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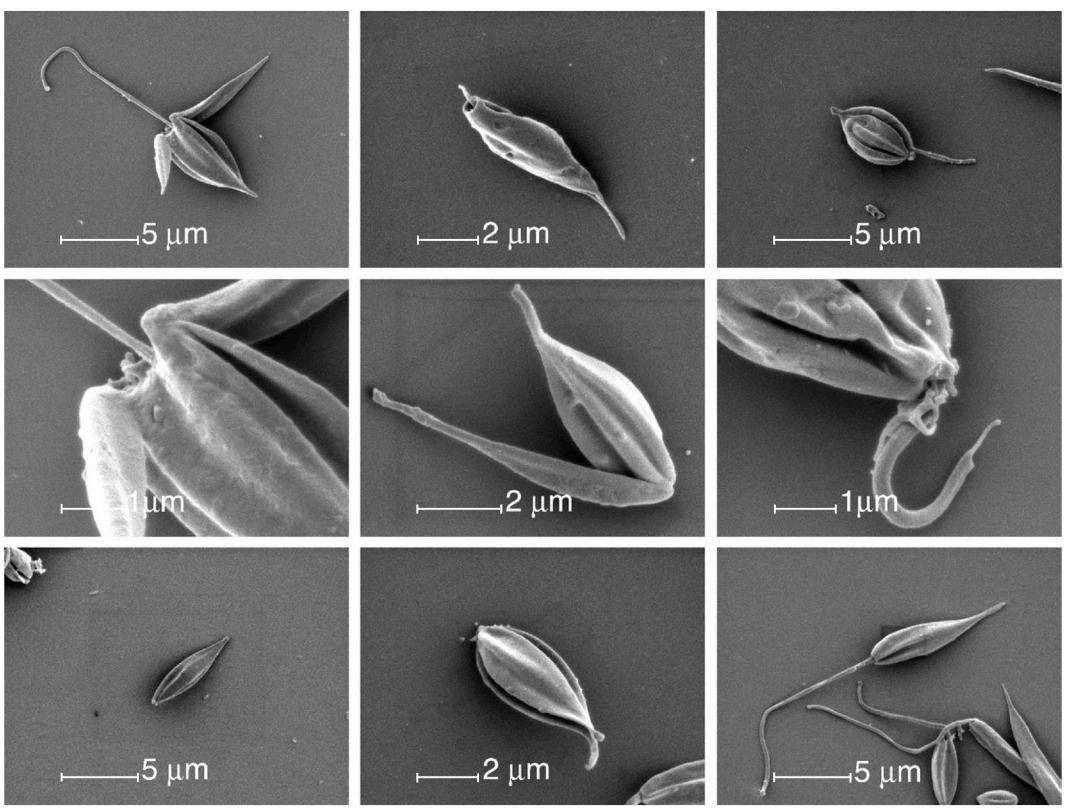

Morphological abnormalities in MIX knockdown *Leishmania major* promastigotes

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A mitochondrial protein affects cell morphology, mitochondrial segregation and virulence in *Leishmania* ☆

Alessandro D. Uboldi [a], Franziska B. Lueder [b,d], Peter Walsh [b,d,1], Timothy Spurck [c], Geoffrey I. McFadden [c], Joan Curtis [a], Vladimir A. Likic [d], Matthew A. Perugini [b,d], Mary Barson [a], Trevor Lithgow [b,d,2], Emanuela Handman [a,*,2]

[a] *The Walter and Eliza Hall Institute of Medical Research, Vic., Australia*
[b] *Department of Biochemistry and Molecular Biology, The University of Melbourne, Vic., Australia*
[c] *Department of Botany, The University of Melbourne, Vic., Australia*
[d] *Bio21 Molecular Science and Biotechnology Institute, Vic., Australia*

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Abstract

The single mitochondrion of kinetoplastids divides in synchrony with the nucleus and plays a crucial role in cell division. However, despite its importance and potential as a drug target, the mechanism of mitochondrial division and segregation and the molecules involved are only partly understood. In our quest to identify novel mitochondrial proteins in *Leishmania*, we constructed a hidden Markov model from the targeting motifs of known mitochondrial proteins as a tool to search the *Leishmania major* genome. We show here that one of the 17 proteins of unknown function that we identified, designated mitochondrial protein X (MIX), is an oligomeric protein probably located in the inner membrane and expressed throughout the *Leishmania* life cycle. The *MIX* gene appears to be essential. Moreover, even deletion of one allele from *L. major* led to abnormalities in cell morphology, mitochondrial segregation and, importantly, to loss of virulence. MIX is unique to kinetoplastids but its heterologous expression in *Saccharomyces cerevisiae* produced defects in mitochondrial morphology. Our data show that a number of mitochondrial proteins are unique to kinetoplastids and some, like MIX, play a central role in mitochondrial segregation and cell division, as well as virulence.

Keywords: Cell division; *Leishmania*; Mitochondria; *Trypanosoma*; Yeast

1. Introduction

Leishmaniasis, the disease caused by the protozoan parasite *Leishmania*, threatens the populations of 88 countries around the world, with about 2 million new cases annually (WHO, 2005). Symptoms range from disfiguring cutaneous and mucocutaneous ulcers to fatal visceral disease. At present, there are no vaccines against leishmaniasis and only a few drugs are available. Unfortunately, their efficacy is limited due to toxicity and lack of effectiveness against all *Leishmania* species (Croft and Coombs, 2003; Croft et al., 2005). In addition, drug resistance is increasing, prompting an urgent need for replacement or supplementary drugs.

Some of the current anti-trypanosome and *Leishmania* drugs act on the mitochondrion (Mukherjee et al., 2006), signifying the therapeutic potential of disruption of mitochondrial function. Mitochondria are essential organelles in most eukaryotes. Each round of the cell cycle generally requires a doubling of the mitochondrial mass and faithful segregation of the mitochondria into each daughter cell (Shaw and Nunnari, 2002; Osteryoung and Nunnari,

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* Corresponding author. Tel.: +61 3 9345 2476; fax: +61 3 9347 0852.
E-mail address: handman@wehi.edu.au (E. Handman).
[1] Present address: St. Vincent's Institute, Victoria, Australia.
[2] These two authors have contributed equally to this work.

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2003; Jensen, 2005). In yeast and in mammals, mitochondrial connectivity and number are maintained by equilibrium in on-going fusion and fission events. A number of proteins, including Fis1, Dnm1 and Mdv1 are required for mitochondrial fission, and others such as Fzo1, Ugo1 and Mgm1 are required for fusion (Shaw and Nunnari, 2002; Okamoto and Shaw, 2005). To date, the only related molecule found in *Leishmania* and trypanosomes is a homologue of the dynamin GTPase Dnm1 (Morgan et al., 2004). This dynamin is involved in mitochondrial fission in *Trypanosoma brucei* and probably also in the related *Leishmania*. BLAST analysis of the *Leishmania major* and *T. brucei* genomes has not revealed homologues of any of the other known eukaryotic fission and fusion proteins, either because these proteins are very divergent in sequence or because different mechanisms control mitochondrial division in kinetoplastids.

Kinetoplastid protozoa like *Leishmania* and trypanosomes have a single mitochondrion housing a massive genome in close association with the flagellar basal body. This genome, the kinetoplast DNA, is composed of a few dozen catenated maxicircles and thousands of minicircles linked together in a single network condensed into a disk-shaped nucleoid (Liu et al., 2005). The division of the kinetoplast is tightly coordinated with the division of the flagellar basal body, the flagellum, and the cell itself (Robinson and Gull, 1991; Robinson et al., 1995; Ploubidou et al., 1999; Ogbadoyi et al., 2003) but many of the proteins involved in these events are not known. The integral link between mitochondrial segregation and cell division suggests that the proteins involved in this process would make attractive drug targets, particularly if they were exclusive to parasites and critical to parasite biology.

Using a bioinformatics approach to search the *Leishmania* genome for new mitochondrial proteins, we have identified the *MIX* gene (for mitochondrial protein X), which is unique to *Leishmania* and *Trypanosoma*. Here we show that MIX is an oligomeric, inner mitochondrial membrane protein expressed throughout the parasite life cycle. Our data suggest that MIX has an influence on cell morphology, mitochondrial segregation and cell division in *Leishmania*.

2. Materials and methods

2.1. Parasite culture

Leishmania major (MHOM/IL/80 FRIEDLIN) parasites were grown at 26 °C in M199 medium containing Hanks' salts (Gibco BRL, USA) and 10% (vol/vol) foetal bovine serum (FBS) (Trace Biosciences, Australia). Parasites in which one allele of the *MIX* gene was deleted by homologous recombination and transgenic parasites maintaining plasmids episomally for over-expression of MIX were grown in the same medium with appropriate antibiotics.

2.2. Bioinformatics

Twenty four *Leishmania* mitochondrial proteins sharing sequence similarity in their N-terminal segments were used to construct a hidden Markov model (HMM) for screening the open-reading frames (ORFs) in the *L. major* genome database. The mitochondrial targeting sequence was assumed to be nine residues long, including the first methionine residue. To build the HMM, we excluded the first methionine residue, and included an extra two residues at the C-terminal end (a total of 10 residues). The package HMMER 2.3.2 (Eddy, 1998) was used for building the model and for searching the *L. major* genome database. Because we were interested only in proteins that carry a similar motif at their N-termini, the search was confined to the first 12 residues of each sequence in the database. The best hits with E-value < 0.018 are shown in Table S1.

