Interaction of mitochondria with microtubules in the filamentous fungus *Neurospora crassa*

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Summary

The establishment and maintenance of the 3D structure of eukaryotic cells depends on active transport and positioning of organelles along cytoskeletal elements. The biochemical basis of these processes is only poorly understood. We analysed the interaction of mitochondria with microtubules in the filamentous fungus *Neurospora crassa*. Mitochondria were fluorescently labelled by expression of matrix-targeted green fluorescent protein. Upon isolation, mitochondria collapsed to round spherical structures that were still able to interact with microtubules in vitro. Binding of mitochondria to microtubules was dependent on peripherally associated proteins on the organellar surface, and was sensitive to adenine nucleotides. MMM1, a mitochondrial outer membrane protein important for maintenance of normal mitochondrial morphology, was not required. This suggests that the interaction of mitochondria with the cytoskeleton is independent of MMM1. We conclude that mitochondrial morphology is maintained by a complex interplay of extrinsic and intrinsic factors, including ATP-dependent proteins on the organellar surface.

Key words: Green fluorescent protein, Microtubules, Mitochondria, *Neurospora*, Organelle biogenesis

Introduction

Mitochondria are essential organelles of eukaryotic cells. They cannot be formed de novo but grow continuously throughout the cell cycle, a process that requires the products of both the nuclear and the mitochondrial genome. Upon cell division, they must be delivered to their proper location in the daughter cell. Mitochondrial inheritance is an ordered process that depends on active transport along cytoskeletal elements (Warren and Wickner, 1996; Yaffe, 1999). However, little is known about the cellular mechanisms mediating this process.

Transport of mitochondria has been most extensively studied in the budding yeast *Saccharomyces cerevisiae*. In this organism, the actin cytoskeleton is of major importance for mitochondrial movement, whereas the microtubule system is not involved (Hermann and Shaw, 1998). In contrast, mitochondria are associated with microtubules in mammalian cells (Heggeness et al., 1978) and transport depends on kinesin-like motor proteins in *Drosophila* and in mice (Nangaku et al., 1994; Pereira et al., 1997; Tanaka et al., 1998). Similarly, cytoplasmic microtubules are required for mitochondrial distribution in the fission yeast *Schizosaccharomyces pombe* (Yaffe et al., 1996). It was shown that mitochondrial movement in the filamentous fungus *Neurospora crassa* is a microtubule-dependent process (Steinberg and Schliwa, 1993) and that disruption of microfilaments has no effect on mitochondrial distribution and morphology (Prokisch et al., 2000). This, combined with its distinct genetic and biochemical advantages, makes *Neurospora* an ideal model organism to study microtubule-dependent organelle transport processes.

In a pioneering study, Steinberg and Schliwa observed vectorial saltatory movement of mitochondria with a mean velocity of 1.4 µm/second in *Neurospora* hyphae, protoplasts, cell fragments and a cell wall-less mutant. In all cell systems tested, disruption of microtubules with nocodazole impaired directional mitochondrial motility (Steinberg and Schliwa, 1993). The molecular components mediating mitochondrial movement in *Neurospora* are not known. Nkin, a distantly related member of the 'conventional' kinesin family (Steinberg and Schliwa, 1995), is not involved in movement and positioning of mitochondria (Seiler et al., 1997). Recently, we described the outer membrane protein MMM1, a component required for mitochondrial morphogenesis in *Neurospora* (Prokisch et al., 2000). However, it was not known whether MMM1 was required for an interaction of mitochondria with microtubules.

Previous work using *Neurospora* to study mitochondrial behaviour relied on the use of electron microscopy (Alberghina et al., 1974; Grad et al., 1999; Hawley and Wagner, 1967), computer-enhanced video microscopy (Steinberg and Schliwa, 1993) or fluorescent dyes (Minke et al., 1999; Prokisch et al., 2000). In recent years, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been developed as a vital marker for the specific labelling of intracellular structures (Tsien, 1998). Although labelling of mitochondria by expression of mitochondria-targeted GFP is a straight forward approach in many experimental systems, including mammalian cells and yeast (Rizzuto et al., 1995; Westermann and Neupert, 2000), expression of GFP turned out to be much more difficult in other organisms including many plants, fungi and protozoa (e.g. Fernandez-Abalos et al., 1998; Haseloff et al., 1997; Hauser et al., 2000; Spellig et al., 1996). Only very recently could expression of GFP be demonstrated in *Neurospora*; however, GFP labelling of specific subcellular structures was never reported (Freitag et al., 2001).

