Role of Unc104/KIF1-related Motor Proteins in Mitochondrial Transport in *Neurospora crassa*^V

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Eukaryotic cells use diverse cytoskeleton-dependent machineries to control inheritance and intracellular positioning of mitochondria. In particular, microtubules play a major role in mitochondrial motility in the filamentous fungus *Neurospora crassa* and in mammalian cells. We examined the role of two novel Unc104/KIF1-related members of the kinesin family, Nkin2 and Nkin3, in mitochondrial motility in *Neurospora*. The Nkin2 protein is required for mitochondrial interactions with microtubules in vitro. Mutant hyphae lacking Nkin2 show mitochondrial motility defects in vivo early after germination of conidiospores. Nkin3, a member of a unique fungal-specific subgroup of small Unc104/KIF1-related proteins, is not associated with mitochondria in wild-type cells. However, it is highly expressed and recruited to mitochondria in $\Delta nkin-2$ mutants. Mitochondria lacking Nkin2 require Nkin3 for binding to microtubules in vitro, and mitochondrial motility defects in $\Delta nkin-2$ mutants disappear with up-regulation of Nkin3 in vivo. We propose that mitochondrial transport is mediated by Nkin2 in *Neurospora*, and organelle motility defects in $\Delta nkin-2$ mutants are rescued by Nkin3. Apparently, a highly versatile complement of organelle motors allows the cell to efficiently respond to exogenous challenges, a process that might also account for the great variety of different mitochondrial transport systems that have evolved in eukaryotic cells.

INTRODUCTION

Mitochondria are ubiquitous and essential organelles of eukaryotic cells. As membrane-bounded organelles, they cannot be generated de novo, but they grow and divide from preexisting organelles (Nunnari and Walter, 1996). In many cell types, mitochondrial inheritance during the cell division cycle is coordinated by ordered partitioning mechanisms involving interactions with the cytoskeleton (Warren and Wickner, 1996). These interactions are also crucial to control the intracellular distribution of mitochondria, which are often positioned at intracellular sites of high demand for ATP (Bereiter-Hahn, 1990; Yaffe, 1999). However, the cellular machinery mediating mitochondrial motility and distribution is not well understood.

The molecular mechanisms used by eukaryotic cells to control mitochondrial transport are strikingly diverse (Yaffe, 1999; Westermann and Prokisch, 2002). Mitochondrial movement in budding yeast depends on the actin cytoskeleton (Hermann and Shaw, 1998; Jensen *et al.*, 2000; Boldogh *et al.*, 2001b). It is a matter of debate whether myosin-related motor proteins are involved (Itoh *et al.*, 2002) or whether it is driven by actin polymerization involving the Arp2/3 complex (Boldogh *et al.*, 2001a). In contrast, mitochondrial distribution in fission yeast is dependent on microtubules (Yaffe *et al.*, 1996), but movement is apparently not mediated by kinesin-like motor proteins (Brazer *et al.*, 2000; Weir and

Yaffe, 2004). On the other hand, kinesin family members have been shown to be involved in microtubule-dependent mitochondrial transport in mammalian cells and *Drosophila* (Nangaku *et al.*, 1994; Pereira *et al.*, 1997; Khodjakov *et al.*, 1998; Tanaka *et al.*, 1998). Fundamental differences in the molecular machinery underlying mitochondrial motility can be found even in closely related organisms. The filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* share many similarities in their life cycle and cell biological functions. However, *Neurospora* uses microtubule tracks for mitochondrial movement (Steinberg and Schliwa, 1993; Prokisch *et al.*, 2000; Fuchs *et al.*, 2002), whereas *Aspergillus* relies exclusively on microfilaments to mediate this process (Oakley and Rinehart, 1985; Suelmann and Fischer, 2000). Functional or evolutionary reasons that might account for these remarkable differences are not known.

The filamentous fungus *N. crassa* is an excellent model system to study microtubule-dependent mitochondrial motility. Cells grow very fast, at a speed of up to 1 $\mu\text{m/s}$ (Kirchner *et al.*, 1999), and in a polarized manner. This requires an efficient transport system for cell organelles, which is provided by microtubule tracks (Steinberg and Schliwa, 1993). Mitochondria show saltatory movement in vivo with a mean velocity of up to 2 $\mu\text{m/s}$ (Steinberg and Schliwa, 1993). We recently reported that mitochondrial binding to microtubules is mediated by adenine nucleotide-sensitive proteins on the organellar surface. These observations point to a possible involvement of kinesin-related motor proteins (Fuchs *et al.*, 2002). However, conventional kinesin, Nkin, is not required for mitochondrial distribution and motility in *Neurospora* (Seiler *et al.*, 1997), and the identity of molecular motors mediating this process remained obscure. To identify motor proteins involved in mitochondrial motility, we searched the *Neurospora* genome (Galagan *et al.*, 2003) for genes encoding putative kinesin-related organelle motors. Functional analysis of two novel members of

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the Unc104/KIF1 subfamily revealed important roles of these proteins in mediating mitochondrial interactions with microtubules in vitro and in vivo.