2.3. Plasmid construction, cloning and transfections

The LmjF08.1200 ORF encoding MIX (*L. major* GeneDB) was amplified from *L. major* (MHOM/IL/80 FRIEDLIN) genomic DNA using the primers 5′-ACC GGGATCCATGCTCCGCCAC-3′ and 5′-TGCATCTA GACGTGTCACGTGTGGGTCG-3′ and ligated to the pGEM-T (easy) vector (Promega, USA).

A green fluorescent protein (GFP)-tagged construct of the putative mitochondrial targeting motif containing amino acids 1–9 designated MIX(1–9)::GFP was created in the pXG-'GFP vector (Ha et al., 1996) for expression in *Leishmania*. To generate a yeast expression constructs for full-length MIX, the *MIX* gene was cut with *Bam*HI and *Eco*RI from the [pGEM-T *MIX*] construct and inserted into pRS413Gal1 yeast expression vector (Mumberg et al., 1995). For fluorescence microscopy, cells were visualized directly or after staining with MitoTracker (MitoTracker Red CM-H2X Ros, Molecular Probes, USA) according to the manufacturer's protocol.

For the deletion of the *MIX* gene in *L. major*, a 1.72 kbp PCR product was amplified using the primers 5′-CT TCGAGGTGTACGCTTGGGTGTC-3′ and 5′-GGGA TAGCAGCAGCAGCAGCAGCG-3′ and ligated to pGEM-T (easy). *Xba*I and *Bam*HI sites were introduced into the 1.72 kbp fragment by Kunkel mutagenesis (Kunkel et al., 1991), using primers 5′-GGTGTGGCGGAGTCTAGAT TGCGGTAAGAGGCACTA-3′ and 5′-GTAGTAGTGT TTCGTCGGATCCCACATGAGCGCGAT-3′. Incorporation of antibiotic resistance genes into these sites eliminated the start codon and a further 299 nucleotides of the *MIX* ORF. Constructs were made that housed the antibiotic resistance gene for puromycin, neomycin and phleomycin. Approximately 20 μg of knockout cassette DNA was used for each transfection, as previously described (Cruz et al., 1991).

2.4. MitoTracker staining and immunofluorescence detection of parasites

Parasites were resuspended in M199 medium containing 5% FBS and 100 nM MitoTracker Red (Molecular Probes, USA) and allowed to settle onto poly-L-lysine-coated coverslips (Sigma, USA). For immunofluorescence, the parasites were fixed with 4% paraformaldehyde/PBS for 15 min at room temperature, washed with PBS, followed by incubation in PBS containing 1% FBS for 1 h at room temperature. The parasites were then incubated for 1 h at room temperature with affinity purified anti-MIX antibodies, followed by fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG antibodies (Silenus Laboratories, Australia). The coverslips were washed and mounted onto slides using DAKO mounting medium (DAKO, Denmark) and stored at 4 °C in the dark until viewed.

2.5. Parasite growth kinetics

On day 0, stationary phase wild-type and Δ*mix*/*MIX* parasites were diluted to 10^5 cells/ml in 10 ml cultures to allow cells to enter logarithmic phase. For each time point, the parasites were pelleted by centrifugation, washed with PBS, fixed in PBS containing 1% formalin, 100 mM glucose and 0.05% sodium azide, and counted by light microscopy.

2.6. Staining of parasite nuclei and kinetoplasts

Log phase wild-type and Δ*mix*/*MIX* parasites were pelleted by centrifugation; washed with PBS and resuspended in PBS at 10^6 parasites/ml. The parasite suspension (100 μl) was cytocentrifuged at 1,500 rpm for 4 min in a Shandon Cytospin 3 cytocentrifuge. Slides were air-dried, fixed in methanol and stained with Diffquik stain (Lab Aids, Australia) to visualize the nucleus and kinetoplast. The number of nuclei and kinetoplasts in each cell were counted by light microscopy.

2.7. Scanning electron microscopy

Log phase promastigotes were washed in PBS, fixed for 1 h with 2.5% glutaraldehyde (ProSciTech) in PBS, washed three times in PBS and a drop of cell suspension was placed onto circular 1% polyethylenimine (Sigma, Chemical Company)-coated coverslips for several minutes. Affixed cells were rinsed in distilled H_2O and slowly dehydrated in a graded series of ethanol. Samples in 100% ethanol were then dried using a Baltec CPD 030 critical point dryer, gold-coated with an Edwards S150B sputter coater and observed at 20 kV with a Philips XL30 FEG Field Emission Scanning Electron Microscope.

2.8. Protein expression and generation of antibodies

The *MIX* gene was sub-cloned from pGEM-T (easy) into the pGEX-2T vector (Pharmacia Biotech, Sweden) for expression as a fusion protein with the *Schistosoma japonicum* glutathione *S*-transferase (GST) in BL-21 (DE3) pLysS *Escherichia coli* or Rosetta pLysS *E. coli* (Novagen, USA). Following induction of expression with 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG) (Bio Vectra, Canada) for 4 h at 37 °C, cells were lysed in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 1 mg/ml lysozyme (Boeringer Mannheim, Germany) and 30 μg/ml DNase I (Pharmacia Biotech, Sweden). Treatment of the inclusion bodies containing MIX for 30 min at 4 °C with TBS containing 1.5% *N*-lauroylsarcosine (Sigma, USA) and 1 mM EDTA resulted in greatly increased solubility of the GST::MIX fusion protein. *N*-lauroylsarcosine-solubilized GST::MIX was purified by affinity chromatography on glutathione–Sepharose 4B (Pharmacia Biotech, Sweden) and eluted with 10 mM reduced glutathione (Sigma, USA) and 5 mM dithiothreitol (DTT). To improve the yield of soluble protein we produced a truncated construct lacking the N-terminal 45 amino acids of MIX in the pET15b vector carrying a hexa-Histidine (6-His) tag (Novagen, USA) designated [pET-15b *MIX*(Δ1–45)]. This protein was purified using Talon metal affinity beads as recommended by the manufacturer (Clontech, USA). The 6-His::MIX(Δ1–45) protein was eluted from the column with 200 mM imidazole in lysis buffer. For analytical ultracentrifugation analysis, the protein was further purified by size exclusion chromatography on a Sepharose 200 HR 16/60 column (Amersham Biosciences, UK) at a flow rate of 1.0 ml/min.

The recombinant GST::MIX and 6-His-MIX(Δ1–45) fusion proteins were used to generate antibodies in rabbits. A column of recombinant MIX protein covalently coupled to Sepharose was used to affinity-purify anti-MIX antibodies from the immune serum.

2.9. Analytical ultracentrifugation

Sedimentation experiments with MIX (0.5 mg/ml) dissolved in 20 mM Tris, pH 7.5, were performed using a Beckman model XL-A analytical ultracentrifuge equipped with a photoelectric optical absorbance system. Samples (380 μl) and reference solutions (400 μl) were loaded into a conventional double sector quartz cell and mounted in a Beckman An-60 Ti rotor. Data were collected at 290 nm in continuous mode, at a temperature of 20 °C and a rotor velocity of 40,000 rpm using a time interval of 300 s and a step-size of 0.003 cm without averaging. Solvent density (0.999 g/ml at 20 °C) and viscosity (1.007 cp), as well as estimates of the partial specific volume (0.733 ml/g at 20 °C) were computed using the program SEDNTERP (Laue et al., 1992). Sedimentation velocity data at multiple time points were fitted to a continuous size-distribution model (Schuck, 2000) using the program SEDFIT (available from http://www.analyticalultracentrifugation.com). Continuous size distribution analysis was performed using the parameters described in the legend for Fig. 7.

2.10. Triton X-114 phase separation of promastigote proteins

Approximately 3×10^8 promastigotes were lysed in 500 μl of ice-cold PBS/0.5% Triton X-114 (Sigma Chemical Company) containing protease inhibitors and 0.1% bromophenol blue to aid in visualization of the detergent phase. The lysate was incubated for 5 min at 37 °C and centrifuged at 1,750*g* to allow the separation of the water and detergent phases (Bordier, 1981). The upper fraction containing the water-soluble proteins and the oily droplet in the lower fraction containing the detergent soluble proteins were collected and analysed by Western blotting as described previously (Towbin et al., 1979).

2.11. Sodium carbonate extraction

Protein extraction was carried out according to the method of Fujiki et al. (1982) with some modifications. Approximately 3×10^8 promastigotes were resuspended in 300 μl of 10 mM triethanolamine, pH 7.5, 0.5 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 25 U/ml Benzonase (Novagen, USA) and protease inhibitors. The suspension was freeze-thawed three times followed by repeated passaging through a 26.5 gauge needle until essentially all parasites had been lysed. Remaining cells and nuclei were removed by centrifugation at 1,000*g* for 5 min at 4 °C and the supernatant was used for sodium carbonate extraction as follows: 375 μl of ice-cold 100 mM sodium carbonate, pH 11.5, was added to the supernatant and swirled gently on ice for 2 h. After the addition of sodium carbonate, the pH was checked to ensure that it was greater than 11. The solution was centrifuged at 100,000*g* at 4 °C for 1 h and the pellet (membrane fraction) and supernatant (soluble fraction) were retained and analysed by Western blotting.