We constructed a mitochondria-targeted GFP (mtGFP) and used this protein to examine the behaviour of mitochondria in vivo and in vitro and to show directly binding of isolated mitochondria to microtubules. Furthermore, we characterised the biochemical basis of the interaction of mitochondria with microtubules in vitro. Interaction of mitochondria is ATP-sensitive and depends on peripherally associated mitochondrial proteins. MMM1 is not required for this process, ascribing to this component a more general role for mitochondrial morphogenesis.

Materials and Methods

Recombinant DNA techniques and plasmid constructions
Standard methods were used for cloning procedures (Sambrook et al., 1989). PCR was performed using *Pfu* Polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

The *mgfp5* allele (Siemering et al., 1996) (GenBank U87973) was amplified by PCR using primers GFP-N (5′ CGG GTA CCA GAT CTA TGA GTA AGG GTG AAG AAC TTT TC) and GFP-C (5′ CGG AAT TCT TAT TTG TAT AGT TCA TCC) and cloned into the *Kpn*I/*Eco*RI sites of pGEM3Su9(1-69) (Rapaport et al., 1998) yielding plasmid pGEM3Su9(1-69)-GFP5. A *Bam*HI/*Eco*RI fragment containing the GFP-coding region plus some upstream restriction sites was transferred to vector pBluescript KS(–) (Stratagene) yielding plasmid pBS-GFP5. The promoter and presequence-coding region of the *atp-1* gene (Bowman and Knock, 1992) (GenBank M84191) was amplified by PCR from genomic *Neurospora* DNA using primers F1a-1 (5′ AAA TCT AGA GAT ATC TTG GAA CGG CCC GG) and F1a-2 (5′ AAA GGA TCC GGC GTA GGT GCG GGC CTG) and cloned into the *Xba*I/*Bam*HI sites of pBS-GFP5 yielding plasmid pBS-mtGFPa. The 3′ coding and terminator region of the *atp-1* gene was amplified by PCR from genomic *Neurospora* DNA using primers F1a-3 (5′ AAA AGT CGA CGT GGT GAG CGT GTA AGT GC) and F1a-4 (5′ AAA GGG CCC TAC TGT GAT CCG CAA ATT CAG) and cloned into the *Sal*I/*Apa*I sites of pBS-mtGFPa yielding plasmid pBS-mtGFPb. Finally, a hygromycin-resistance-conferring cassette was amplified by PCR from plasmid pCB1179 (Sweigard et al., 1997) using primers *Not*I-Hyg (5′ AAA GCG GCC GCA GGG AAT AAG GGC GAC ACG G) and Hyg-*Not*I (5′ AAA GCG GCC GCT GCC GAT TTC GGC CTA TTG G) and cloned into the *Not*I site of pBS-mtGFPb yielding plasmid p*Nc*-mtGFP.

Neurospora genetic methods and isolation of mitochondria
Neurospora wild-type strain was St. Lawrence 74*A* (Fungal Genetics Stock Center, Kansas City, KS), and *mmm-1* mutant strain was *mmm-1*[RIP23] (Prokisch et al., 2000). Standard genetic and microbiological techniques were used for the growth and manipulation of *Neurospora* strains (Davis and de Serres, 1970). *Neurospora* was grown at 25°C in Vogel's minimal medium under continuous aeration and illumination with white light, with the exception that cultures for isolation of GFP-labelled mitochondria were grown in the dark in order to avoid extensive photobleaching. Transformation of *Neurospora* was carried out as described (Staben et al., 1989; Vollmer and Yanofsky, 1986). Selection of hygromycin-resistant strains was on Vogel's minimal medium supplemented with 150 µg/ml hygromycin B (Boehringer, Mannheim, Germany). Isolation of microconidia was according to published procedures (Ebbole and Sachs, 1990). Mitochondria were isolated by differential centrifugation as described (Sebald et al., 1979).

Preparation of microtubules
Preparation of tubulin from porcine brain was according to published procedures (Williams and Lee, 1982). To prepare microtubules, 1 mg tubulin was thawed on ice and centrifuged for 10 minutes at 250,000 *g* in a Beckman TLA 100 rotor at 4°C. The supernatant was transferred to a new centrifuge tube, supplemented with 1 mM GTP and incubated for 12 minutes at 37°C. After addition of 20 µM taxol (Molecular Probes, Eugene, OR) to stabilise microtubules, polymerisation was continued for 30 minutes. Microtubules were purified by sedimentation through a sucrose cushion (10 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM PMSF, 4 mM MgCl$_2$, 40% w/v sucrose) by centrifugation for 10 minutes at 250,000 *g* in a Beckman TLA 100 rotor at 25°C. Pelleted microtubules were washed carefully and resuspended in buffer A (10 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM PMSF, 4 mM MgCl$_2$, 250 mM sucrose) supplemented with 20 µM taxol. For rhodamine-labelled microtubules, tubulin was mixed with rhodamine-labelled tubulin (a kind gift of Günther Woehlke, Universität München) in a ratio of 5:1 and polymerisation was performed as above.

