells were electroporated with supercoiled p21PHLEO, and transformed cells were selected on BHI plates containing high levels of Zeocin (90 μg/ml). The presence of multiply catenated forms of the p21PHLEO plasmid in both wild type and selected mutant clones transformed with p21PHLEO was visible on Southern blots probed with the *KAP1* gene (Fig. 2, lanes 2 and 5). The presence of the p21PHLEO plasmid in the Zeocin-resistant double disruption strain was also confirmed by hybridization of Southern blots with a probe derived from the phleomycin-resistance gene (data not shown). Faint bands at approximately 8 kb in all lanes are likely due to weak hybridization to distantly related genes.

3.3. Expression of plasmid-encoded KAP1

Western blot analysis was performed to detect the expression of plasmid-encoded KAP1 protein in the null mutant strain of *C. fasciculata*. The wild-type KAP1 protein is detected on SDS gels migrating as a 21 kDa species. Although the predicted size of the KAP1 protein based on the *KAP1* DNA sequence is 13.9 kDa [17], the highly basic protein (p$I = 13.0$) migrates with an apparent molecular mass of 21 kDa. Disruption of both alleles of *KAP1* resulted in the disappearance of the *KAP1* band. A faint band representing a fragment of the KAP1 protein derived from the disruption of the second allele can be seen on an overexposed blot, but no full-length KAP1 protein can be seen (Fig. 3 lane 2). This fragment of the KAP1 protein lacks the C-terminal third of KAP1 and appears to be unstable based on the very low level of the fragment detected on Western blots. However, in the mutant strain rescued by expression of the *KAP1* gene from the p21PHLEO plasmid, the full-length KAP1 protein is observed at a level approximately half that of the wild-type strain (Fig. 3, lanes 1 and 3), demonstratiing that the protein is expressed from the extrachromosomal *KAP1* gene.

3.4. Immunolocalization of KAP1 protein

It was shown previously that the KAP1 protein co-localizes with the kinetoplast in wild-type cells [17]. Immunolocalization of KAP1 in wild-type cells counterstained with DAPI shows that the protein is present exclusively in the kinetoplast (Fig. 4A–C). This fluoresence labeling pattern differs slightly from that observed by immunofluorescence microscopy earlier in which the fluorescence appeared to localize to the two faces of the disc [17]. This difference possibly reflects an incomplete penetration of the horse-radish peroxidase-conjugated

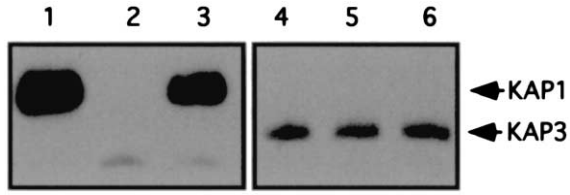

Fig. 3. Western blot of cell extracts (2×10^6 cell equivalents) from wild-type (lanes 1 and 4), double-disruption (lanes 2 and 5) and a double-disruption strain transformed with p21PHLEO expressing KAP1 (lanes 3 and 6). Identical blots were probed with antisera against either KAP1 (lanes 1–3) or KAP3 (lanes 4–6) recombinant proteins [8,17].

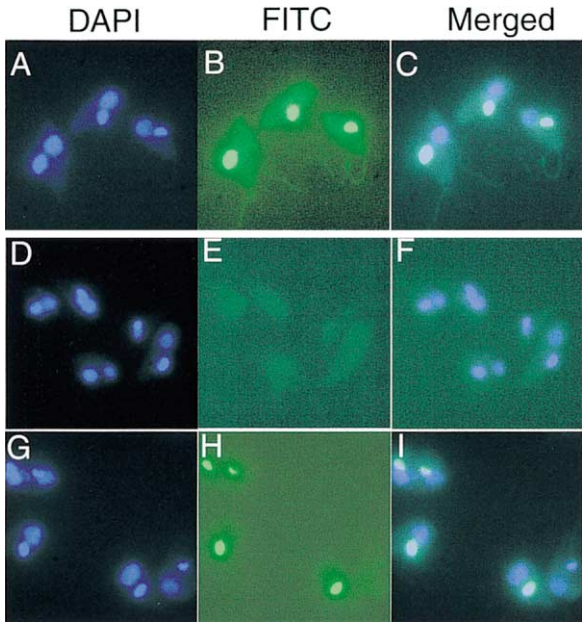

Fig. 4. Immunolocalization of KAP1 protein in wild-type and mutant cells. (A–C) Wild-type cells; (D–E) double-disruption strain; (G–I) double-disruption strain transformed with p21PHLEO.

antibody molecules into the disc in the earlier study. Electron microscopic immunolocalizations presented later also show the presence of KAP1 protein throughout the kinetoplast disc.