2.12. Sub-cellular fractionation of L. major parasites

Preparation of crude mitochondrial and soluble/microsomal fractions was carried out as described (Mahapatra et al., 1994) with slight modifications. Parasites were washed twice with PBS/20 mM glucose and resuspended at 1.6×10^9 parasites/ml in 1 mM Hepes, pH 7.4, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.1% BSA and protease inhibitors. The suspension was repeatedly passed through a 26-gauge hypodermic needle until more than 80% of the cells had been lysed. Lysis was monitored microscopically. Sucrose was added immediately to a final concentration of 250 mM. The lysate was then incubated on ice for 30 min with 25 U/ml of Benzonase, before centrifuging for 5 min at 1000*g* at 4 °C to pellet whole cells and nuclei. The supernatant was centrifuged twice for 10 min at 10,000*g* at 4 °C to produce a supernatant containing the soluble/microsomal fraction and a pellet containing the mitochondria.

Fractionation of mitochondria into mitoplast and intermembrane space/outer membrane fractions was carried out as described (Mukherjee et al., 1999). To separate the inner membrane fragments from soluble matrix components, mitoplasts were resuspended in hypotonic buffer (1 mM Tris, pH 8.0, 1 mM EDTA) and sonicated for six bursts of 10 s duration, with cooling on ice for 30 s between each burst, using a Branson Sonifier 250 with duty cycle set to 100% and power set to 50%. This was followed by centrifugation at 144,000*g* for 1 h to produce the inner membrane pellet and matrix supernatant.

2.13. Sub-cellular fractionation of yeast cells

W303a cells transformed with [pRS413413Gal1 *MIX*] were grown in minimal medium (100 ml) containing lactate for 16 h at 30 °C, at which time the culture was in mid-log phase. Expression of MIX was induced by addition of 1% galactose. After 3 h of induction, cells were collected by centrifugation at 3,000*g* at 4 °C for 10 min and mitochondrial and post-mitochondrial cytosol/microsomal fractions prepared as previously described (George et al., 2002) The fractions were analysed by SDS–PAGE and immuno-blotting as previously described (Beilharz et al., 1998) using anti-MIX antibodies. Antibodies to porin, a yeast mitochondrial outer membrane protein, hexokinase, a cytosolic protein and sec61, a protein of the endoplasmic reticulum were used as controls for the purity of the mitochondrial fraction.

For assessing sub-mitochondrial localization of MIX in yeast cells, mitochondria were prepared and analysed as previously described (Beilharz et al., 1998) using antibodies to MIX for detection. Antibodies to the matrix protein mtHsp70, the intermembrane space protein cytb2 and the outer membrane protein Tom20 were used as controls for the mitochondrial sub-compartments.

2.14. Infection of macrophages and mice with MIX heterozygous (Δmix/MIX) parasites

BALB/c-derived bone marrow and peritoneal macrophages were grown on coverslips (Stewart et al., 2005) and infected with stationary phase Δ*mix*/*MIX* or wild-type parasites at a parasite-to-macrophage ratio of 2:1. The extent of uptake into macrophages and parasite survival was assessed over several days of culture. Macrophages were fixed in methanol and stained with Giemsa, and the number of attached and internalised parasites counted by light microscopy. For in vivo studies, highly susceptible BALB/c mice were infected with 10^6 wild-type or Δ*mix*/*MIX* parasites intradermally at the base of the tail and the rate of development of skin lesions was monitored weekly by measuring the lesion diameter as previously described (Elso et al., 2004; Stewart et al., 2005). The survival of parasites in mice was determined by isolation and quantitation of parasites from the lymph nodes draining the site of infection (Stewart et al., 2005). Experiments were performed in accordance with Institutional Animal Ethics approval AEC-2005-012.

2.15. Image acquisition and processing

Confocal fluorescence images were captured using a Biorad MRC1024 Confocal Scanning Laser microscope mounted on a Zeiss Axioscop or a Leica DMIRE2 microscope and a Leica TCS SP2 imaging system. Immunofluorescence staining was visualized with a Zeiss Axioskop2 fluorescence microscope. Images were assembled with Adobe Photoshop CS 8.0 and Macromedia Freehand MX. For Western blots and DNA gel, the images were acquired from films or Kodak paper with an Epson 1680 scanner and processed with Adobe Photoshop and Macromedia Freehand. For densitometric scanning, X-ray films were scanned using a BIORAD GS-800 calibrated densitometer and quantitated using BIORAD Quantity One 4.6.1 software.

3. Results

3.1. MIX is a mitochondrial protein unique to Leishmania and trypanosomes

The genome of *L. major* has recently been sequenced and a few putative mitochondrial proteins have been annotated based on homology to known mitochondrial proteins (Ivens et al., 2005). Analysis of the mitochondrial targeting sequences revealed an unusual conservation in primary structure. The N-terminal sequences of 20 of these proteins (data not shown) yield the motif shown in Fig. 1A. These 20 sequences were used to construct a hidden Markov model to screen all of the ORFs in the *L. major* genome. Hidden Markov models are a class of probabilistic models that turn a multiple sequence alignment into a position-specific scoring system suitable for searching large databases for distantly homologous sequences (Krogh et al., 1994; Eddy, 2004). A ranked list of sequences that match the model is shown in Table S1 (see on-line supplementary material). Seventeen proteins of unknown function show very good correspondence to the pattern and have a homologue in *T. brucei*, but many of these have no obvious homologue in other eukaryotes. Based on the conservation of sequence between *Leishmania* and *T. brucei* as a measure of functional importance and the absence of a human homologue, we chose one of these (LmjF08.1200) for further study. We designated the protein MIX for mitochondrial protein X.

MIX is a nuclear gene encoding a protein of 195 amino acids with a predicted molecular weight of 22.2 kDa. The alignment of *L. major* MIX protein with orthologs from *T. brucei* and *Trypanosoma cruzi* is shown in Fig. 1B. The three sequences are highly conserved: *T. brucei* and *T. cruzi* MIX sequences are 72.6% and 74.2% identical to the *Lmj*MIX sequence, respectively, using LALIGN (Huang and Miller, 1991) (available at http://www.ch.embnet.org/cgi-bin/LALIGN_form.html). MIX has a predicted N-terminal mitochondrial targeting sequence nine amino acids in length (Nakai and Horton, 1999) and a single predicted transmembrane domain (residues 28–42, Fig. 1B) (Cserzo et al., 1997). Using either the conserved domain search tool CDART (Geer et al., 2002) or pair-wise alignment searches with PSI-BLAST (Altschul et al., 1997) we were unable to find any convincing relatedness between MIX and proteins from other organisms.

In order to test whether the predicted mitochondrial targeting sequence of MIX is functional, we fused its sequence to the N-terminus of the GFP. The first nine amino acids of MIX were sufficient to target the reporter GFP to the mitochondrion of *Leishmania* as judged by co-localization with the mitochondrion-specific marker MitoTracker Red (Fig. 2A). Immunofluorescence staining (Fig. 2B) and subcellular fractionation and Western blotting using anti-MIX antibodies (Fig. 2C) showed that MIX itself has mitochondrial localization in *Leishmania*. Sub-mitochondrial fractionation and Western blotting indicated that MIX is located in the inner mitochondrial membrane of *Leishmania* based on co-localization with the inner membrane protein Cytochrome *c* (Fig. 2D).

3.2. MIX single-allele knockout (Δmix/MIX) parasites show aberrant cellular morphology and defects in kinetoplast segregation and cell division

Since MIX has no obvious functional motifs that allude to function, we decided to create *MIX* gene deletion mutant *L. major* and look for phenotypic effects as pointers to function. *Leishmania* are diploid and therefore require two rounds of gene inactivation with constructs carrying different drug cassettes to select homozygous deletion mutants. The schematic of the *MIX* knockout construct is shown in Fig. 3A. In two independent experiments we successfully deleted the *MIX* gene from one allele of the *Leishmania* diploid genome, but we were unable to obtain viable cells in which both copies of the *MIX* gene were inactivated. The presence of the drug cassette and its correct integration into one allele was demonstrated by PCR with specific primers (Fig. 3B). Heterozygous parasites (Δ*mix*/*MIX*) showed significantly reduced expression of MIX protein by Western blotting (Fig. 3C). The expression of MIX protein was only 38%, 27% and 24% of wild-type levels in clones 1, 2 and 3, respectively, indicating that removal of one copy of the *MIX* gene is sufficient to significantly reduce the cellular expression of MIX protein. All of the Δ*mix*/*MIX* clones showed identical phenotypes.