Incubation of microtubules with mitochondria for fluorescence microscopy
Rhodamine-labelled microtubules (1 mg/ml) were incubated together with GFP-labelled mitochondria (1 mg/ml) for 15 minutes on ice in buffer A (see above) in the presence of 20 µM taxol and 20 U/ml apyrase. Samples were examined directly by fluorescence microscopy.

Floatation of microtubules with mitochondria
For pretreatment of mitochondria with high salt concentration, isolated organelles were incubated for 10 minutes on ice in buffer A (see above) supplemented with 1 M KCl. Then, organelles were pelleted by centrifugation for 8 minutes at 12,000 *g*, washed twice with SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) and resuspended in SEM at a final concentration of 10 mg/ml. For pretreatment of mitochondria with protease, isolated organelles were incubated for 20 minutes on ice in buffer A supplemented with 100 µg/ml trypsin (PMSF was omitted). Protease treatment was stopped by the addition of 1 mg/ml soybean trypsin inhibitor (STI) and 15 minutes incubation on ice. Then, mitochondria were washed and resuspended as above.

To allow binding of mitochondria to microtubules, 40 µg mitochondria were incubated with 40 µg taxol-stabilised microtubules in 250 µl buffer A supplemented with 20 µM taxol and 20 U/ml apyrase and incubated for 20 minutes on ice (for trypsin-pretreated mitochondria 0.5 mg/ml STI were added). To study adenine nucleotide-dependent interactions, 2 mM ATP or ADP were supplemented instead of apyrase.

For floatation, samples were adjusted to a sucrose concentration of 1.8 M by addition of 650 µl 2.5 M sucrose and loaded at the bottom of a SW60 ultracentrifugation tube. This was overlaid with 2 ml buffer A adjusted to 1.7 M sucrose and 1 ml buffer A. The gradient was centrifuged for 4 hours at 200,000 *g* in a Beckman SW60 rotor at 4°C. After centrifugation, a 900 µl fraction from the upper part of the gradient was removed. Four fractions of 0.5 ml and one fraction of 1 ml were harvested, and sedimented material at the bottom of the tube was resuspended in SEM. Proteins were precipitated with TCA and analysed by Western blotting. Floatation of mitochondria was controlled using a polyclonal antibody against porin, whereas floatation of microtubules was detected using monoclonal anti-tubulin antibody WA3 (kindly provided by Ursula Euteneuer and Manfred Schliwa, Universität München). Signals were quantified by densitometry (Pharmacia ImageScanner with Image Master 1D Elite software package, Amersham Bioscience, Uppsala, Sweden).

Miscellaneous
Standard fluorescence and phase contrast microscopy and processing of images were performed as described (Prokisch et al., 2000). SDS-
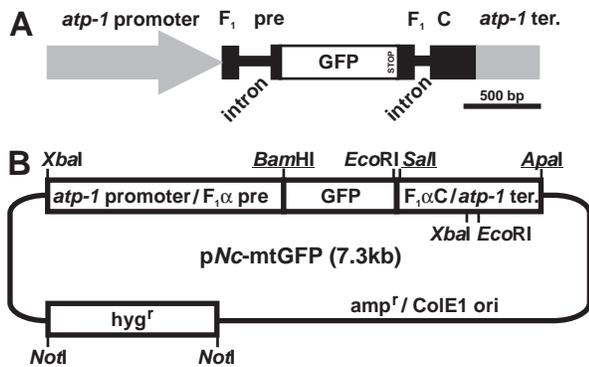
Fig. 1. Plasmid for expression of mitochondria-targeted GFP in *Neurospora crassa*. (A) Structure of the mtGFP cassette. The sequence coding for GFP including a stop codon is symbolised by a white box, regulatory sequences of the *atp-1* gene are depicted in grey, introns are delineated by black lines, and regions coding for parts of the $F_1\alpha$ protein are depicted by black boxes. $F_1\alpha$ pre, region of the *atp-1* gene coding for the mitochondrial presequence; $F_1\alpha C$, region of the *atp-1* gene coding for the C-terminus of the $F_1\alpha$ protein (not translated in the chimeric construct); *atp-1* ter., *atp-1* terminator. (B) Plasmid map for p*Nc*-mtGFP. All relevant functional elements are indicated. The ampicillin resistance gene (amp[r]) and the origin of replication for *E. coli* (ColE1 ori) are derived from the pBluescript vector. The plasmid map is not drawn to scale. Unique restriction sites are underlined. See text for cloning details.