Consistent with the absence of the KAP1 protein in cell lysates prepared from the double disrupted strain, no immunofluorescence was detected in kinetoplasts of the mutant strain (Fig. 4E–F). Upon introduction of the p21PHLEO plasmid into the mutant strain, the KAP1 protein is observed to be present in the kinetoplast by immunofluorescence (Fig. 4H–I). Immunolocalization of the plasmid encoded KAP1 protein to the kinetoplast in the mutant cells clearly shows that the protein derived from the introduced gene is not only expressed but is also correctly processed and imported into the kinetoplast. A small percentage of the cells transformed by p21PHLEO failed to show any immunolocalization and possibly represent cells that have lost the plasmid since the cells were grown in the absence of drug selection for this experiment.

3.5. Structural rearrangement of the kinetoplast

We have used transmission electron microscopy of glutaraldehyde-fixed cells to examine the detailed organization of the kinetoplast in the null mutant strain. Preservation of the double layer structure of both the cell membrane and mitochondrial membrane (Fig. 5) indicates good preservation of the ultrastructure of the analyzed samples. The mitochondrial tube with its plate-like cristae contained a disc-shaped kinetoplast close to the base of the flagellum. In longitudinal sections of the cell, the DNA fibers in the kinetoplast appear aligned in parallel array (Fig. 5A, C, E). In wild-type cells, the kDNA disc was approximately 250–300 nm thick and measured up to 850 nm in diameter (Fig. 5A, data not shown). As seen in longitudinal and transverse sections of wild-type cells (Fig. 5A and B), the DNA fibers were tightly packed throughout the structure. In the transverse sections of the kinetoplast disc, thin DNA fibers of mutually interlocked minicircles were regularly spaced (Fig. 5B). The diameter of the fibers was about 10 nm, as judged from highly magnified pictures.

The fine structure of the kinetoplast in the double disruption cells (Fig. 5C and D) clearly differed from that of the wild type. Although the disc-shaped kinetoplast retained its wild-type shape and dimensions, the internal organization was significantly altered. Inside the disc, the DNA fibers were still aligned in parallel to its axis (Fig. 5C), but they appeared to be packed into thicker strands approximately 30 nm in diameter (Fig. 5D). These fibers were separated from each other by electron-lucent zones, and their number was significantly lower than that of the DNA fibers in the wild-type structure (Fig. 5D). Another prominent feature of the strain lacking the KAP1 protein was the presence of a central electron-dense layer situated in the middle of the disc (Fig. 5C). These unusual structural features of the kinetoplasts in the mutant strain were present in all cells examined.

Interestingly, the kinetoplast ultrastructure of the rescued strain was similar to that of wild-type cells indicating that episomal expression of KAP1 protein was sufficient for the restoration of a normal kinetoplast structure (Fig. 5E). A kinetoplast morphology intermediate to those of the wild-type and mutant cells was observed in only a small fraction of the rescued cells (less than 10% in 70 analyzed cells).

Kinetoplast localization of the plasmid expressed KAP1 protein was also confirmed by immunoelectron microscopy. In wild-type cells, the KAP1 protein is present throughout the kinetoplast (Fig. 5F). The intensity of labeling was invariably strong and evenly spread, with the protein present also in peripheral sections of the disc. However, no signal was observed outside the disc, including the mitochondrial lumen surrounding the kDNA. While no labeling was observed in the kinetoplast in cells with the disrupted *KAP1* genes (data not shown), an intense and homogeneous labeling was observed in kinetoplasts of the rescued strain (Fig. 5G).