The morphology of Δ*mix*/*MIX* parasites was assessed by SEM. Early logarithmic phase parasites (procyclic promastigotes) were used for the analysis since more striking morphological abnormalities were seen during this phase of growth. The population of Δ*mix*/*MIX* promastigotes was morphologically heterogeneous (Fig. 4). Only 28% of the parasites appeared similar to wild-type cells, while the rest showed serious morphological defects. These included elongated posterior ends (29%), stumpy, tapered or absent flagella, deformed flagellar pockets and shorter, rounded body dimensions (44%). "Wing-like" protrusions from

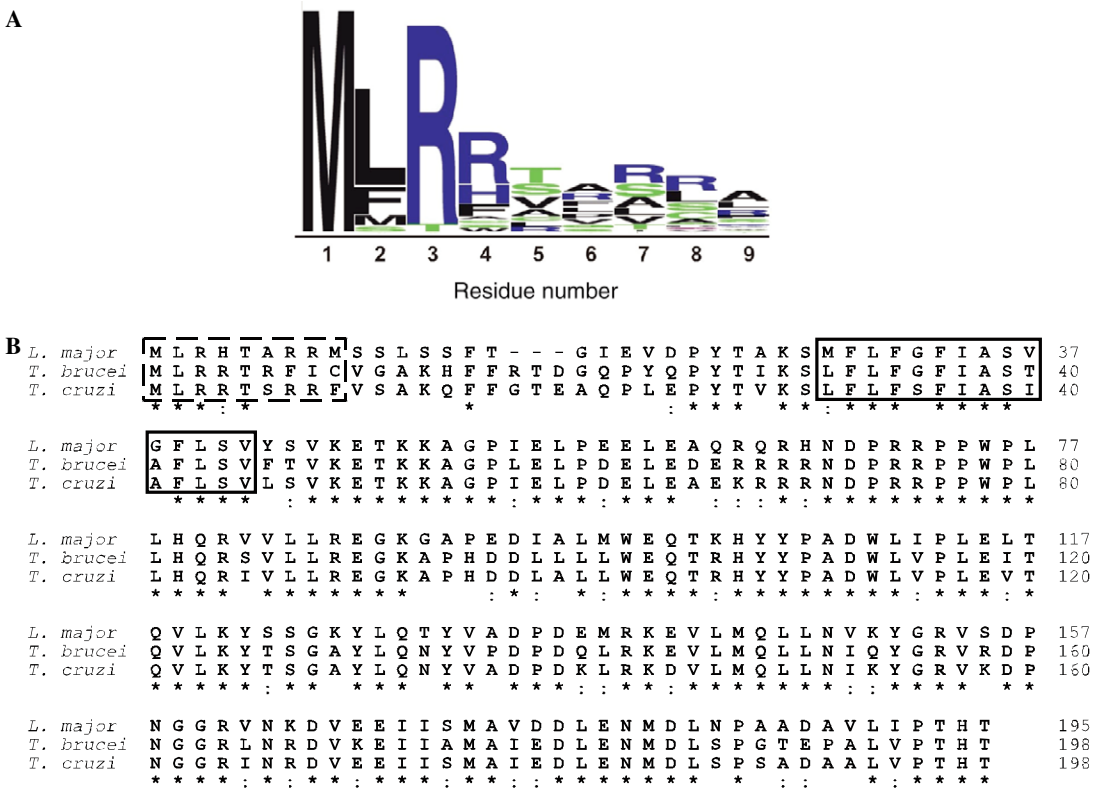

Fig. 1. Mitochondrial protein X (MIX) is unique to kinetoplastids. (A) The mitochondrial targeting sequence is conserved in primary structure in many mitochondrial proteins from *Leishmania*. A sequence logo (Crooks et al., 2004) compiled from the first nine residues of 24 mitochondrial proteins from *Leishmania* is shown. The height of the letters in the sequence logo represents the degree of conservation of a given residue at each position within the sequence motif. Hydrophobic amino acids are shown in black, basic amino acids in blue and hydroxylated amino acids in green. (B) The amino acid alignment of *Leishmania. major*, *Trypanosoma brucei* and *Trypanosoma cruzi* MIX sequences. This alignment was generated using Clustal W (http://www.ebi.ac.uk/clustalw/). Identical residues between the three sequences are marked with an asterisk, while conservative substitutions are marked with a colon. The N-terminal mitochondrial targeting sequence and putative transmembrane domain are indicated by dashed and solid boxes, respectively.

the parasite's anterior ends were also observed, which likely represent parasites that failed to develop or separate appropriately. None of these morphological defects were seen in wild-type cells.

To investigate if the morphological abnormalities in the Δ*mix*/*MIX* mutants were accompanied by defects in kinetoplast or nuclear segregation, the number and distribution of nuclei and kinetoplasts were quantitated during the early logarithmic phase of growth by Giemsa staining. Apart from the normal N1K1, N1K2 and N2K2 phenotypes representing cells in various stages of the cell cycle (Ploubidou et al., 1999; Tyler et al., 2001), a small percentage of cells (approximately 7%) displayed abnormalities in nuclear and kinetoplast number and position in the cell. These abnormalities were either absent or extremely rare in wild-type cells (Fig. 5A). Examples of these phenotypes are shown in Fig. 5B. These include cells with two nuclei and one kinetoplast (N21K), cells with several nuclei and a single kinetoplast (N[n+2]K1), cells with one or two nuclei and no kinetoplast (N1K0 and N2K0), and enucleate cells harbouring only a single kinetoplast known in *T. brucei* as zoids (N0K1). Many of the 2N1K cells had a visible cleavage furrow separating one of the nuclei from the other nucleus and kinetoplast, and some had one of the two nuclei positioned in the posterior region of the cell. In addition, for some cells cytokinesis originated from the posterior region of the cell instead of the expected anterior region near the flagellum. Moreover, for some of these cells (NnKn cells) cytokinesis was incomplete, resulting in cellular extensions that harboured either a nucleus or kinetoplast or were empty. The percentage of cells harbouring this defect (approximately 3%) is consistent with the percentage of incompletely formed or incompletely separated parasites observed by SEM. Finally, there was a small population of cells that had two kinetoplasts, one on either side of a single nucleus, and only one flagellum instead of the expected two flagella, one for each kinetoplast.

3.3. When expressed in yeast, MIX localizes to the inner mitochondrial membrane and leads to over-divided mitochondria

To explore the role of MIX in mitochondrial tubule morphology we turned to the yeast experimental system. This system has the advantage that it is relatively easy to monitor changes in mitochondrial morphology in response to overexpression of fission or fusion proteins and has been used successfully to elucidate the role of many of the

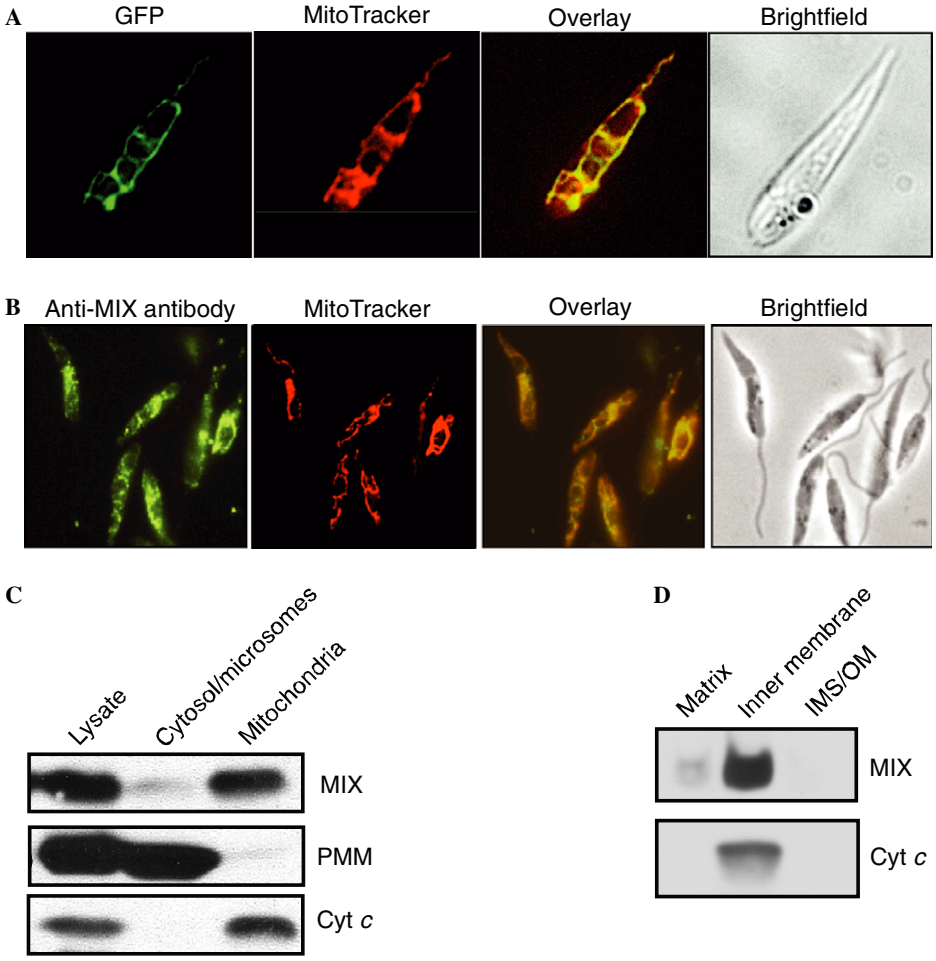

Fig. 2. MIX is a mitochondrial protein. (A) Confocal microscopy images showing the co-localization of MitoTracker Red and MIX(1–9)::GFP (green fluorescent protein) fusion protein to the mitochondrion of *Leishmania major*. (B) Co-localization of MitoTracker and MIX detected by immunofluorescence using rabbit anti-MIX antibodies and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG. (C) Sub-cellular fractionation and Western blotting using anti-MIX antibodies indicates that MIX is localized to the mitochondrion of *L. major*. Cytochrome *c* (Cyt *c*) and phosphomannomutase (PMM) were used as controls for the mitochondrial and cytosolic/microsomal fractions, respectively. (D) Inner membrane localization of MIX in a preparation of purified *L. major* mitochondria detected by Western blotting. Cytochrome *c* was used as a control for the inner membrane. IMS, intermembrane space; OM, outer membrane.

proteins involved in control of mitochondrial shape and division (Okamoto and Shaw, 2005).

Although, MIX has no homologue in yeast, it targeted to mitochondria when yeast cells were transfected with a *MIX* construct as evidenced by immuno-blot analysis showing MIX in the mitochondrial fraction but not in the cytosolic/microsomal fraction (Fig. 6A). Control proteins were detected in the expected fractions.

