Accumulation of Cytoplasmic Dynein and Dynactin at Microtubule Plus Ends in *Aspergillus nidulans* Is Kinesin Dependent[□]

Jun Zhang,* Shihe Li,* Reinhard Fischer,[†] and Xin Xiang*[‡]

*Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; and [†]Max-Planck-Institute for Terrestrial Microbiology, D-35043 Marburg, Germany

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The mechanism(s) by which microtubule plus-end tracking proteins are targeted is unknown. In the filamentous fungus *Aspergillus nidulans*, both cytoplasmic dynein and NUDF, the homolog of the LIS1 protein, localize to microtubule plus ends as comet-like structures. Herein, we show that NUDM, the p150 subunit of dynactin, also forms dynamic comet-like structures at microtubule plus ends. By examining proteins tagged with green fluorescent protein in different loss-of-function mutants, we demonstrate that dynactin and cytoplasmic dynein require each other for microtubule plus-end accumulation, and the presence of cytoplasmic dynein is also important for NUDF's plus-end accumulation. Interestingly, deletion of NUDF increases the overall accumulation of dynein and dynactin at plus ends, suggesting that NUDF may facilitate minus-end-directed dynein movement. Finally, we demonstrate that a conventional kinesin, KINA, is required for the microtubule plus-end accumulation of cytoplasmic dynein and dynactin, but not of NUDF.

INTRODUCTION

In eukaryotic cells, the microtubule cytoskeleton is essential for cell cycle progression, the establishment of cell polarity, and cell migration. In most interphase cells, microtubules are polarized in such a way that the minus ends are located at the microtubule organizing center near the nucleus while the plus ends extend to the periphery. Microtubule plus ends are very dynamic, constantly exploring the cytoplasmic space with alternate growing and shrinking phases (reviewed by Desai and Mitchison, 1997; Gundersen, 2002). Kinesin superfamily members and cytoplasmic dynein together play important roles in intracellular trafficking of various proteins, vesicles, and organelles. Because conventional kinesin is a plus-end-directed motor and dynein is a minus-end-directed motor, it is thought that conventional kinesin is responsible for moving materials from the cell body toward cell periphery, whereas cytoplasmic dynein moves materials from the cell periphery back to the cell body (reviewed by Goldstein and Yang, 2000).

We have shown that cytoplasmic dynein is required for the proper distribution of nuclei within the hyphae of the filamentous fungus *Aspergillus nidulans* (reviewed by Morris *et al.*, 1998b). Cytoplasmic dynein is a complex that consists of heavy chains (HCs), intermediate chains (ICs), light intermediate chains, and light chains (reviewed by King, 2000; Tynan *et al.*, 2000). The HC contains the motor domain (reviewed by Asai and Koonce, 2001), and the other chains may target the motor to various cargoes (Steffen *et al.*, 1997; Tai *et al.*, 1999; Young *et al.*, 2000). The function of cytoplasmic dynein requires dynactin (reviewed by Holleran *et al.*, 1998; Ma *et al.*, 1999; Roghi and Allan, 1999; King and Schroer, 2000; Kumar *et al.*, 2000), a complex that contains multiple subunits, including the 150-kDa dynactin protein and the actin-related proteins Arp1 and Arp11 (reviewed by Holleran *et al.*, 1998; Eckley *et al.*, 1999). Our genetic analyses of nuclear distribution identified multiple nuclear distribution (*nud*) genes encoding components of the cytoplasmic dynein and dynactin complexes. For example, the *nudA*, *nudI* and *nudG* genes encode the HC, the IC and the 8-kDa light chain of cytoplasmic dynein (Xiang *et al.*, 1995a; Beckwith *et al.*, 1998; Zhang *et al.*, 2002). The *nudK* gene encodes the actin-related protein Arp1 of the dynactin complex (Xiang *et al.*, 1999). Novel proteins have also been discovered such as NUDC, NUDF, and NUDE (Osmani *et al.*, 1990; Xiang *et al.*, 1995b; Efimov and Morris, 2000). NUDF functions in the cytoplasmic dynein pathway and is homologous

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[‡] Corresponding author. E-mail address: xxiang@usuhs.mil.