Preliminary electronmicroscopic examination of purified kDNA networks by the cytochrome c spreading technique has not revealed any significant differences in the size or topology of networks from the *KAP1* mutant strain (data not shown).

4. Discussion

The *C. fasciculata* KAP proteins are small, highly basic proteins associated with the kinetoplast DNA. The high degree of condensation of the kDNA in vivo and the ability of purified KAP proteins to condense kDNA networks in vitro suggest that these proteins play a role in the condensation and organization of the kDNA in vivo [8]. Studies presented here using gene-disruption techniques are consistent with such a role for one of these proteins, the KAP1 protein.

Since *C. fasciculata* grows as an asexual diploid organism in culture, it was necessary to disrupt both alleles of the *KAP1* gene. The development of plasmid vectors conferring drug resistance in *Leishmania major* and their ability to replicate and express drug resistance in *C. fasciculata* [18,19] provided the means for constructing the *KAP1* disruption strain. The lack of expression of the full length KAP1 protein in the disrupted strain was demonstrated both by Western blotting and by immunofluorescence.

Although the mutant strain is viable, indicating that the KAP1 protein is non-essential, the kinetoplast structure is dramatically rearranged in the strain. Surprisingly, the mutant cells appear to grow indefinitely and have been grown for over 50 generations in our lab. The inability of the KAP2, 3 or 4 proteins to complement the KAP1 function in the organization of the kinetoplast structure is consistent with a role for KAP1 distinct from those of the KAP2, 3 and 4 proteins. This difference is not unexpected in light of the distinctive properties of the KAP1 protein [17]. KAP1 protein is the most basic of the four proteins with a calculated p*I* of 13, whereas KAP2, 3 and 4