To further define the location of MIX within the mitochondria, yeast expressing MIX were subjected to treatments designed to selectively permeabilize the outer and inner mitochondrial membranes and expose particular sub-compartments (Fig. 6B). Isolated mitochondria were treated with proteinase K to shave off protein fragments on the outer membrane that are exposed to the cytosol. Since MIX protein remained intact after this treatment, an outer membrane, cytosolic-facing location can be ruled out. Second, the mitochondrial outer membrane was disrupted osmotically, followed by proteinase K treatment. This treatment should leave intact only matrix proteins and those inner membrane proteins that are not exposed to the intermembrane space. Since, MIX was degraded by this treatment, MIX must be exposed to the intermembrane space. Finally, MIX was not detected when mitochondria were resuspended in hypotonic buffer plus Triton X-100 to disrupt all membranes and expose all proteins to protease K digestion. The control proteins behaved as expected following the various treatments.

To determine if MIX overexpression in yeast could affect mitochondrial morphology, we used an expression construct with the *MIX* gene under the control of the inducible GAL promoter, which provides for high-level expression when the cells are grown on medium containing galactose (Mumberg et al., 1994). After 4–5 h growth on medium containing the inducer galactose, MitoTracker staining of the yeast cells expressing MIX revealed clear

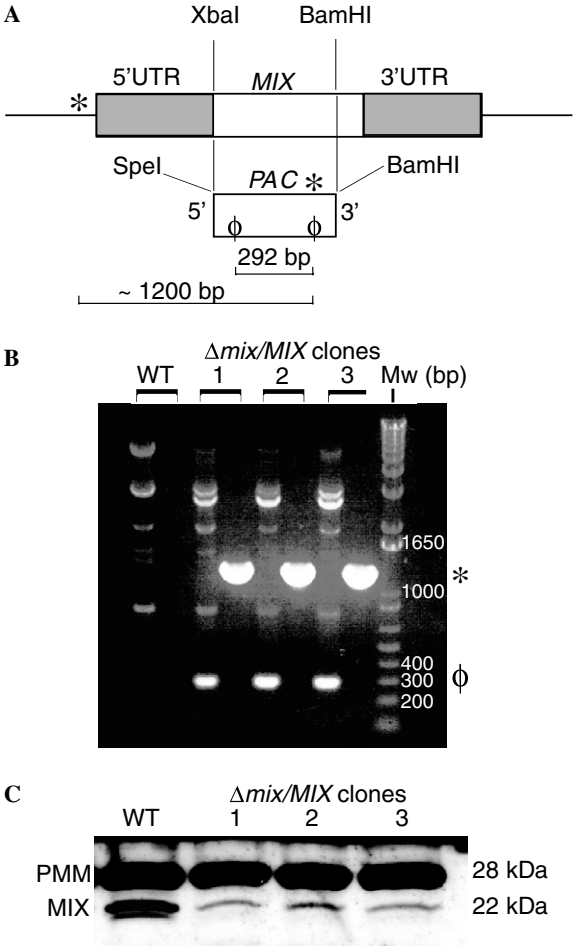

Fig. 3. Generation of *MIX* heterozygous (Δ*mix*/*MIX*) parasites by gene deletion. (A) Schematic diagram showing the replacement of the *MIX* gene by the puromycin acetyl transferase (*PAC*) gene in the knockout construct. (B) PCR analysis of genomic DNA to verify gene disruption. PCR analysis was carried out on three Δ*mix*/*MIX* clones and wild-type (WT) parasites using two sets of primers: one to amplify a fragment of the PAC gene (Φ) to confirm the presence of the drug cassette and one to amplify a fragment spanning the PAC gene and the 5′ untranslated region (UTR) outside of the construct DNA (*) to confirm correct integration of the PAC gene into the genomic DNA of the Δ*mix*/*MIX* clones. (C) Δ*mix*/*MIX* clones showed significantly reduced expression of MIX protein by Western blotting. Expression of phosphomannomutase (PMM) was used as a control for equal loading.

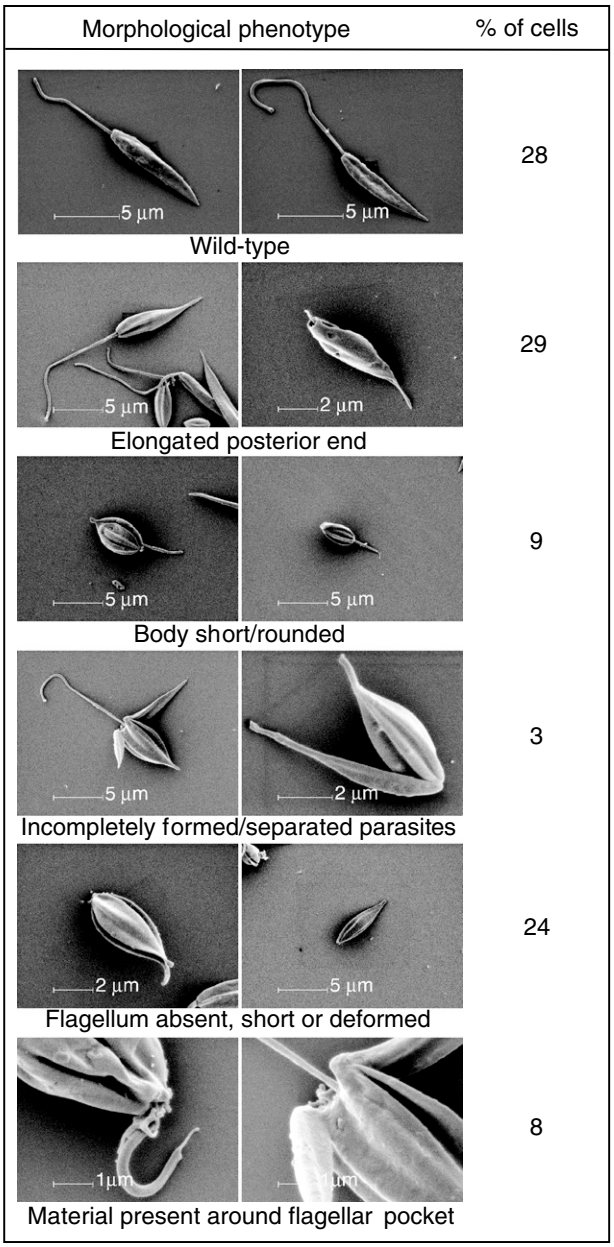

Fig. 4. Δ*mix*/*MIX* parasites display morphological abnormalities. SEM was used to assess 182 Δ*mix*/*MIX* parasites. The percentage values do not add to 100% because some of the cells harboured more than one morphological abnormality. None of these defects were detected in wild-type cells.

defects in mitochondrial morphology (Fig. 6C). Control cells, transformed with the empty vector displayed the reticular network of mitochondrial tubules typical of wild-type yeast cells. However, 70% of yeast cells expressing MIX displayed a fractured reticulum indicative of excessive mitochondrial fission, with a further 13% of cells having mitochondria in fuzzy clumps.

3.4. Self-association of MIX protein

Since a distinguishing feature of many proteins involved in mitochondrial division is their ability to form homo-oligomers (Sossong et al., 1999; Fukushima et al., 2001; Gonzalez et al., 2003; Messerschmitt et al., 2003; Okamoto and Shaw, 2005;), we tested the ability of MIX to oligomerize. The predicted molecular weight of MIX is approximately 22 kDa, but Western blotting of promastigote lysate also detected additional forms of approximately 40 and 80 kDa which could represent oligomeric forms of the protein (Fig. 7A). Moreover, oligomerization of recombinant MIX[Δ1–45], which lacks the predicted transmembrane domain, was also detected when the protein was stored in the presence of 10% glycerol (Fig. 7A). The difference between the oligomerization pattern of endogenous and recombinant MIX protein is possibly due to the absence