PAGE and blotting of proteins to nitrocellulose were performed according to standard methods. The ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for western blotting.

Results

Construction of a mitochondria-targeted GFP-expressing plasmid

Labelling of mitochondria by expression of GFP fused to a mitochondrial presequence has been reported for a number of experimental systems, yet it turned out that it is not a simple task to achieve the same in *Neurospora*. After several attempts we reasoned that the following parameters might be most critical: (1) the choice of the GFP allele; (2) the choice of the promoter; and (3) the presence of introns.

It was reported that mRNA transcribed from the wild-type GFP coding region of *Aequorea victoria* is aberrantly spliced in *Arabidopsis*. Successful GFP expression in plants was only achieved with a gene that was optimised to the codon usage of *Arabidopsis,* whereby the cryptic intron was removed (Haseloff et al., 1997). *Arabidopsis*-optimised alleles were used for expression of GFP in the basidiomycete fungus *Ustilago maydis* (Spellig et al., 1996) and the filamentous ascomycete fungus *Aspergillus nidulans* (Suelmann et al., 1997). We chose the *mgfp5* allele (Siemering et al., 1996) to construct a mitochondria-targeted GFP for expression in *Neurospora*. In addition to altered codons, this variant contains some amino acid exchanges that improve protein folding at elevated temperatures.

We reasoned that optimal expression of GFP will be best achieved if all regulatory elements are derived from a highly expressed *Neurospora* gene. The α subunit of the F_1 ATP synthase is an abundant protein of mitochondria. In
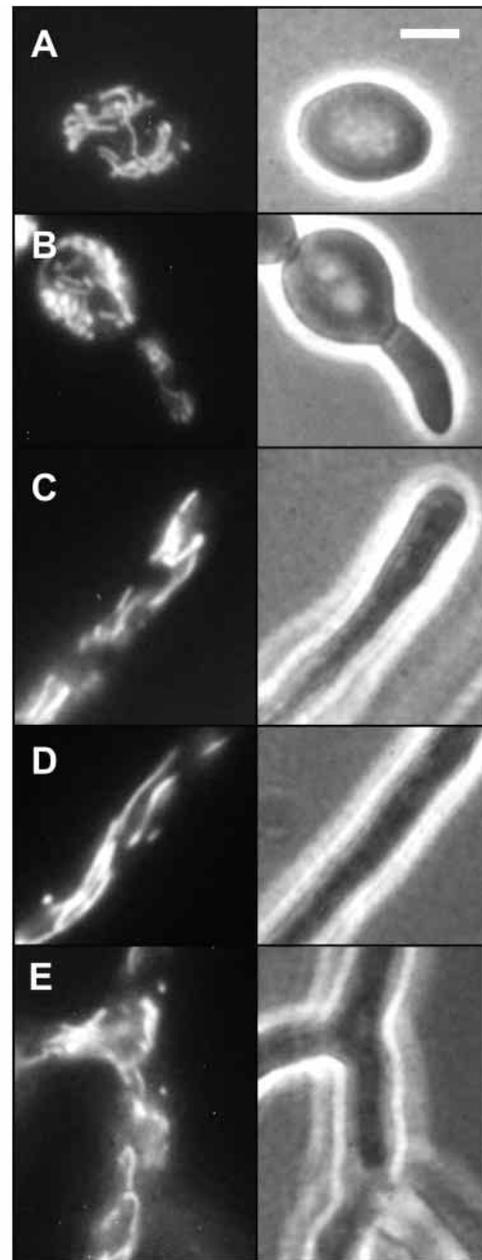

Fig. 2. GFP-labelled mitochondria in living *Neurospora* cells. Conidiospores of a strain expressing mtGFP were grown for different time periods in Vogel's liquid minimal medium at 30°C under agitation and subjected to fluorescence (left) and phase contrast (right) microscopy. (A) Conidiospore before germination; (B) newly germinated conidiospore; (C) hyphal tip after 4 hours incubation; (D) hypha after overnight incubation; (E) hyphal branch point after overnight incubation. Bar, 5 μm.