MATERIALS AND METHODS

Plasmid Constructions

Standard methods were used for the manipulation of DNA. For construction of a plasmid for expression of Nkin3 fused to an N-terminal glutathione S-transferase (GST) moiety in *Escherichia coli*, the *nkin-3* coding region was amplified from genomic *Neurospora* DNA by polymerase chain reaction (PCR) by using primers 5' AAA AGC TTG GAT CCA TGT GTT TAT TCC ACC ACC and 5' AAA GGA TCC GAA TTC TCA TAT GAT ATT AGT CCT CAC CC and cloned into the *Bam*HI site of vector pETGEXCT (Sharrocks, 1994), yielding pFF10. For construction of a plasmid for expression of the C-terminal half of Nkin3 fused to an N-terminal GST moiety in *E. coli*, a DNA fragment covering 711 base pairs of the *nkin-3* coding region was amplified from genomic *Neurospora* DNA by using primers 5' AAA AGA TCT CGC ATT CGT ACC CGT GCC G and 5' AAA GGA TCC GAA TTC TCA TAT GAT ATT AGT CCT CAC CC, digested with *Bgl*II and cloned into the *Bam*HI site of vector pETGEXCT, yielding pFF11. For construction of a plasmid for expression of an Nkin2 fragment fused to a C-terminal hexahistidine tag in *E. coli*, a DNA fragment covering 885 base pairs of the *nkin-2* coding region was amplified from genomic *Neurospora* DNA by using primers 5' GGG CCA TGG AGG ACG CCA ATG CCC GCA and 5' AAA AGA TCT GGC TTT CGC TTC AAG AGG ATG, and cloned into the *Nco*I and *Bgl*II sites of vector pQE60 (QIAGEN, Hilden, Germany), yielding pFF12. A plasmid for disruption of the *nkin-2* gene, pFF15, was constructed in three consecutive cloning steps. First, a DNA fragment covering 2000 base pairs of the terminator region of the *nkin-2* gene was amplified from genomic *Neurospora* DNA by using primers 5' CAA CGT AGT AAC CCG TTG and 5' AAT AGG CCG AAA TCG GCA GTG CAT TCA GGG ATA CCC AAT GGG, cloned into the PCR product cloning vector pCRII-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, yielding plasmid pFF13. Second, a cassette conferring resistance to hygromycin B was amplified from plasmid pCB1179 (Sweigard *et al.*, 1997) by using primers 5' AAA GCG GCC GCA GGG AAT AAG GCG GAC ACG G and 5' AAA GCG GCC GCT GCC GAT TTC GGC CTA TTG G and cloned into the *Not*I site of pFF13, yielding plasmid pFF14. Third, a DNA fragment covering 2041 base pairs of the promoter region of the *nkin-2* gene was amplified from genomic DNA by PCR by using primers 5' GGG TCT AGA CGT CTG GAG TCT CGA CCG and 5' GGG CTC GAG TCC GGA CGC GAT CAC AGC and cloned into the *Xba*I and *Xho*I sites of pFF14, yielding plasmid pFF15. pFF15 was linearized by restriction digestion with *Sac*I before it was transformed into *Neurospora*.

Antibodies

An antigen for Nkin2 was generated by expression of an Nkin2 fragment (amino acids 372–666) carrying a C-terminal hexahistidine tag in *E. coli* by using pFF12 (pQE60 expression system; QIAGEN) and purification on nickel-nitrilotriacetic acid (Ni-NTA) according to the manufacturer's instructions. Soluble protein was eluted from a Ni-NTA column. Antigens for Nkin3 were generated by expression of either full-length Nkin3 or the C-terminal half fused to an N-terminal GST moiety in *E. coli* by using pFF10 and pFF11 (pETGEXCT expression system). Insoluble recombinant protein was purified as inclusion bodies. Antigens were injected into rabbits. Affinity purification of antibodies was according to published procedures (Harlow and Lane, 1988).

Strains, Growth Conditions, and Isolation of *Neurospora* Mutants

Standard genetic and microbiological techniques were used for the growth and manipulation of *Neurospora* strains (Davis and de Serres, 1970). *Neurospora* strains used were wild-type strain St. Lawrence 74A (Fungal Genetics Stock Center, Kansas City, KS), a wild-type strain expressing mitochondria-targeted green fluorescent protein (mtGFP) (Fuchs *et al.*, 2002), and HP1, a parental strain used for sheltered disruption (Nargang *et al.*, 1995). *Neurospora* was grown in Vogel's minimal medium under continuous aeration and illumination with white light at 25°C (Davis and de Serres, 1970). Transformation of *Neurospora* was carried out as described previously (Vollmer and Yanofsky, 1986; Staben *et al.*, 1989).