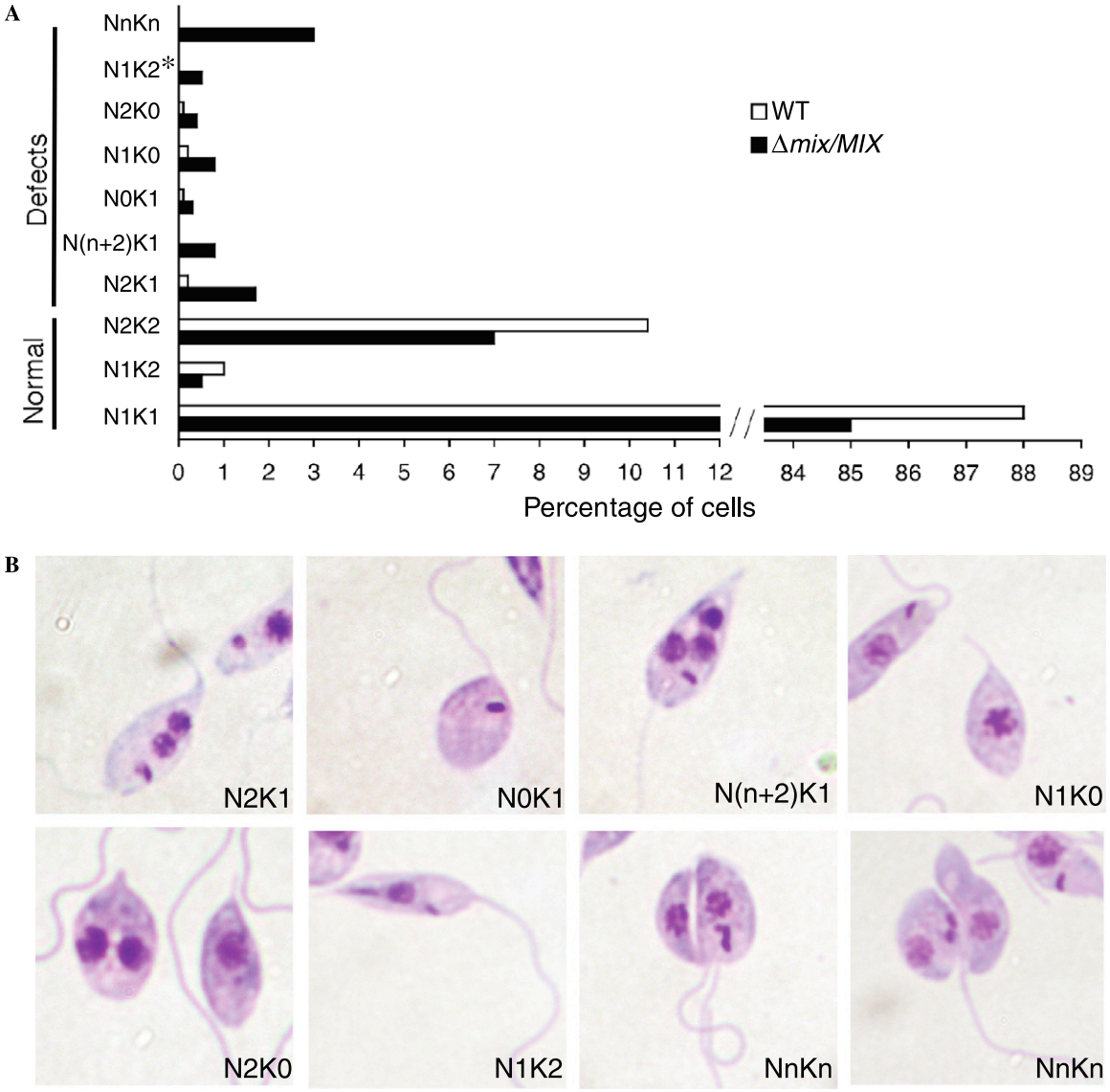

Fig. 5. Δ*mix*/*MIX* parasites display abnormalities in number and distribution of nuclei and kinetoplasts. (A) Giemsa staining was used to quantify the number of nuclei and kinetoplasts in 1,488 Δ*mix*/*MIX* parasites and 1,256 wild-type parasites. The N1K2* signifies that NK complement is normal, but the positioning of kinetoplasts in the cell is abnormal. NnKn parasites contain cellular extensions and a variable number of nuclei and kinetoplasts. (B) Examples of the various NK phenotypes described above.

of the first 45 amino acids (including the transmembrane domain) from the recombinant protein.

To determine the stability and quaternary structure of recombinant MIX [Δ1–45], sedimentation velocity studies were performed in the analytical ultracentrifuge under native solution conditions. When the data was fitted to a continuous-size distribution model (Schuck, 2000), a single peak with an apparent molecular mass of 41 kDa was obtained (Fig. 7B). This is in close agreement with the theoretical mass of the MIX dimer. Similar data were obtained following incubation of MIX at 4 °C for 22 days, which indicates that the MIX dimer is stable over an extended time period. This result was confirmed when MIX [Δ1–45] was separated by size exclusion chromatography, where it also eluted as a dimer (data not shown). Thus, MIX likely exists as a dimer, but has the ability to homo-oligomerize under certain conditions.

3.5. Unusual membrane interaction properties of MIX protein

Some mitochondrial division proteins, such as mgm1, FtsZ and Mdm33 are associated with the inner mitochondrial membrane (Wong et al., 2000; Messerschmitt et al., 2003; Wong et al., 2003; Nishida et al., 2004). To establish if the predicted transmembrane segment of MIX does indeed function as a transmembrane segment, parasite cell extracts were subjected to detergent phase separation in Triton X-114 (Bordier, 1981). Surprisingly, MIX partitioned into both the aqueous and detergent fractions in

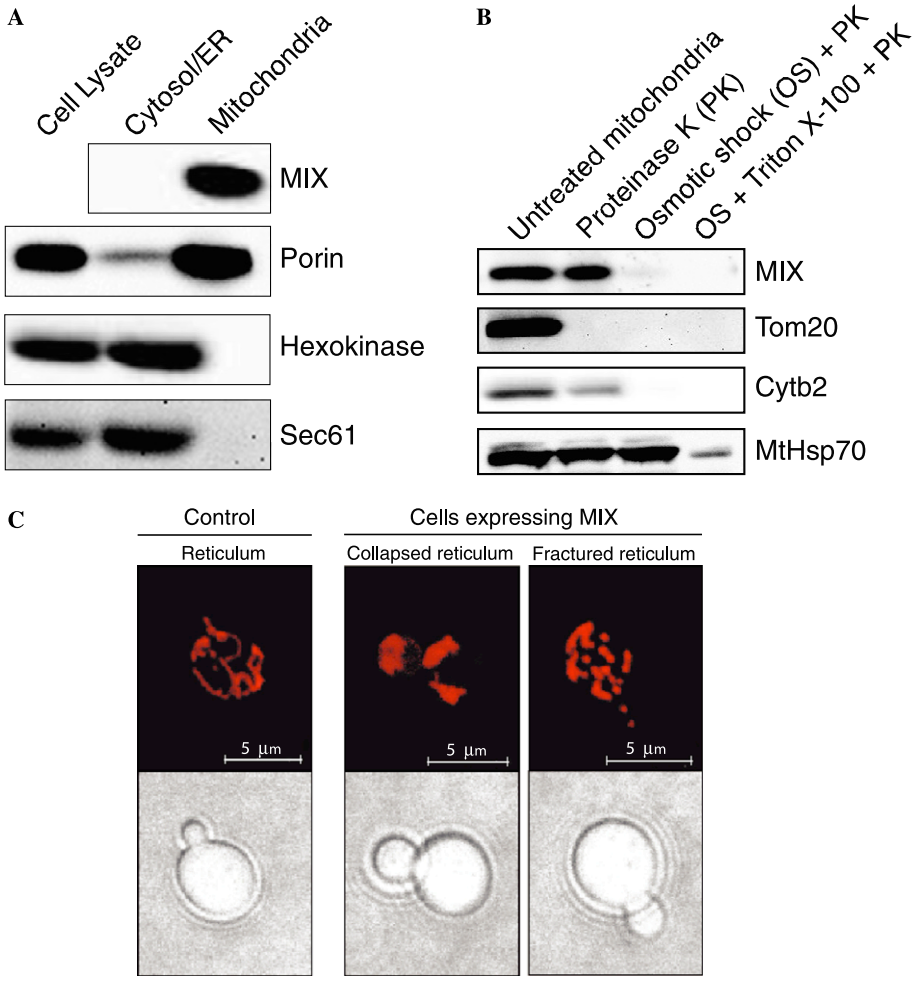

Fig. 6. Mitochondrial protein X (MIX) expressed in *Saccharomyces cerevisiae* localizes to the mitochondria and affects mitochondrial morphology. (A) Sub-cellular fractionation of yeast and Western blotting using anti-MIX antibodies reveal that MIX is localized to the mitochondria of yeast cells. Porin, Hexokinase and Sec61 were used as controls for the mitochondrial, cytosolic and ER fractions, respectively. (B) Inner membrane/intermembrane space localization of MIX in yeast mitochondria detected by Western blotting. Tom20, Cytb2 and MtHsp70 were used as controls for the outer membrane, intermembrane space and matrix, respectively. (C) Expression of MIX in *Saccharomyces cerevisiae* produces defects in mitochondrial morphology. Confocal images of yeast cells stained with MitoTracker showing the typical reticular structure in wild-type cells and the morphological defect of "collapsed" and "fractured" organelles induced by expression of MIX. Seventy percent of cells expressing MIX displayed a fractured reticulum and 13% had mitochondria in fuzzy clumps ($n = 100$).

approximately equal proportions, whereas control proteins, integral membrane protein Parasite Surface Antigen-2 (PSA-2) and soluble phosphomannomutase (PMM), partitioned into the detergent and aqueous phases, respectively (Fig. 7C). Solubilization of MIX with Triton X-114 was complete since no MIX could be detected in the insoluble pellet. Since some proteins are known to behave anomalously during Triton X-114 phase separation (Maher and Singer, 1985; Pryde and Phillips, 1986; Hooper and Bashir, 1991; Niehrs et al., 1992), we used a second approach and subjected promastigote lysates to sodium carbonate extraction to separate integral membrane proteins from peripheral membrane proteins and cytosolic proteins (Fujiki et al., 1982). As was the case for Triton X-114 phase separation, MIX partitioned into both soluble and integral membrane fractions, whereas the control PSA-2 and PMM were exclusively present in the membrane and soluble fractions, respectively (Fig. 7C). Thus, both methods indicate that MIX behaves as a membrane protein, as well as a soluble protein, and both forms display the same molecular weight, suggesting that there is no processing of the hydrophobic domain of the protein that could account for this behaviour.