Neurospora it is encoded by the nuclear *atp-1* gene (Bowman and Knock, 1992). The protein is synthesized in the cytosol as a precursor with a cleavable presequence that directs it to the mitochondrial matrix space. The coding sequence is interrupted by five introns, one of which is located within the region coding for the presequence. Several reports propose that introns may have an expression-augmenting activity (e.g. Brinster et al., 1988; Buchman and Berg, 1988; Choi et al.,
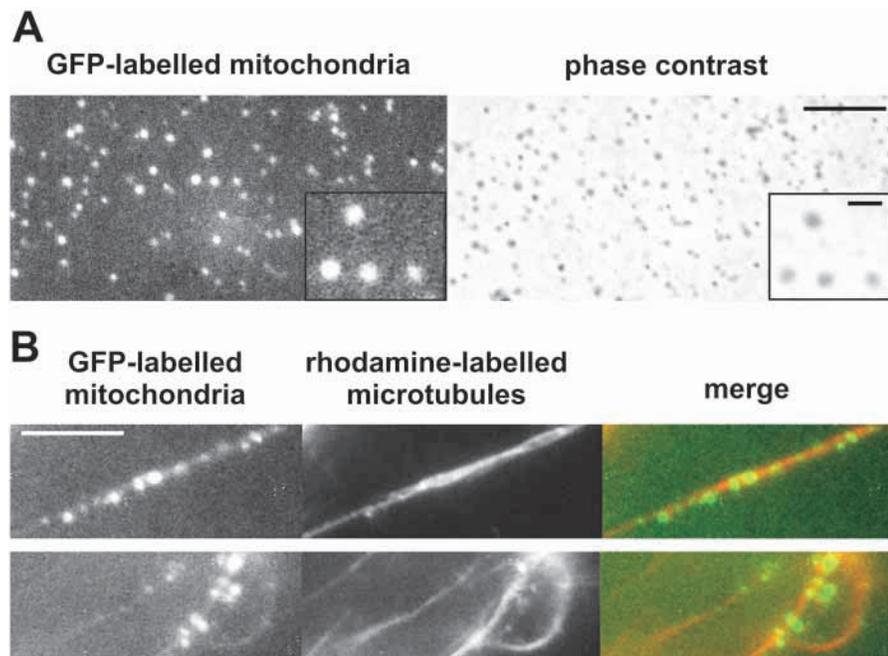
Fig. 3. Behaviour of isolated mitochondria. (A) Mitochondria were isolated from a *Neurospora* strain expressing mtGFP and subjected to fluorescence (left) and phase contrast (right) microscopy. The insert shows an enlarged section from the center of the image. (B) Isolated mtGFP-containing mitochondria were incubated with rhodamine-labelled microtubuli and subjected to fluorescence microscopy. Bars, 5 μm (A,B); 1 μm (A,inset).

1991; Korb et al., 1993; Palmiter et al., 1991). Therefore we decided to include the first and fifth intron of *atp-1* in the chimeric gene.

A DNA fragment covering 1130 bp of the *atp-1* upstream region and the first 40 codons including intron 1 were fused in frame with the *gfp5* coding region including a stop codon. At the 3′ end we added a DNA fragment covering the last 91 codons including intron 5 and 441 bp of the terminator region. The encoded protein, mtGFP, consists of the $F_1\alpha$ presequence (including the processing site for the matrix processing peptidase), two amino acids of the mature $F_1\alpha$ protein and seven additional amino acids (present due to cloning reasons), fused to GFP. The mtGFP expression cassette was combined with a cassette conferring resistance to hygromycin B. The resulting plasmid, p*Nc*-mtGFP, is shown in Fig. 1.

Expression of mitochondria-targeted GFP in *Neurospora*

Upon transformation of p*Nc*-mtGFP into *Neurospora crassa* wildtype, 27 hygromycin B-resistant strains were isolated. Mycelia of 25 transformants exhibited significant green fluorescence. As expected, fluorescent staining was restricted to mitochondria, virtually without background staining. However, for unknown reasons only a small fraction of cells contained fluorescent mitochondria. One strain that showed intensely labelled mitochondria in single hyphae was selected for further analysis. In order to enrich the cells that expressed the mtGFP construct, this strain was passaged seven times on hygromycin B-containing medium. It should be noted that repeated passage on selective media also enriched the fraction of mtGFP-expressing cells in other transformants. After this procedure, the majority of cells contained green mitochondria. We reasoned that most of the progeny that germinated from conidia of a strain that was sufficiently enriched in mtGFP-expressing cells should also express mtGFP. To obtain a homokaryotic strain (i.e. a strain containing only a single type of nuclei), microconidia were isolated at this stage and

germinated on hygromycin B-containing medium. This step resulted in strains containing fluorescent mitochondria in more than 90% of the cells. Specific labelling of mitochondria was observed at all stages of the asexual life cycle. Mitochondria were observed as small thread-like organelles in conidia (Fig. 2A) and newly germinated conidia (Fig. 2B), as well as in hyphal tips (Fig. 2C), old hyphae (Fig. 2D) and at hyphal branch points (Fig. 2E).