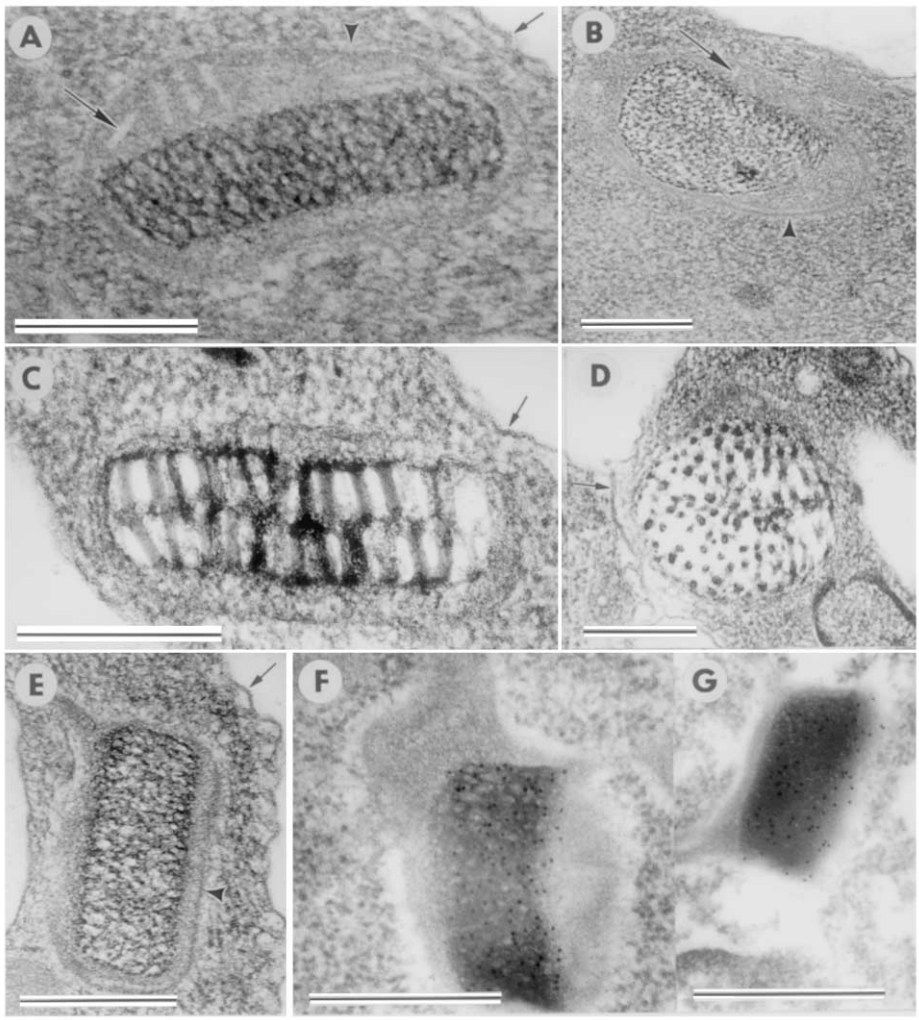

Fig. 5. Transmission electron microscopy and immunogold labeling. (A–E) Epon-Araldite-embedded cells. Longitudinal sections of the kinetoplast disk of wild type (A), mutant (C) and rescued mutant (E) cells. Transverse sections parallel to the face of the kinetoplast disk of wild-type (B) and mutant (D) cells. (F–G), sections of LR White-embedded wild type (F) and rescued mutant (G) cells treated with the anti-KAP1 antibodies and secondary antibodies coupled with colloidal gold. Small arrows, cell membrane; large arrows, cristae; large arrowhead, mitochondrial membrane; bars, 500 nm.

proteins have calculated p*I*s of 11.2, 10.7 and 10.3, respectively. Also, lysine and alanine together account for more than 50% of the residues of KAP1, and 25 out of the 28 lysine residues contained in the mature protein are in the C-terminal half of the protein. Another distinctive feature of KAP1 protein is a high proline content (15 prolines out of 142 residues in the mature protein). Finally, KAP1 protein binds non-specifically to kinetoplast minicircle DNA [17], whereas KAP2, 3 and 4 proteins show preferential binding to a specific region on the minicircle DNA [8].

The rearranged structure of the kinetoplast in the mutant strain implicates the KAP1 protein in the structural organization of the kinetoplast. Although a fragment of the KAP1 protein is produced in the double disruption strain, it is unlikely to contribute to the mutant phenotype since it is present at an extremely low level and is not observed in the kinetoplast by immunolocalization. The deletion of the C-terminal one-third of the KAP1 protein sequence in this fragment possibly results in degradation of the fragment, which would account for its low level.

The current view of kinetoplast DNA topology is similar to that of a knight's chain mail armor with individual minicircles interlocked with three neighbors on average [9]. Each minicircle in the kinetoplast disc is viewed as being stretched taut, arranged in parallel and condensed side to side to form the disc structure. It is unclear how to reconcile this model with the resulting organization of the kinetoplast disc observed here. Transverse sections of the kinetoplast reveal a discrete number of fibers of approximately 10 nm in diameter. Each fiber may possibly represent a side-to-side association of a discrete number of interlocked minicircles stretched taut from opposite ends. The minicircles in each fiber are likely associated with proteins including the KAP 2, 3, and 4 proteins as well as additional unidentified proteins.

One possible model to account for the organization of the minicircles into discrete DNA-protein fibers involves a discrete number of 'nucleation factors' with KAP1 protein as one of the components. Each of the factors might be responsible for the side-to side association of a specific number of minicircles. In the absence of the KAP1 protein, the number of such nucleation factors might be reduced, resulting in fewer but thicker fibers. Possible candidates for other components of such hypothetical factors include the other kinetoplast-associated proteins, KAP 2, 3 and 4 [8]. While this model is highly speculative, it does make specific predictions that are testable. Biochemical and molecular genetic analysis of these H1 histone-like proteins could validate or invalidate the model and possibly provide a clearer picture of the roles of these basic proteins in kinetoplast structure and organization.

Acknowledgements

This work was supported by the National Institutes of Health grant AI45536 (to D.S.R.) and a grant from the Grant Agency of the Czech Republic 204/00/1212 (to J.L.). J.C. was partially supported by an American Cancer Society grant (PRG-99-352-01-GMC). We thank Jana Nebesárová and Maruska Manurová for help with immunoelectron microscopy.