3.6. MIX is expressed during the entire Leishmania life cycle

Amastigotes obtained from skin lesions of infected mice were allowed to differentiate into promastigotes in culture, and the expression of MIX was determined by immunoblotting on lysates from procyclic promastigotes, infective metacyclic promastigotes and amastigotes. MIX was expressed during the entire life cycle (Fig. 8A), but with

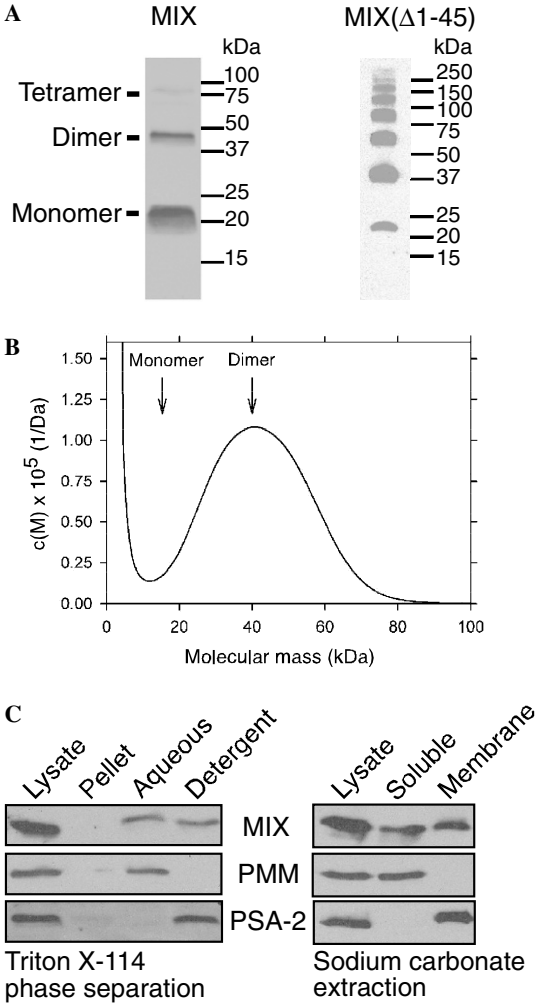

Fig. 7. MIX is an oligomeric protein with unusual membrane interaction properties. (A) MIX can form multimers. Promastigote protein lysate and recombinant MIX(Δ1–45) protein (incubated with 10% glycerol) were separated on 13% SDS–PAGE gels before Western blotting using anti-MIX antibodies. (B) Sedimentation velocity analysis of recombinant MIX(Δ1–45) protein. The continuous mass $[c(M)]$ distribution is plotted as a function of molecular mass (kDa) for MIX. The molecular mass of the recombinant MIX monomer and dimer based on amino acid sequence are indicated with arrows. Sedimentation velocity experiments were conducted in a Beckman model XL-A analytical ultracentrifuge as described in the experimental procedures. Continuous size-distribution analysis was performed using the program SEDFIT at a resolution of 100 species with $M_{min} = 4.0$ kDa, $M_{max} = 130$ kDa, $f/f_0 = 1.81$ and at $P = 0.95$. (C) Analysis of MIX membrane association by Triton X-114 phase separation and sodium carbonate extraction of promastigote lysate. MIX was detected by Western blotting with anti-MIX antibodies. Controls were detected by anti-PSA-2 and phosphomannomutase (PMM) antibodies.

reduced levels in metacyclic promastigotes and amastigotes compared with procyclic promastigotes (approximately 76% and 63% of procyclic levels, respectively, as measured by densitometry). MIX expression in amastigotes varied considerably in different preparations, but was always reduced compared with that in promastigotes, as shown in a representative Western blot (Fig. 8A).

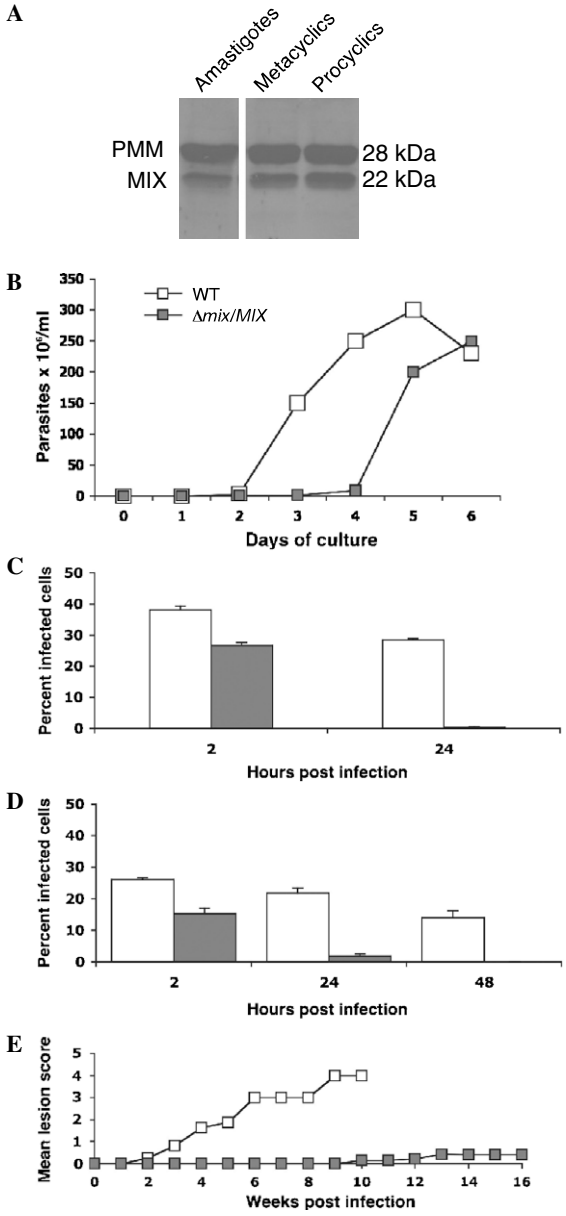

Fig. 8. Mitochondrial protein X (MIX) is expressed throughout the *Leishmania* life cycle and Δ*mix*/*MIX* parasites display reduced growth, reduced infectivity in vitro and attenuated virulence in vivo. (A) MIX is expressed throughout the life cycle of *Leishmania* with reduced expression in amastigotes. Western blotting using anti-MIX and anti-phosphomannomutase (PMM) as loading control was carried out on lysates from procyclic promastigotes, metacyclic promastigotes and amastigotes. (B) Δ*mix*/*MIX* parasites grow more slowly than wild-type parasites with a prolonged lag phase. Growth rate was determined as described in Section 2. MIX knockdown parasites do not survive in bone marrow-derived (C) or peritoneal macrophages (D). Macrophages were infected for the indicated periods and stained with Giemsa. The number of internalised parasites in 500 cells was counted in duplicate slides and the mean and SD calculated. (E) For in vivo studies, BALB/c mice were infected with wild-type or Δ*mix*/*MIX* parasites intradermally at the base of the tail and the rate of development of skin lesions was determined by measuring the diameter of the lesion plus the standard deviation of the mean. The graph represents one experiment with eight mice in each group.

3.7. MIX knockdown parasites grow more slowly than wild-type parasites with a prolonged lag phase and display reduced macrophage infectivity in vitro and attenuated virulence in vivo

Δ*mix*/*MIX* parasites were examined to see if deletion of one allele of *MIX* was sufficient to affect growth in culture. Fig. 8B shows that when Δ*mix*/*MIX* parasites were diluted to 1×10^5 parasites/ml from a stationary phase culture, they remained in the lag phase 2 days longer than wild-type parasites under similar conditions. However, once the Δ*mix*/*MIX* parasites entered into the logarithmic phase, they grew at approximately the same rate as wild-type parasites as evidenced by the approximately parallel growth curves during the logarithmic phase of growth.

To test if deletion of one allele of *MIX* affected parasite virulence, infective stationary phase Δ*mix*/*MIX* and wild-type parasites were used to infect resident peritoneal macrophages or bone marrow-derived macrophages in vitro and their initial uptake into the cells and subsequent survival were assessed over several days of culture. The mutants displayed a significant loss of virulence. There was no significant difference in the initial parasite uptake at 2 h p.i. of bone marrow-derived macrophages but after 24 h only 0.3% of the cells harboured Δ*mix*/*MIX* parasites compared with 28% harbouring wild-type organisms (Fig. 8C).

A similar overall pattern of infection was observed with resident peritoneal macrophages (Fig. 8D). Infection rates for wild-type parasites were 26%, 22% and 14% after 2, 24 and 48 h of infection, respectively. In contrast, infection rates for Δ*mix*/*MIX* parasites were 15% after 2 h and only 4% after 24 h. After 48 h of infection, no Δ*mix*/*MIX* parasites could be detected.

We also infected the highly susceptible BALB/c mice with stationary phase wild-type and Δ*mix*/*MIX* parasites intradermally and monitored the rate of development of skin lesions (Fig. 8E). Mice infected with stationary phase wild-type parasites developed severe lesions and had to be killed after 10 weeks. In contrast, mice infected with stationary phase Δ*mix*/*MIX* parasites showed no lesions for the first 10 weeks of infection and only half of them developed minor lesions over a period of 16 weeks (Fig. 8E).