Table 1. *A. nidulans* strains used in this work (all the strains have the *veA1* marker)

Strain	Genotype*	Source
GR5	<i>pyrG89; wA3; pyroA4</i>	G. S. May
R153	<i>wA3; pyroA4</i>	C. F. Roberts
WX116	<i>nudM116; pyrG89; yA1</i>	Xiang <i>et al.</i> (1999)
GFP- <i>nudA</i>	<i>GFP-nudA-pyr4; pyrG89; wA3; pyroA4</i>	Xiang <i>et al.</i> (2000)
GFP- <i>nudF</i>	<i>GFP-nudF-pyr4; pyrG89; wA3; pyroA4</i>	Han <i>et al.</i> (2001)
JZ10 or J1	<i>GFP-nudI-pyr4; pyrG89; wA3; pyroA4</i>	Zhang <i>et al.</i> (2002)
JZ3	<i>GFP-nudM-pyr4; pyrG89; wA2; pyroA4</i>	This work
SL5	<i>GFP-nudA-pyr4; nudM116; pyrG89; possibly yA1 and/or wA3</i>	This work
SL6	<i>GFP-nudF-pyr4; nudM116; pyrG89; possibly yA1 and/or wA3</i>	This work
ΔF /GFP- <i>nudA</i>	$\Delta nudF$ - <i>pyr4; GFP-nudA-pyr4; pyrG89; wA3; pyroA4</i>	Zhang <i>et al.</i> (2002)
ΔF /GFP- <i>nudI</i>	$\Delta nudF$ - <i>pyr4; GFP-nudI-pyr4; pyrG89; wA3</i>	This work
ΔF /GFP- <i>nudM</i>	$\Delta nudF$ - <i>pyr4; GFP-nudM-pyr4; pyrG89; wA3</i>	This work
JZ105	<i>GFP-nudF-pyr4; nudK317; pyrG89; wA3</i>	This work
JZ108	<i>GFP-nudF-pyr4; $\Delta nudA$-<i>pyrG; pyrG89; possibly wA3</i></i>	This work
ΔA /GFP- <i>tubA</i>	<i>GFP-tubA-pyr4; $\Delta nudA$-<i>pyrG; pyrG89; possibly wA3 and pyroA4</i></i>	This work
XX97	<i>GFP-nudF-pyr4; nudA2; pyrG89; wA3</i>	This work
XX108	<i>GFP-nudF-pyr4; pyrG89; nudI416; wA3</i>	This work
JZ4	<i>GFP-nudA-pyr4; $\Delta kinA$-<i>pyr4; pyrG89; wA3</i></i>	This work
SL1	<i>GFP-nudF-pyr4; $\Delta kinA$-<i>pyr4; pyrG89; yA1</i></i>	This work
SL7	<i>GFP-nudM-pyr4; $\Delta kinA$-<i>pyr4; pyrG89; wA3</i></i>	This work
SL9	<i>GFP-nudI-pyr4; $\Delta kinA$-<i>pyr4; pyrG89; wA3</i></i>	This work
SNR7a	<i>$\Delta kinA$-<i>pyr4; yA2; pyroA4</i></i>	R. Fischer

to a human protein, LIS1, required for neuronal migration (Xiang *et al.*, 1995b; Willins *et al.*, 1997). Both NUDC and NUDE interact with NUDF/LIS1 (Morris *et al.*, 1998a; reviewed by Morris *et al.*, 1998b; Efimov and Morris, 2000). The human Lis-1 gene was identified as a causal gene for a type 1 lissencephaly, a human disease characterized by brain malformation due to neuronal migration defects (Reiner *et al.*, 1994). Since the discovery that LIS1 homologs participate in dynein function in fungal systems (Xiang *et al.*, 1995b; Geiser *et al.*, 1997), LIS1 and its homologs also have been found to have dynein-related functions in higher eukaryotes (Swan *et al.*, 1999; Faulkner *et al.*, 2000; Liu *et al.*, 2000; Smith *et al.*, 2000; Dawe *et al.*, 2001). More importantly, a direct physical interaction between LIS1/NUDF and the motor domain of cytoplasmic dynein heavy chain has been demonstrated in both mammalian cells and *A. nidulans* (Sasaki *et al.*, 2000; Hoffmann *et al.*, 2001; Tai *et al.*, 2002), suggesting that LIS1 may regulate dynein motor activity. However, because LIS1 was not isolated as a component of the dynein complex, interaction between LIS1 and dynein may be transient and restricted to special sites in the cell.