Behaviour of isolated mitochondria

In order to examine the behaviour of isolated organelles, we prepared mitochondria from the mtGFP-expressing *Neurospora* strain. Isolated mitochondria were small spherical organelles of about 0.5 μm diameter or less (Fig. 3A). In contrast to mitochondria observed in vivo (see above), the diameter of the isolated organelles was rather diverse, but all organelles were spherical, indicating that the isolation procedure resulted in a loss of structural features. Under the conditions used, no co-isolated tubulin could be detected by Western blotting (not shown), which indicates that the interaction of mitochondria with the cytoskeleton was lost upon isolation. We consider it likely that this is the reason for a collapse of the thread-like organelles seen in vivo to round spherical structures. When rhodamine-labelled microtubules were added, specific binding of mitochondria was observed (Fig. 3B), indicating that isolated mitochondria retain their ability to interact with the cytoskeleton without the requirement of soluble cytosolic factors.

Interaction of mitochondria with microtubules in vitro

To define the biochemical basis of mitochondrial binding to microtubules we developed an assay that allows this interaction to be monitored in vitro. Isolated organelles were incubated with taxol-stabilised porcine brain microtubules and then floated in a sucrose density gradient. In the absence of ATP, a
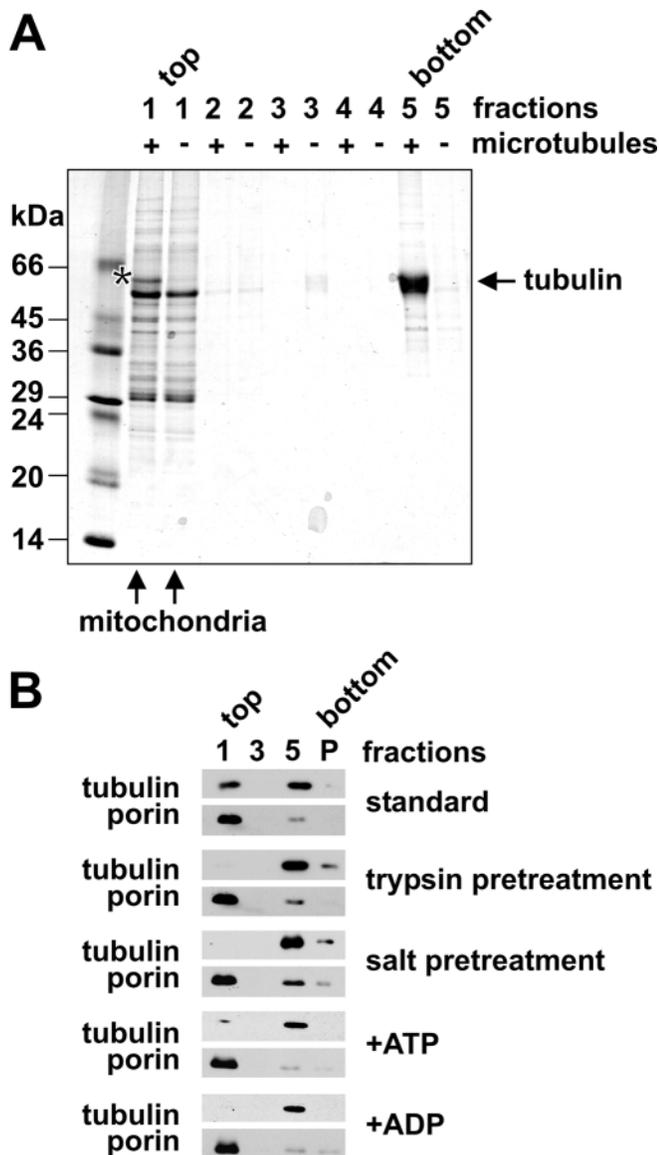
Fig. 4. Binding of microtubules to mitochondria in vitro. (A) Isolated mitochondria were incubated with and without an excess of microtubules and subjected to floatation centrifugation in a sucrose density gradient. After harvesting fractions of the gradient were precipitated with TCA and analysed by SDS-PAGE and Coomassie staining. Tubulin floated with mitochondria is marked with an asterisk. (B) Isolated mitochondria were incubated with microtubules, and floated in a sucrose density gradient; fractions were harvested, proteins were precipitated and analysed by SDS-PAGE and western blot. Only fractions 1 (containing floated mitochondria as controlled by blotting against porin), 3 (from the middle of the gradient) and 5 (containing non-floated proteins) are shown. P, pellet from the bottom of the gradient. Top panel, standard: mitochondria incubated with microtubules under standard conditions (i.e. in the absence of adenine nucleotides and without pretreatment of organelles). Panels 2 and 3: trypsin-pretreated mitochondria and salt-washed mitochondria, respectively, which were incubated with microtubules under standard conditions. Panels 4 and 5: non-pretreated mitochondria that were incubated with microtubules in the presence of ATP or ADP, respectively.
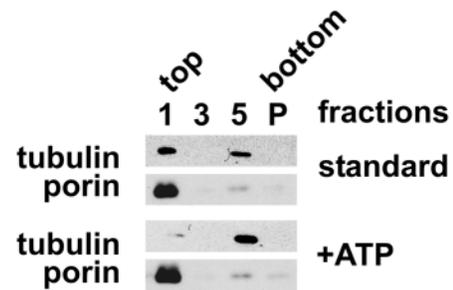