4. Discussion

We identified 17 *Leishmania* genes predicted to encode mitochondrial proteins and which appear to have no homologues in humans. Little is known about the mitochondrial protein import machinery in these organisms but it is intriguing that many of the proteins have targeting sequences that are short and conserved in primary structure (Hausler et al., 1997; Hines and Ray, 1998; this paper). The significance of the small pre-sequences is unknown but in the case of MIX, this sequence is sufficient to target the protein not only to the parasite mitochondrion, but also to yeast mitochondria. In mammals and yeast, the presence of a N-terminal amphipathic targeting sequence is associated with transport of proteins to the mitochondrial matrix via the TOM and Tim23 complexes (Herrmann and Neupert, 2003; Rehling et al., 2003; Truscott et al., 2003; Herrmann and Hell, 2005). Thus, we may expect matrix localization for these short pre-sequence proteins and this is indeed true for some of these proteins (Hines and Ray, 1998). However, proteins such as MIX, that harbour bipartite targeting sequences consisting of an N-terminal amphipathic α-helix followed by a transmembrane segment are often laterally inserted into the mitochondrial inner membrane (Herrmann and Neupert, 2003; Wiedemann et al., 2004; Herrmann and Hell, 2005). We have shown that this is likely to be the case for MIX expressed in both *Leishmania* and yeast cells, implying that MIX carries out its functions at the level of the inner membrane.

The observed phenotype of collapsed and fractured mitochondrial reticulum following over-expression of MIX in yeast is similar to that observed for over-expression of Mdm33, a yeast mitochondrial inner membrane protein thought to be involved in mitochondrial fission (Messerschmitt et al., 2003). Mdm33 shows additional similarities to MIX, which may have functional relevance. Like MIX, Mdm33 is thought to undergo homo-oligomeric interactions. The fragmented and collapsed mitochondrial phenotype caused by over-expression of MIX in yeast is suggestive of excess mitochondrial fission. In *Leishmania*, the occurrence of N2K1 and N(n+2)K1 multinucleate-single kinetoplast parasites in the under-expressing Δ*mix*/*MIX* knockdown population, in which there is nuclear but not kinetoplast division, provides support for the role of MIX in mitochondrial fission.

Although, MIX does not occur naturally in yeast, *T. brucei* proteins with short N-terminal mitochondrial targeting sequences similar to that of MIX have been successfully expressed in yeast and, like MIX, correctly localize to mitochondria (Hausler et al., 1997). Moreover, there are precedents for specific functional effects of proteins expressed in heterologous systems. For example, the protein FtsZ from the brown alga *Mallomonas spendens*, was shown to interact with endogenous proteins and cause defects in yeast mitochondrial morphology (Beech et al., 2000). Similarly, the Bcl-2 family of apoptosis regulators absent in yeast have similar effects when expressed in yeast as they do in mammalian cells (Sato et al., 1994; Hanada et al., 1995) and the pro-apoptotic protein Bax, which participates in mitochondrial fission in mammalian cells (Karbowski et al., 2002), can cause fission of mitochondria when expressed in trypanosomes, where it does not have homologues (Esseiva et al., 2004).

Thus, the fact that MIX affects mitochondrial tubule morphology in yeast points to a potential role for MIX in mitochondrial division. The availability of defined yeast mutants with well-characterized mitochondrial defects as an adjunct to our studies in the homologous *Leishmania* system should facilitate the elucidation of the role of MIX in controlling mitochondrial tubule dynamics.

Although, we showed that MIX is most likely a mitochondrial inner membrane protein exposed to the intermembrane space, the behaviour of MIX during both Triton X-114 phase separation and sodium carbonate extraction identified a membrane associated form as well as a soluble form of the protein of similar molecular weight. Some mitochondrial proteins with bipartite signal sequences are initially laterally inserted into the inner membrane before being released into the intermembrane space by proteolytic cleavage (Herrmann and Hell, 2005). However, cleavage of MIX after the transmembrane segment would produce a protein of approximately 17 kDa, which we have not observed. It is possible that two different conformations of MIX with differing solution properties may exist, as is the case for the pro-apoptotic Bax protein, where a conformational change which exposes the transmembrane domain allows membrane insertion and oligomerization (Roucou and Martinou, 2001).

The unusual behaviour of MIX during Triton X-114 phase separation is reminiscent of that of the GRA3 protein from *Toxoplasma gondii*, which also distributes equally between the aqueous and detergent phases during Triton X-114 phase separation (Ossorio et al., 1994). The detergent fraction is thought to represent homo-oligomerized, membrane-associated protein.

In trypanosomatids, the division of the kinetoplast is tightly coordinated with the division of the flagellar basal body, the flagellum and the cell itself (Robinson and Gull, 1991; Robinson et al., 1995; Ploubidou et al., 1999; Ogbadoyi et al., 2003). Coordination of mitochondrial segregation depends on the "tripartite attachment complex" that links the kinetoplast DNA to the basal body of the flagellum (Schneider, 2001; Liu et al., 2005). The segregation of the kinetoplast and nucleus takes place in synchrony, although the relative timing and order of the kinetoplast and nuclear division varies depending on the species (Steinert and Steinert, 1962; Cosgrove and Skeen, 1970; Simpson and Braly, 1970; Van Assel and Steinert, 1971; Woodward and Gull, 1990; Ploubidou et al., 1999; Tyler et al., 2001). This preference may be a reflection of the morphology of the cell types. In *T. brucei* segregation of the kinetoplast takes place before nuclear segregation (Woodward and Gull, 1990; Ploubidou et al., 1999; Tyler et al., 2001). We observed that wild-type *L. major* cells (like *T. brucei*) early in the cell cycle have a N1K1 complement, those that have undergone kinetoplast segregation have a N1K2 complement, while after mitosis and prior to cytokinesis cells have a N2K2 complement.

The majority of Δ*mix*/*MIX* parasites had normal NK numbers, but a small proportion displayed defective NK number and distribution. The most common defect observed consisted of cells with defective cytokinesis, such that formation of the division cleavage furrow occurred from the posterior end, rather than the anterior end of the parasite. This process results in the formation of incompletely separated parasites visible as cellular extensions (referred to as NnKn cells). These extensions may be empty, house either a nucleus or kinetoplast, or a normal NK complement. It is interesting that following microtubule inhibition in *T. brucei*, the formation of 0N1K zoids from 1N2K cells occurs from the posterior region of the cell (Robinson et al., 1995).

Although approximately 93% of cells appeared normal when judged by their NK complement, only about 30% appeared normal by morphological criteria detected by SEM. This discrepancy between aberrant NK number and morphology has been observed for *T. brucei* CRK1 and CRK2 knockdown procyclic parasites, where approximately half of 1N1K cells (normal by NK complement), harboured the morphological defect of elongated posterior ends (Tu and Wang, 2005). Elongated posterior ends are also a relatively common morphological abnormality of Δ*mix*/*MIX* cells (29% of cells). In the case of parasites with elongated posterior ends observed in *T. brucei*, the cells were arrested in the G1 phase of the cell cycle (Li and Wang, 2003; Tu and Wang, 2005). Similarly, *T. brucei* cells treated with a high concentration of the anti-microtubule agent rhizoxin produced a high proportion of 1N1K cells with unusual morphologies, which were interpreted to be blocked in both kinetoplast and nuclear segregation (Ploubidou et al., 1999). Whether any of the Δ*mix*/*MIX* 1N1K cells are blocked or delayed during the cell cycle remains to be determined.

It is also possible that some of the effects of Δ*mix*/*MIX* parasites on the cell cycle may be a consequence of disruption of other cellular functions, such as mitochondrial metabolism or protein import. For example, it has been shown that the flagellum has a role in controlling cell size, shape, polarity and division of *T. brucei* and determines the point where cytokinesis is initiated (Tyler et al., 2001; Kohl et al., 2003). Since the flagellum is linked to the mitochondrial membranes and kinetoplast DNA by a system of microfilaments (Ogbadoyi et al., 2003), one can envisage secondary effects on flagellar biogenesis resulting from dysfunction within the kinetoplast region of the mitochondrion of Δ*mix*/*MIX*, leading to the observed morphological defects.

It is surprising that reduction of MIX expression by deletion of one allele of the *MIX* gene was sufficient to cause such significant effects. However, gross morphological defects have also been observed in *T. cruzi* UDP-glucose 4′-epimerase single-allele deletion mutants (MacRae et al., 2006). For haploid Δ*mix*/*MIX* cells, MIX protein expression levels are expected to fluctuate around a mean of 50%, but MIX expression varied from 24% to 38% of wild-type levels, depending on the Δ*mix*/*MIX* clone. This lower level of expression is likely due to the stochastic nature of gene expression. Gene expression is subject to random probabilistic fluctuations, such that levels can be substantially lower than 50% and will vary over time (Santarosa and Ashworth, 2004). In addition, the MIX expression levels may be sustained at low levels if feedback mechanisms apply. Thus, depending on the levels of MIX protein at any point in time within the cell cycle, a cell

may continue on to complete a normal cell cycle or may display the observed defects. This variability within a clonal population of cells was also seen in single-allele deletion mutants of *T. cruzi* UDP-glucose 4′-epimerase (MacRae et al., 2006).

In conclusion, we have identified a kinetoplastid-specific mitochondrial protein that has an influence on cell morphology, mitochondrial segregation and cell division, and importantly, on parasite virulence. Our data suggest that MIX has a role in regulating mitochondrial membrane dynamics, although the exact mechanism remains to be elucidated. Regardless of whether *MIX* knockdown causes the observed defects directly or indirectly, the dramatic phenotype of *MIX* heterozygotes, the uniqueness of MIX to kinetoplastids and the observed decrease in virulence of Δ*mix*/*MIX* parasites suggest that MIX function is critical.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2006.08.006.

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