Recently, we have shown that GFP-labeled cytoplasmic dynein and its putative regulator NUDF form comet-like structures that accumulate at the dynamic plus ends of microtubules in *A. nidulans* (Han *et al.*, 2001). This is consistent with discoveries made in mammalian cells that dynactin, and in some cases dynein, localize at the plus ends of microtubules near the cell periphery (Vaughan *et al.*, 1999; Valetti *et al.*, 1999; Habermann *et al.*, 2001; Vaughan *et al.*, 2002). These proteins belong to a growing family of proteins named "plus-end tracking proteins" or +TIPs, which also include the cytoplasmic linker protein CLIP170, the tumor suppressor protein APC, and its binding protein EB1 (Perez *et al.*, 1999; Tirnauer *et al.*, 1999; Brunner and Nurse, 2000; Mimori-Kiyosue *et al.*, 2000a,b; Lin *et al.*, 2001; reviewed by Schuyler and Pellman, 2001; Schroer, 2001). How +TIPs

accumulate at microtubule plus ends in vivo is unknown. In this study, we show that in *A. nidulans*, the p150 subunit of dynactin, which is encoded by the *nudM* gene, is also localized to microtubule plus ends. We used loss-of-function mutants of cytoplasmic dynein, dynactin, NUDF, and the conventional kinesin KINA to investigate the genetic requirements for dynein, dynactin, and NUDF/LIS1 plus-end localization. Our data support a mutually cooperative role for dynein and dynactin in each other's plus-end localization. Our results also demonstrate that the KINA kinesin is required for cytoplasmic dynein and dynactin localization to microtubule plus ends. However, NUDF most likely uses a KINA-independent mechanism for its plus-end association. Interestingly, the *nudF* deletion mutant increases the plus-end accumulation of dynein and dynactin, suggesting that NUDF may facilitate dynein's departure from the plus ends of microtubules.

MATERIALS AND METHODS

Aspergillus nidulans Strains, Media, and Techniques

A. nidulans strains used in this study are listed in Table 1. Growth media used were YAG, YAG + UU, and MM + glycerol. *Aspergillus* growth and transformation were as described previously (Osmani *et al.*, 1987; Waring *et al.*, 1989; Xiang *et al.*, 1995 a,b). MM medium for strains with GR5 background was supplemented with 5 μ g/ml pyridoxine. *Aspergillus* protein isolation and Western analysis was done as described previously (Zhang *et al.*, 2002).

Cloning of the *nudM* p150 Dynactin Gene from *A. nidulans*

We first searched the *A. nidulans* genome database (<http://microbial.cereon.com/>) for sequence homologs of the RO3 protein, the p150 dynactin of *Neurospora crassa* (Tinsley *et al.*, 1996). Several sequences were identified. We then designed two oligos: AAATGCGC-

CGAGCTTACCATC (underlined sequence indicates the first codon ATG) and TTCGAGACCCACAACCTC for amplifying the genomic DNA encoding the p150 dynactin. By using the GR5 (wild-type strain) genomic DNA as a template, we amplified a region of 4 kb and cloned it into the PCR2.1 vector (Invitrogen, Carlsbad, CA). Sequencing of this clone confirmed that it encodes the p150 dynactin from *A. nidulans* because it shows high sequence homology with dynactin genes in other organisms. Strong protein sequence homology between the *A. nidulans* dynactin and the *Neurospora crassa* Ro-3 dynactin also helped us in identifying the only intron at the 5'-coding region of the gene. For further sequence analysis, the intron sequence was removed based on the presence of the conserved splicing sites. For sequencing the whole p150 dynactin coding region, we also designed two oligos, CTCGATGCACCTCTTACG and AGATGATTGACATAGTGG, and used them for polymerase chain reaction on genomic DNA template. This allowed us to obtain a clone of the C terminus of dynactin that overlaps with the 4-kb clone.

We transformed the 4-kb fragment of p150 dynactin into various new *nud* mutants (Xiang *et al.*, 1999). The autoreplicating plasmid pAid was cotransformed for selection. We found that the 4-kb p150 dynactin fragment complemented the *nudM116* mutation. Our cloned p150 dynactin gene most likely represents the *nudM* gene rather than an extragenic suppressor of the *nudM116* mutation, based on the following evidence. The 4-kb fragment used to transform the *nudM116* mutant is not a full-length gene containing promoter and termination sequences. It starts from ATG and ends in the middle of the C terminus. Thus, it is most likely that this fragment repaired the mutation by homologous recombination rather than integrated into another site as an extracopy number suppressor. Furthermore, we analyzed a cross between a *ts*⁺ transformant and a wild-type strain R153. (To facilitate such a cross, we first streaked the *ts*⁺ transformant on YUU plates to allow the spontaneous loss of the cotransformed extrachromosomal vector pAid carrying the *pyrG* gene). This cross yielded 100% *ts*⁺ progeny (*n* = 420), consistent with the notion that the original *nudM116* mutation was repaired by the p150 dynactin genomic DNA that underwent homologous recombination.