A heterokaryotic *nkin-2* mutant ($\Delta nkin-2/nkin-2^{WT}$) was constructed by sheltered disruption, essentially as described previously (Nargang *et al.*, 1995). Spheroplasts of HP1 were transformed with pFF15, and transformants were selected on minimal medium containing hygromycin. Isolates were purified by single-colony isolation on hygromycin-containing medium, genomic DNA was isolated, and transformants harboring a nucleus containing a targeted deletion of the *nkin-2* gene were identified by Southern blotting. A strain containing a single integration of the disrupting DNA fragment was chosen for further analysis. Growth of this strain on minimal medium containing 0.2 mg/ml histidine and 400 μ M p-fluoro-DL-phenyl-alanine (fpa)

forced the disruptant-containing nucleus, as determined by Southern and Western blotting.

To isolate a homokaryotic $\Delta nkin-2$ mutant, the heterokaryotic strain was passaged three times on plates with selective medium forcing the disruptant-containing nucleus. Then, conidia were harvested and used to inoculate single colonies on plates containing minimal medium with 2% sorbose (instead of sucrose), 0.2 mg/ml histidine, and 10 μ g/ml pantothenic acid. Cells from these colonies were transferred onto solid minimal medium supplemented with histidine and pantothenic acid and grown until conidia were formed. Conidia were then used to test the requirement of histidine and pantothenic acid. Isolates that could grow in the absence of pantothenic acid, but not in the absence of histidine, were homokaryotic and contained the $\Delta nkin-2$ allele. Genotypes were confirmed by Southern and Western blotting.

Cell Fractionation

Isolation of mitochondria, postmitochondrial supernatant, and cell extracts (Sebald *et al.*, 1979), preparation of outer membrane vesicles (Mayer *et al.*, 1993) and sucrose gradient purification of mitochondria (Rowley *et al.*, 1994) were performed according to published procedures. For preparation of extracts of conidiospores, 30 OD₆₀₀ units of cells were resuspended in 100 μ l of SDS-PAGE sample buffer, glass beads were added, and the sample was incubated for 5 min at 95°C and vortexed for 5 min. Then, 400 μ l of SDS-PAGE sample buffer was added, the sample was centrifuged for 10 min at 13,000 $\times$ g, and 15 μ l of the supernatant was used for SDS-PAGE. Salt extraction of mitochondria was performed by resuspension of mitochondria in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2 (SEM) with 1 M KCl, incubation for 30 min on ice, and centrifugation for 12 min at 4°C at 13,000 $\times$ g. The supernatant was precipitated with trichloroacetic acid (TCA), and the mitochondrial pellet was resuspended in SEM.

Assay of Mitochondrial Binding to Microtubules

Mitochondrial binding to microtubules was assayed by flotation in sucrose gradients and by fluorescence microscopy as described previously (Fuchs *et al.*, 2002). For antibody inhibition experiments, 500 ng of affinity-purified antibodies was added to 100 μ g of mitochondria and incubated for 30 min on ice in 250- μ l volume assay buffer in the absence of ATP (Fuchs *et al.*, 2002). Then, mitochondria were reisolated by centrifugation for 12 min at 4°C at 13,000 $\times$ g, and 100 μ g of microtubules was added. After an incubation for 30 min on ice, mitochondria were floated by sucrose gradient centrifugation (Fuchs *et al.*, 2002). For microscopy, reisolation of mitochondria was omitted.

Microscopy

Staining of mitochondria and fluorescence microscopy was carried out as described previously (Prokisch *et al.*, 2000; Fuchs *et al.*, 2002). Mitochondrial motility was analyzed in intact cells by computer-enhanced phase contrast videomicroscopy. Under these conditions, mitochondria can be readily identified by their elongated shape (Steinberg and Schliwa 1993; Wu *et al.*, 1998). Conidiospores were inoculated on microscope slides that were covered with a thin layer of minimal medium containing 0.2 mg/ml histidine and 15% gelatin. Spores were allowed to germinate and grow overnight at room temperature. Then, a few microliters of fresh medium was added, and the sample was covered with a coverslip. Mitochondrial movement was analyzed using a Zeiss Axiophot microscope equipped with a 100 $\times$ oil immersion objective. Images were recorded with a Hamamatsu Orca ER 1394 camera and processed with SimplePCI software (Compix, Tualatin, OR).

RESULTS

Nkin2 and Nkin3 Are Novel Kinesin-related Motor Proteins of the Unc104/KIF1 Subfamily

The *Neurospora* genome harbors 10 genes that are predicted to encode kinesin-related motor proteins (Fuchs *et al.*, 2002; Schoch *et al.*, 2003; Xiang and Plamann, 2003; Borkovich *et al.*, 2004). Two of these predicted proteins, Nkin2 (genome annotation number NCU06733.1) and Nkin3 (NCU03715.1), are closely related to members of the Unc104/KIF1 subfamily. Because this group contains many known organelle motors (Hirokawa, 1998), Nkin2 and Nkin3 are likely candidates for motor proteins mediating mitochondrial motility.