Fig. 5. Binding of microtubules to mitochondria lacking MMM1. Mitochondria isolated from a *mmm-1*[RIP] mutant were incubated with microtubules under standard conditions or in the presence of ATP and analysed as described for Fig. 4B.

significant amount of microtubules floated together with mitochondria to the top of the gradient, which indicates binding to the organelle (Fig. 4A). Under these conditions, 40-60% of the recovered tubulin fractionated with mitochondria, as determined by quantification of western blot signals (Fig. 4B). Binding of mitochondria to microtubules could not be observed when the organelles were pretreated with protease (Fig. 4B), indicating that the interaction is mediated by proteins on the organellar surface. Similarly, mitochondria that were pre-treated with a high-salt wash and then incubated with microtubules under standard (i.e. low-salt) conditions did not show stable binding to microtubules (Fig. 4B). This suggests that proteins peripherally associated with the mitochondrial outer membrane are required. Addition of cytosol or dialysed salt extract did not restore binding of salt-washed mitochondria to microtubules (not shown), suggesting that the proteins mediating this interaction are not cycling on and off the organelle. Also, the addition of ATP- or ADP-dissociated microtubules from mitochondria (Fig. 4B) suggesting an involvement of adenine nucleotide-dependent factors. We conclude that the binding of mitochondria to microtubules is mediated by ATP-dependent proteins that are peripherally associated with the organellar surface.

Interaction of *mmm-1* mutant mitochondria with microtubules

MMM1 is integral to the mitochondrial outer membrane. Null mutants in yeast and *Neurospora* exhibit giant mitochondria that are largely immotile (Boldogh et al., 1998; Prokisch et al., 2000). It has been proposed that the Mmm1 protein of yeast is required for coupling of the organelle to the actin cytoskeleton (Boldogh et al., 1998). We asked whether *Neurospora* mitochondria lacking MMM1 retain their ability to interact with microtubules. Mitochondria isolated from an *mmm-1* mutant strain were incubated with microtubules and floated in a sucrose density gradient as above. Microtubules were observed to bind to the organelles in an ATP-dependent manner similar to wild-type mitochondria (Fig. 5). We conclude that the interaction of mitochondria with microtubules is independent of MMM1.

Discussion

Eukaryotic cells use ordered partitioning mechanisms to inherit

mitochondria (Warren and Wickner, 1996). However, different organisms use different cytoskeletal systems to move and position their organelles. Microtubule-dependent transport of mitochondria appears to be of major importance in a great variety of species from the fungal and animal kingdoms (Yaffe, 1999). In this respect, the extensively studied yeast *S. cerevisiae* is an exception because mitochondrial transport is mediated by the actin cytoskeleton (Boldogh et al., 2001; Hermann and Shaw, 1998). We set out to investigate microtubule-dependent mitochondrial behaviour in *Neurospora*, a model organism that allows biochemical and genetic approaches to be combined.