Construction of GFP-*nudM* (p150 Dynactin) Strain

To construct the GFP-*nudM* strain in which the endogenous *nudM* gene is replaced by a functional GFP-*nudM* fusion gene, we used a strategy similar to what has been described previously for the construction of the GFP-*nudI* strain (Zhang *et al.*, 2002). For construction of the GFP-*nudM* plasmid, a 1.4-kb DNA sequence of the N-terminal region of *nudM* was amplified with oligos D-*NotI* AAGCGGCCGCTGGCCGAGCTTACCATC and D-*SmaI* AAC-CCGGGTCTTCTCATCTGCAAC as primers. The polymerase chain reaction product was digested with *NotI*/*SmaI* and ligated to the *NotI* and *SmaI* sites of pLB01 (Liu and Morris, 2000). The resulting plasmid contains sequence of a codon-modified GFP (Fernandez-Abalos *et al.*, 1998), fused to the N terminus of NUDM and the expression of the fusion protein is under the control of the *alcA* promoter (Waring *et al.*, 1989). On transformation, homologous integration of the *nudM* sequence on the plasmid into the genomic *nudM* sequence generates a truncated *nudM* gene with its own promoter and a GFP-*nudM* fusion gene under the control of the *alcA* promoter. The ability to repress the *alcA* promoter by using glucose allows us to select for transformants in which homologous integration occurred based on their *nud*-like colony morphology on glucose (similarly described by Zhang *et al.*, 2002). One such transformant for which the single integration event was confirmed by Southern analysis was designated the GFP-*nudM* strain and used for further observation. GFP-NUDM is functional *in vivo* because the GFP-*nudM* cells grow as well as the wild-type cells and exhibit normal nuclear distribution on glycerol medium that is nonrepressing to the *alcA* promoter (our unpublished data). A similar strategy was also used to create a GFP-*nudK* (Arp1 of the dynactin complex)

strain. However, adding GFP to the N terminus of NUDK Arp1 causes Arp1 to lose its function as the GFP-*nudK* strain grows like a *nud* mutant on a glycerol plate. Therefore, we focused on the GFP-*nudM* (p150 dynactin) strain to study dynactin localization in *A. nidulans*.

Placing the GFP-*nudA*, GFP-*nudI*, GFP-*nudM*, and GFP-*nudF* Fusion Alleles into Various Mutants

Standard genetic techniques in *A. nidulans* were used for genetic crosses. To place a GFP fusion in the background of a specific *nud* mutation, a *nud* mutant carrying the *pyrG89* mutation was crossed to the strain harboring the GFP fusion. The GR5 parental strain carrying the GFP fusions has the *pyrG89* marker, and therefore requires uridine and uracil for growth. The GFP fusions are genetically marked by the *Neurospora pyr4* gene that was integrated into the genome along with GFP. This *pyr4* gene allows the cells to grow without uridine and uracil. Thus, if any *pyrG89* strain is crossed to the GFP strains, only those progeny that contain the GFP-fusion can grow without uridine and uracil. Progeny carrying both the GFP fusion gene and the *nud* mutation were identified by their ability to grow without uridine and uracil supplementation and by their temperature-sensitive *nud* phenotype at 42°C. To identify progeny carrying a GFP fusion in the background of a particular *nud* deletion strain in which the deletion allele is also marked by *pyr4* or *pyrG*, we rely on phenotypic analyses. Specifically, for the cross between the Δ *nudF* or Δ *nudA* strain and the GFP-*nudA*, GFP-*nudI*, GFP-*nudM* or GFP-*nudF* strains, the progeny that carry the GFP fusion proteins in the Δ *nudF* or Δ *nudA* background were selected by the presence of the *nud* phenotype on a glycerol plate at 32°C and the presence of green fluorescence under the microscope using Δ *nudF* or Δ *nudA* as negative control, and confirmed by Western analysis demonstrating the presence of the GFP-fusion proteins. For the crosses between the Δ *kinA* strain and the GFP-*nudA*, GFP-*nudI*, GFP-*nudM*, or GFP-*nudF* strains, the progeny that carry the GFP fusion proteins in the Δ *kinA* background were selected by the presence of the *nud* phenotype of the GFP strains on YUU plates (repressive medium for the *alcA* promoter) at 32°C and the smaller colony phenotype of the Δ *kinA* mutant on a glycerol plate. In addition, Southern blot analyses were used to confirm the presence of the Δ *kinA* allele in strains with various GFP fusion proteins.