Nkin2 shares a conserved domain structure with other bona fide members of the Unc104/KIF1 subfamily (Figure 1A). It has an N-terminal kinesin motor domain, a forkhead associated (FHA) domain, which is thought to be involved in interactions with phosphopeptides (Durocher *et al.*, 1999), and a C-terminal pleckstrin homology (PH) domain, which has been shown to play an important role in the interaction

Figure 1. Nkin2 and Nkin3 are novel members of the Unc104/KIF1 subfamily of kinesin-related motor proteins. (A) Domain structure of Nkin2 and Nkin3. Homologies to known protein domains were revealed by searching the Pfam database (Bateman *et al.*, 2002). Kinesin motor domains are depicted in black, FHA domains in light gray, and PH domains in white. Numbers indicate amino acid residues defining the borders of the predicted domains, and the C termini of the proteins, respectively. Protein domains are drawn to scale. (B) Homology tree of representative kinesin family members. Fungal homologues sharing the same domain structure with Nkin2 are highlighted by a white box. This group includes predicted proteins from *Magnaporthe grisea* (genome annotation number MG09255.4), *Gibberella zeae* (FG10189.1), *Botryotinia fuckeliana* (gene KLP8), *Cochliobolus heterostrophus* (KLP8), and *Aspergillus nidulans* (AN7547.2). Fungal homologues closely resembling Nkin3 are highlighted by a light gray box. This group includes predicted proteins from *Gibberella moniliformis* (KLP7), *A. nidulans* (AN6863.2), and *Cochliobolus heterostrophus* (KLP7). Other members of the KIF1/Unc104 family sharing the same domain structure with Nkin2 are Kin3 of *Ustilago maydis* (Wedlich-Söldner *et al.*, 2002), KIF1b of *Mus musculus* (Nangaku *et al.*, 1994), Unc104 of *C. elegans* (Otsuka *et al.*, 1991), and Unc104 of *D. discoideum* (Pollock *et al.*, 1999). Nkin (Steinberg and Schliwa, 1995) and Kif5b (Tanaka *et al.*, 1998) are conventional kinesins. The tree was constructed using DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada).

with cargo membranes (Klopfenstein *et al.*, 2002). This conserved domain organization is also found, e.g., in the founding member of the subfamily, the *Caenorhabditis elegans* Unc104 protein, which serves as a vesicle transporter in neurons (Hall and Hedgecock, 1991).

Nkin3 is a rather unusual kinesin family member because of its relatively low molecular mass (75.8 kDa). It has an N-terminal kinesin motor domain, and a C-terminal domain that does not share homology to any other known protein sequence motif (Figure 1A). Similar protein-coding sequences can be found in the genomes of related filamentous fungi. Based on extensive phylogenetic analysis it has been suggested that Nkin3 and its relatives constitute a unique fungal subgroup of "truncated" Unc104/KIF1-like proteins (Schoch *et al.*, 2003). Cellular functions of members of this new subgroup are not known. Because Nkin3-related proteins belong to the Unc104/KIF1 family, it can be speculated that they act as organelle motors. However, members of this subgroup lack a PH domain, and possible mechanisms of binding to cellular membranes are unknown. A homology tree of putative *Neurospora* organelle motors and representative Unc104/KIF1 and conventional kinesin-like proteins is shown in Figure 1B.

Nkin2 Is Peripherally Associated with Mitochondria

We asked whether Nkin2 and Nkin3 are bound to mitochondria. *Neurospora* wild-type cells were fractionated by differential centrifugation into postmitochondrial supernatant (PMS) and a crude mitochondrial fraction. Then, mitochondria were further purified on a sucrose gradient, and outer membrane vesicles (OMVs) were prepared. All fractions were analyzed by immunoblotting by using specific antisera against Nkin2, Nkin3, and Nkin. Tom40 was used as a marker for the mitochondrial outer membrane, and the peroxisomal protein Pex14 was used as a marker for nonmitochondrial membranes (Figure 2A). Nkin3 and Nkin were found exclusively in the postmitochondrial supernatant, which contains cytosolic proteins and light cellular membranes. The fractionation behavior of Nkin2 was similar to the integral mitochondrial outer membrane protein, Tom40. It was highly enriched in purified mitochondria and outer membrane vesicles. In contrast, only trace amounts of Pex14 could be detected in these fractions. We suggest that a major fraction of Nkin2 is associated with mitochondria.

Nkin2 was less enriched in outer membrane vesicles than Tom40 (Figure 2A, lane 4). A possible explanation is that Nkin2 is only peripherally associated with the mitochondrial outer membrane, resulting in the loss of a certain amount of protein during outer membrane vesicles preparation. Furthermore, it cannot be excluded that a subfraction of Nkin2 also might be associated with other membranous organelles. To investigate the nature of the association of Nkin2 with mitochondrial membranes more directly, mitochondria were washed with 1 M KCl and reisolated. High salt treatment released Nkin2 into the supernatant, whereas Tom40 and the soluble intermembrane space protein cytochrome *c* heme lyase (CCHL) remained in the organellar pellet (Figure 2B). We conclude that Nkin2 is a peripheral membrane protein that binds to mitochondrial outer membrane lipids and/or receptor proteins involving ionic interactions.