Our results indicate that binding of mitochondria to microtubules depends on peripherally associated organellar proteins and is sensitive to adenine nucleotides. It does not require binding of soluble cytosolic factors to the organelle. This interaction might be mediated by a microtubule-dependent motor protein, such as a kinesin family member or a dynein-related protein. Also GTP and GDP were observed to dissociate microtubules from mitochondria in the binding assay (not shown). This can be explained by the fact that kinesin proteins are capable of using different nucleotides, as demonstrated, for example, by in vitro microtubule gliding assays (Steinberg and Schliwa, 1996). Alternatively, this observation may point to an involvement of GTPases that might regulate organelle binding to the cytoskeleton. Homology searches on the completely sequenced *Neurospora* genome (*Neurospora* Sequencing Project, Whitehead Institute/MIT Center for Genome Research; www-genome.wi.mit.edu) reveal the existence of at least 10 putative kinesin-related proteins (F.F. and B.W., unpublished). We consider it likely that one of these predicted proteins is involved in mitochondrial transport, as it was demonstrated for the kinesin family members KIF1B, KLP67A and KIF5B in animal cells (Nangaku et al., 1994; Pereira et al., 1997; Tanaka et al., 1998). The availability of genomic sequence data should aid in the identification of the *Neurospora* mitochondria motor.

What are the cellular mechanisms that determine the shape of an organelle? The outer membrane protein MMM1 is a key component for maintenance of normal mitochondrial morphology (Burgess et al., 1994; Prokisch et al., 2000). It was suggested that it establishes mitochondrial structure by recruiting actin binding proteins to the organelle (Boldogh et al., 1998). Surprisingly, it was found that *mmm-1* null mutants of *Neurospora* display giant mitochondria very similar to the yeast mutant (Prokisch et al., 2000). At this time it could not be excluded that MMM1 acts as a receptor for microtubule-binding proteins in *Neurospora* and thereby determines mitochondrial structure (Prokisch et al., 2000). The data presented here argue against this possibility because mitochondria lacking MMM1 can still bind microtubules. Our results favour a function of MMM1 in an internal organellar scaffold-like structure, as recently proposed (Aiken Hobbs et al., 2001). This study also proposed a role for Mmm1p in mitochondrial DNA inheritance in yeast. However, unlike budding yeast, *Neurospora* is an obligate aerobic organism. As *mmm-1* is not essential for viability in *Neurospora* (Prokisch et al., 2000), mechanisms must exist that facilitate transmission of the mitochondrial genome and, therefore, maintenance of respiratory competence, in the absence of MMM1.

Two lines of evidence suggest that organellar morphology is

determined by a complex interplay of internal and external factors. First, isolated mitochondria, which have lost their interaction with the cytoskeleton, collapse into round spherical structures. This indicates that intrinsic organellar factors are not sufficient to maintain an elongated shape of mitochondria and, furthermore, suggests that an alignment along cytoskeletal tracks is important. Second, an elongated shape is apparently not a prerequisite for a mitochondria/cytoskeleton interaction because isolated wild-type and *mmm-1* mutant mitochondria are still able to (re)bind to microtubules. By contrast, null mutants of *mmm-1* have a severely affected mitochondrial structure, although their capability to interact with microtubules is maintained. These observations suggest that the ability to bind to the cytoskeleton is not sufficient to establish normal mitochondrial morphology. It will be a challenge in the future to identify the organellar and cytoskeletal components involved. This will certainly improve our understanding of the molecular mechanisms that contribute to the establishment of the 3D structure of eukaryotic cells.

Another achievement reported here is the use of GFP as a fluorescent marker in *Neurospora crassa*, one of the classic model organisms used by geneticists, biochemists and cell biologists. While this manuscript was in preparation, another group described the visualisation of cytosolic GFP expressed from the heterologous *ToxA* promoter in *Neurospora* (Freitag et al., 2001). However, this group reported that it was not possible to find *Neurospora* promotors sufficiently strong to drive high level expression of GFP, and that GFP had to accumulate for at least 16 hours in hyphae before visualisation was possible. Our approach demonstrates that: (1) it is possible to express sufficient amounts of GFP from the endogenous *Neurospora atp-1* promoter; (2) GFP labelling can be observed in conidia and in newly germinated hyphae; and (3) GFP can be used as a marker of specific intracellular structures. The availability of molecular genetic tools and the possibility of producing large amounts of biomass at low cost make *Neurospora* an attractive model in which to study many different topics ranging from organelle biogenesis to molecular clocks or developmental processes (Davis, 2000; Perkins, 1992). The strategies described here and in previous studies (Freitag et al., 2001), preferably together with additional improvements, might help to establish GFP as a valuable tool for the exploration of a variety of different aspects of *Neurospora* cell biology.

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