References

[1] Donelson JE, Gardner MJ, El-Sayed NM. More surprises from Kinetoplastida. Proc Natl Acad Sci USA 1999;96:2579–81.
[2] Arts GJ, Benne R. Mechanism and evolution of RNA editing in kinetoplastida. Biochim Biophys Acta 1996;1307:39–54.
[3] Alfonzo JD, Thiemann O, Simpson L. The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. Nucleic Acids Res 1997;25:3751–9.
[4] Lukeš J, Jirků M, Avliyakulov N, Benada O. Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helicis*. EMBO J 1998;17:838–46.
[5] Shapiro TA, Englund PT. The structure and replication of kinetoplast DNA. Annu Rev Microbiol 1995;49:117–43.
[6] Perez MD, Englund PT. Microtechnique for electron microscopy of DNA. Nucleic Acids Res 1993;21:1327–8.
[7] Ferguson M, Torri AF, Ward DC, Englund PT. In situ hybridization to the *Crithidia fasciculata* kinetoplast reveals two antipodal sites involved in kinetoplast DNA replication. Cell 1992;70:621–9.
[8] Xu CW, Hines JC, Engel ML, Russell DG, Ray DS. Nucleus-encoded histone H1-like proteins are associated with kinetoplast DNA in the trypanosomatid *Crithidia fasciculata*. Mol Cell Biol 1996;16:564–76.
[9] Chen J, Rauch CA, White JH, Englund PT, Cozzarelli NR. The topology of the kinetoplast DNA network. Cell 1995;80:61–9.
[10] Perez-Morga DL, Englund PT. The attachment of minicircles to kinetoplast DNA networks during replication. Cell 1993;74:703–11.
[11] Perez-Morga D, Englund PT. The structure of replicating kinetoplast DNA networks. J Cell Biol 1993;123:1069–79.
[12] Kitchin PA, Klein VA, Fein BI, Englund PT. Gapped minicircles. A novel replication intermediate of kinetoplast DNA. J Biol Chem 1984;259:15532–9.
[13] Kitchin PA, Klein VA, Englund PT. Intermediates in the replication of kinetoplast DNA minicircles. J Biol Chem 1985;260:3844–51.
[14] Birkenmeyer L, Sugisaki H, Ray DS. Structural characterization of site-specific discontinuities associated with replication origins of minicircle DNA from *Crithidia fasciculata*. J Biol Chem 1987;262:2384–92.
[15] Sheline C, Melendy T, Ray DS. Early intermediates in the replication of DNA minicircles in isolated kinetoplasts from *Crithidia fasciculata*. Mol Cell Biol 1988;9:169–76.
[16] Hines JC, Ray DS. Tandem arrangement of two genes encoding kinetoplast-associated H1 histone-like proteins. Mol Biochem Parasitol 1997;89:41–9.
[17] Hines JC, Ray DS. The *Crithidia fasciculata* KAP1 gene encodes a highly basic protein associated with kinetoplast DNA. Mol Biochem Parasitol 1998;94:41–52.
[18] Freedman DJ, Beverley SM. Two more independent selectable markers for stable transfection of Leishmania. Mol Biochem Parasitol 1993;62:37–44.

[19] Cruz A, Coburn CM, Beverley SM. Double targeted gene replacement for creating null mutants. Proc Natl Acad Sci USA 1991;88:7170–4.
[20] Pasion SG, Brown GW, Brown LM, Ray DS. Periodic expression of nuclear and mitochondrial DNA replication genes during the trypanosomatid cell cycle. J Cell Sci 1994;107:3515–20.
[21] Pasion SG, Hines JC, Aebersold R, Ray DS. Molecular cloning and expression of the gene encoding the kinetoplast-associated type II DNA topoisomerase of *Crithidia fasciculata*. Mol Biochem Parasitol 1992;50:57–67.
[22] Ray DS, Hines JC. Disruption of the *Crithidia fasciculata* RNH1 gene results in the loss of two active forms of ribonuclease H. Nucleic Acids Res 1995;23:2526–30.
[23] Lukes J. Life cycle of *Goussia pannonica* (Molnar, 1989) (Apicomplexa, Eimeriorina), an extracytoplasmic coccidium from white bream *Blicca bjoerkna*. J Protozool 1992;39:484–94.