Nkin2 Is Required for Binding of Mitochondria to Microtubules

The mitochondrial association points to a possible role of Nkin2 as a mitochondrial motor protein. To investigate whether Nkin2 is required for mitochondrial binding to

Figure 2. Nkin2 is peripherally associated with the mitochondrial outer membrane. (A) Subfractionation of cells. Wild-type cells were fractionated by differential centrifugation into PMS (lane 1) and a crude mitochondrial fraction (lane 2). Crude mitochondria were either further purified by sucrose gradient centrifugation (lane 3) or used for preparation of OMVs (lane 4). Fifty micrograms of protein of each fraction was analyzed by SDS-PAGE and immunoblotting by using specific antisera against Nkin2, Nkin3, and Nkin. The integral outer membrane protein, Tom40, served as a marker for mitochondria, and the peroxisomal protein, Pex14, served as a marker for nonmitochondrial membranes. (B) Salt-extraction of mitochondria. Isolated mitochondria were left untreated on ice (lane 1) or extracted with 1 M KCl and reisolated by centrifugation (lane 2). Proteins were precipitated from the salt extract with TCA (lane 3). Fifty micrograms of protein of each fraction was analyzed by SDS-PAGE and immunoblotting. Tom40 served as a marker for the mitochondrial outer membrane, and CCHL, a soluble protein of the intermembrane space, was used to control for integrity of the outer membrane.

microtubules we used an assay that allows this interaction to be monitored in vitro. Isolated mitochondria were incubated with taxol-stabilized microtubules, floated in a sucrose density gradient, and fractions harvested from the gradient were analyzed by immunoblotting. Flotation of microtubules with mitochondria indicates a specific interaction, which is adenine nucleotide sensitive and mediated by peripherally associated mitochondrial proteins (Fuchs *et al.*, 2002). To investigate whether Nkin2 is required for this interaction, mitochondria were preincubated with antibodies directed against Nkin2 before microtubules were added.

This treatment abolished binding of mitochondria to microtubules (Figure 3A). Preincubation of mitochondria with antibodies against other kinesin-related proteins, Nkin and Nkin3, or nonrelated outer membrane proteins, Tom20 and Tom22, had no effect compared with nontreated mitochondria (Figure 3A).

To corroborate the observation that Nkin2 antibodies inhibit mitochondrial binding to microtubules, we used a microscopic in vitro assay that allows the visualization of the behavior of individual organelles (Fuchs *et al.*, 2002). Mitochondria were isolated from a strain expressing mtGFP. These mitochondria were preincubated with antibodies against Nkin2 or Nkin3, or left untreated, before rhodamine-labeled microtubules were added. Binding of mitochondria to microtubules was visualized by fluorescence microscopy. Again, Nkin2 antibodies efficiently inhibited the interaction of mitochondria with microtubules, whereas Nkin3 antibodies had no effect (Figure 3B). We conclude that Nkin2 plays a crucial and specific role in mediating mitochondrial binding to microtubules.

Depletion of Nkin2 Results in an Increase of Nkin3 Levels and the Recruitment of Nkin3 to Mitochondria

To examine the role of Nkin2 in vivo, we constructed a null mutant by using the "sheltered disruption" method (Nargang *et al.*, 1995), which takes advantage of the fact that *Neurospora* has multinucleate cells. In brief, the *nkin-2* gene was deleted via homologous recombination in a heterokaryotic strain. The resulting mutant harbors two kinds of nuclei, one with a null allele ($\Delta nkin-2$) and another one with a wild-type allele (*nkin-2*^{WT}). These nuclei contain selectable markers that enable the shift of nuclear ratios in heterokaryotic cells. Growth on medium containing benomyl and pantothenic acid favors propagation of the nucleus containing the *nkin-2*^{WT} allele. Under these conditions, Nkin2 protein levels in cell extracts are normal (Figure 4A). In contrast, growth of heterokaryotic cells on medium containing fpa and histidine forces the $\Delta nkin-2$ nucleus to predominate in the growing mycelium and thus leads to depletion of the Nkin2 protein (Figure 4A). These conditions allow the phenotype of cells lacking Nkin2 to be studied. Surprisingly, cells depleted of Nkin2 did not show any obvious phenotype. The growth behavior of cells depleted of Nkin2 was normal, and mitochondrial distribution, morphology, and motility resembled wild-type cells (our unpublished observations).

Intriguingly, the level of Nkin3 present in mutant cells was strongly elevated after depletion of Nkin2. The Nkin3 protein is barely visible in immunoblots of total cell extracts of wild-type cells. However, after 3 d growth in the presence of fpa and histidine, the time required to completely deplete Nkin2 from the heterokaryon, Nkin3 was visible as a strong band (Figure 4B). Levels of Nkin and Tom40 were not changed under these conditions. When cells were fractionated into postmitochondrial supernatant and mitochondria, a significant portion of Nkin3 was recovered with mitochondria (Figure 4B). Again, this effect was specific for Nkin3, and Nkin was not affected. This suggests that Nkin3 levels are increased and the protein is recruited to mitochondria when Nkin2 levels become limiting.

Nkin3 Is Up-Regulated during Growth of Cells in the Absence of Nkin2

Next, we isolated a homokaryotic mutant containing exclusively the $\Delta nkin-2$ nucleus. In brief, the heterokaryotic strain was grown under conditions favoring propagation of the nucleus harboring the $\Delta nkin-2$ allele. Conidiospores were

Figure 3. Antibodies against Nkin2 inhibit mitochondrial binding to microtubules in vitro. (A) Flotation assay. Isolated mitochondria were preincubated in the absence or presence of the antibodies indicated on the right-hand side. Then, mitochondria were reisolated by centrifugation, incubated with taxol-stabilized microtubules in the absence of ATP and floated in a sucrose density gradient. Fractions were harvested from the gradient, and proteins were precipitated and analyzed by SDS-PAGE and immunoblotting by using antibodies against the proteins indicated on the left-hand side. Only fractions 1 (containing floated mitochondria as controlled by

Figure 4. Nkin3 is up-regulated and recruited to mitochondria in the absence of Nkin2 in heterokaryotic cells. (A) Depletion of Nkin2 in heterokaryotic cells. The HP1 wild-type strain (*nkin-2^{WT}/nkin-2^{WT}*) and a heterokaryotic mutant strain (Δ *nkin-2/nkin-2^{WT}*) were grown for 3 d in liquid minimal medium supplemented either with benomyl and pantothenic acid (ben/pan) or p-fluoro-DL-phenylalanine and histidine (fpa/his). Equal amounts of cell extracts were analyzed by SDS-PAGE and immunoblotting. (B) Up-regulation and mitochondrial association of Nkin3 upon depletion of Nkin2. Heterokaryotic Δ *nkin-2/nkin-2^{WT}* cells were grown for the indicated time periods in liquid minimal medium supplemented with fpa/his. Total cell extracts, PMSs, and mitochondria were prepared, and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting.

harvested from this culture and plated on sorbose medium to obtain single colonies. These were then screened for clones lacking the markers of the *nkin-2^{WT}* nucleus. Absence of the wild-type allele in homokaryotic Δ *nkin-2* strains was confirmed by Southern blot analysis (our unpublished observations) and immunoblotting (Figure 5A). Similar to heterokaryotic strains, homokaryotic mutants did not show any growth defects (Figure 5B). Mitochondria resembled the wild type under most conditions, and motility defects could be observed only in newly germinated hyphae (see below).

Also in homokaryotic Δ *nkin-2* strains, high levels of Nkin3 were found (Figure 5A). On subcellular fractionation of Δ *nkin-2* cells, a significant fraction of Nkin3 was associated with mitochondria and could be recovered with outer mem-

blotting against porin), 3 (from the middle of the gradient), and 5 (containing nonfloated proteins) are shown. P, pellet from the bottom of the gradient. (B) Visual assay. Mitochondria were isolated from a *Neurospora* strain expressing mtGFP and preincubated in the absence or presence of the indicated antibodies. Then, rhodamine-labeled microtubules (rho-MT) were added, incubation was continued in the absence of ATP, and samples were analyzed by fluorescence microscopy. Bar, 5 μ m.

Figure 5. Nkin3 is up-regulated and binds to mitochondria in growing homokaryotic cells lacking Nkin2. (A) Up-regulation and mitochondrial association of Nkin3 in homokaryotic $\Delta nkin-2$ mutants. The HP1 wild-type strain ($nkin-2^{WT}/nkin-2^{WT}$), a heterokaryotic mutant ($\Delta nkin-2/nkin-2^{WT}$), and two independently isolated homokaryotic mutants ($\Delta nkin-2$) were grown for 16 h in fpa/his-containing medium. Then, cells were analyzed as in Figure 4B. (B) Growth of $\Delta nkin-2$ cells. Wild-type (WT) and $\Delta nkin-2$ cells were inoculated in the middle of a petri dish containing Vogel's minimal medium agar and grown over night at 25°C. Bar, 1 cm. (C) Mitochondrial association of Nkin3 in $\Delta nkin-2$ cells. $\Delta nkin-2$ cells were subfractionated and analyzed as in Figure 2A. (D) Nkin3 level in conidiospores. Cell extracts were prepared from conidiospores of the homokaryotic $\Delta nkin-2$ strain and its isogenic wild-type (WT) and analyzed by SDS-PAGE and immunoblotting. (E) Up-regulation of Nkin3 in newly germinated $\Delta nkin-2$ cells. Conidiospores of the homokaryotic $\Delta nkin-2$ strain were allowed to germinate and grow for the indicated time periods. Then, total cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting. For comparison, a cell extract of a 14 h old wild-type culture is shown.

brane vesicles (Figure 5C). We asked whether Nkin3 is constitutively up-regulated in cells lacking Nkin2 or whether this is an adaptation of actively growing cells. To discriminate between these possibilities, we first analyzed Nkin3 levels in cell extracts of wild-type and $\Delta nkin-2$ conidiospores. Intriguingly, spores lacking Nkin2 contained only

Figure 6. Antibodies against Nkin3 inhibit binding of $\Delta nkin-2$ mitochondria to microtubules in vitro. Mitochondria were isolated from a $\Delta nkin-2$ mutant and subjected to the microtubule flotation assay as in Figure 3A.

basal levels of Nkin3 (Figure 5D). However, when conidiospores were germinated in liquid culture, a strong increase of Nkin3 was observed in vegetatively growing cells after 6–10 h (Figure 5E). We conclude that up-regulation of Nkin3 is a response of growing hyphae to limiting levels of Nkin2.

Mitochondria Lacking Nkin2 Require Nkin3 for Binding to Microtubules

The observations that Nkin3 is recruited to mitochondria in cells lacking Nkin2 and that the $\Delta nkin-2$ mutant does not show severe defects in mitochondrial distribution, point to the possibility that Nkin3 overexpression might compensate for the loss of Nkin2 function. Therefore, we tested whether Nkin3 antibodies would interfere with binding of $\Delta nkin-2$ mitochondria to microtubules in vitro. Remarkably, deletion of the $nkin-2$ gene made mitochondria sensitive to the addition of Nkin3 antibodies in the microtubule flotation assay. Although Nkin3 antibodies had no effect on wild-type mitochondria (compare Figure 3A), they efficiently inhibited binding of $\Delta nkin-2$ mitochondria to microtubules (Figure 6). As expected, Nkin2 antibodies did not interfere with the interaction of mutant mitochondria with microtubules. We conclude that Nkin3 mediates mitochondrial binding to microtubules in the absence of Nkin2.

Mitochondrial Dynamics Is Compromised by Deletion of the $nkin-2$ Gene

We examined the role of Nkin2 in mitochondrial motility by computer-enhanced videomicroscopy in live cells. Wild-type and $\Delta nkin-2$ conidia were allowed to germinate, and the behavior of mitochondria was observed in growing hyphae. Wild-type mitochondria were seen as numerous small, snake-like organelles that showed a complex and dynamic behavior. Continuous movement, fusion and fission resulted in frequent shape changes (Supplemental Movie 1). Within a few seconds, the spatial arrangement of the organelles was

Figure 7. Cells lacking Nkin2 show altered mitochondrial motility and morphology. (A) Wild-type and $\Delta nkin-2$ conidiospores were allowed to germinate and grow over night on medium-covered microscope slides. Then, mitochondrial behavior in growing hyphae was analyzed by computer-enhanced phase contrast videomicroscopy (2 images per second). The complete datasets are available in two movies covering 30 s of hyphal growth (see Supplemental Movie 1 for behavior of wild-type mitochondria and movie 2 for $\Delta nkin-2$ mitochondria). They are representative examples of 10 independent experiments. Differences in cellular growth rate are due to variations between individual cells. Right, representative individual organelles near the hyphal tip have been highlighted. Bar, 5 μ m. (B) Wild-type and $\Delta nkin-2$ cells were grown for 7 h after germination of conidiospores, stained with 10 μ g/ml rhodamine B hexyl ester, and analyzed by phase contrast (left) and fluorescence (right) microscopy. Bar, 5 μ m.

reorganized in wild-type cells, and individual mitochondria were seen that moved significantly faster than the growth speed of the hyphal tip (Figure 7A). In contrast, mutant cells that were examined a few hours after germination of conidiospores showed several defects in mitochondrial morphology and behavior (Supplemental Movie 2). In average, mutant mitochondria were longer than wild-type mitochondria. Almost all mutant mitochondria were oriented parallel to the longitudinal axis of the hypha, whereas wild-type mitochondria moved more freely in the cell. Most strikingly, forward movement of mitochondria in mutant cells was not faster than the growth speed of the hyphal tip. Apparently, mitochondria were moved forward with the growing cell, probably in a passive manner, and their spatial arrangement changed much slower than in wild-type cells (Figure 7A). Very similar changes of mitochondrial distribution and morphology were seen when mitochondria were stained with the membrane potential-sensitive dye rhodamine B hexyl ester. Mitochondria were larger, less numerous and more oriented along the longitudinal axis in $\Delta nkin-2$ cells (Figure 7B). We conclude that Nkin2 is required for normal mitochondrial distribution and motility. It should be noted that it was not possible to exactly measure the velocity of mitochondrial movement and statistically analyze their behavior, because the mitochondria frequently left the focal plane, and it was difficult to track the movement of individual organelles for more than a few seconds. Mitochondrial motility

defects could not be observed any more when cells were allowed to grow for more than 10 h. This is the time required for up-regulation of Nkin3 (compare Figure 5C). We propose that mitochondrial motility defects seen in recently germinated cells are rescued by Nkin3 as growth of hyphae proceeds.

DISCUSSION

Here, we show that Nkin2, a novel kinesin-related protein, mediates mitochondrial interactions with microtubules in *Neurospora*. Intriguingly, a related protein, KIF1B, has been shown to play a similar role in murine cells. KIF1B colocalizes with mitochondria in vivo, is enriched in mitochondrial fractions, and is able to transport mitochondria along microtubules in vitro (Nangaku *et al.*, 1994). Nkin2 and KIF1B belong to the same group of evolutionarily conserved motor proteins, the Unc104/KIF1 family. Thus, mitochondrial transport is mediated by closely related motors in organisms as distantly related as *Neurospora* and mice.

Unc104/KIF1-related proteins function as organelle transporters in a variety of organisms from fungi to mammals. For example, the *Dictyostelium* homologue DdUnc104 serves as a vesicle transporter, but it is not required for mitochondrial transport (Pollock *et al.*, 1999). Unc104 and KIF1A are examples for specialized transporters of neuronal vesicles in

C. elegans (Hall and Hedgecock, 1991) and mice (Yonekawa *et al.*, 1998), and Kin3 is a motor mediating the transport of early endosomes in the basidiomycetous fungus *Ustilago maydis* (Wedlich-Söldner *et al.*, 2002). Apparently, the major cellular functions of Unc104/KIF1 proteins vary between organisms, and, conversely, not all cell types rely on Unc104/KIF1 family members to mediate mitochondrial motility. The involvement of conventional kinesins in mitochondrial transport is similarly diverse between organisms. Disruption of the gene encoding mouse conventional kinesin heavy chain, *kif5B*, results in perinuclear clustering of mitochondria (Tanaka *et al.*, 1998), and a specific form of kinesin light chain is associated with mitochondria in cultured mammalian cells (Khodjakov *et al.*, 1998). Mutants lacking conventional kinesin in the filamentous fungus *Nectria hematococca* display a withdrawal of mitochondria from the growing hyphal tip (Wu *et al.*, 1998). Thus, conventional kinesin and its close relatives are important for mitochondrial distribution in certain mammalian and fungal cell types. In contrast, conventional kinesin, Nkin, is not involved in mitochondrial transport in *Neurospora*. Mitochondria show wild-type-like behavior in *nkin* knockout mutants (Seiler *et al.*, 1997), and here we show that Nkin is not localized to mitochondria. Obviously, the involvement of a particular type of motor protein in mitochondrial motility in different organisms does not reflect the evolutionary relatedness of these species.

How can we reconcile the observations that diverse organisms harbor similar sets of highly conserved kinesin-related proteins, but often rely on different motors—or even on different cytoskeletal systems—to perform the same cellular function? A possible explanation is that eukaryotic cells are equipped with a kit of organelle motors that is, at least in part, redundant and allows rapid responses to physiological and environmental challenges. We observed an astonishingly fast adaptation of mutant cells to the depletion of Nkin2. Within a few hours, expression of Nkin3 was highly increased, and this normally nonmitochondrial motor protein was recruited to mitochondria. Mitochondrial motility defects were manifest only in a small time window a few hours after germination of conidiospores. This effect was seen in several independently isolated mutants and did not require any second mutagenesis event. Thus, it reflects an inherent capacity of the organism to adapt to a mutagenic challenge. Both Nkin2 and Nkin3 are able to mediate mitochondrial binding to microtubules in vitro—Nkin2 on wild-type mitochondria and Nkin3 on $\Delta nkin-2$ mitochondria. If two different motor proteins have the same intrinsic capability, it is likely that only subtle changes are required to favor one over the other to execute a particular cellular function. These changes conceivably occurred many times during evolution and might account for the variability of organellar transport machineries that we observe in eukaryotic cells.

Our current study shows that mitochondrial binding to microtubules in *Neurospora* depends on Nkin2, a protein strikingly similar to the murine mitochondrial motor KIF1B. It will be interesting to see in the future whether organellar transport systems in highly polarized mammalian cells, such as neurons (Brown, 2003), have this and other conserved features in common with the fast growing hyphae of filamentous fungi. Our second major finding is that Nkin3, a member of a fungal-specific subgroup of small Unc104/KIF1-related proteins, mediates mitochondrial motility in the absence of Nkin2. This implies that the cellular transport machinery is remarkably robust against mutagenic challenges. Furthermore, it shows that Nkin3 has the capacity to

act as an organelle motor, even though its normal cellular function remains unknown. With the identification of the motor proteins involved, we can now address further fundamental questions, such as the identity of putative mitochondrial receptors for motor proteins and regulatory factors that are required to tune mitochondrial motor activity.

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