Image Acquisition and Analyses, Immunostaining, and Western Blotting

Cells were grown in Δ TC3 culture dishes (Bioprotechs, Butler, PA) in 1.5 ml of MM + glycerol + pyridoxine medium at 32°C overnight. In contrast to ethanol or threonine that causes overexpression of the *alcA*-driven genes, glycerol is a noninducing but derepressing carbon source (Waring *et al.*, 1989), which does not cause the *alcA*-driven dynein or NUDF to be overproduced compared with the endogenous protein in a wild-type strain (our unpublished data). Images were captured using an IX70 inverted fluorescence microscope (Olympus, Tokyo, Japan) (with a 63 $\times$ objective) linked to a 5-MHz MicroMax cooled charge-coupled device camera (Princeton Scientific Instruments, Monmouth Junction, NJ) as described previously (Xiang *et al.*, 2000). For measuring the intensity of GFP-dynein or dynactin comets in wild-type and Δ *nudF* cells, individual GFP comets were segmented and the intensity sum of each segment was measured using the Measuring Segment tool in the IPLab software. Because the GFP-labeled dynein and dynactin comets that are the closest to the hyphal tip are usually the brightest and have similar signal intensity among wild-type cells, we only used values obtained from these comets in each sequence. Data were processed using SigmaPlot and Microsoft Excel.

For visualizing microtubules in cells expressing GFP-NUDM, we used the DM1A anti- α -tubulin antibody (Sigma-Aldrich, St. Louis, MO) for immunostaining, with a procedure similar to what was described previously (Willins *et al.*, 1995; Han *et al.*, 2001). For

Figure 1. (A) Dynamics of GFP-NUDM dynactin comets in vivo. Bar, $\sim 5 \mu\text{m}$. (B) Immunostaining of GFP-NUDM and microtubules. This image resulted from a merge of a pseudocolored microtubule image (red) and a GFP-NUDM image (green).

digestion of the cell wall, β -D-glucanase (16 mg/ml) and driselase (10 mg/ml) (Interspers Products, Foster City, CA) were used (Jung *et al.*, 2000). Although the GFP comets (dynein, dynactin, and NUDF) are present in nearly 100% of the living cells, after fixation, cell wall digestion, and staining, only a very low percentage of the cells show GFP comets. Because this procedure significantly lowered the GFP-comet signal intensity, we used a polyclonal anti-GFP antibody (Chemicon International, Temecula, CA) to stain GFP after microtubule staining. The anti-GFP antibody was used at 1/60. The anti-tubulin antibody was used at 1/200. Cy3-anti-mouse (1/1000; Sigma-Aldrich) and fluorescein isothiocyanate-anti-rabbit secondary antibodies (1/1000; Sigma-Aldrich) were used to visualize microtubules and GFP-NUDM in the same cell. Using this modified procedure, we were able to see GFP-NUDM signals at microtubule ends near the hyphal tip in $\sim 20\%$ of fixed cells. 4,6-Diamidino-2-phenylindole staining and Western blotting analyses were done as described previously (Zhang *et al.*, 2002).

RESULTS

Cytoplasmic Dynein and Dynactin Require Each Other for Microtubule Plus-End Localization

Our genetic analysis indicated that the *nudM* gene encodes the *A. nidulans* p150 dynactin protein (GenBank accession no. AY158343). NUDM shows 43.9% overall sequence identity to the *N. crassa* dynactin protein RO3 (Tinsley *et al.*, 1996) and contains the CAP-Gly microtubule-binding motif at its N terminus (the GCG program). We have constructed a GFP-*nudM* strain in which the endogenous *nudM* gene was replaced by a functional GFP-*nudM* fusion gene. The GFP-NUDM fusion protein forms dynamic comet-like structures that locate on the distal plus ends of microtubules near the hyphal tip (Figure 1, A and B), a behavior similar to that exhibited by GFP-NUDA (cytoplasmic dynein heavy chain), GFP-NUDI (cytoplasmic dynein IC), and GFP-NUDF (the LIS1-like protein) (Xiang *et al.*, 2000; Han *et al.*, 2001; Zhang *et al.*, 2002).

To test whether cytoplasmic dynein plays a role in the localization of dynactin, we crossed the GFP-*nudM* strain to the *nudA* deletion mutant, $\Delta nudA$. In the $\Delta nudA$ mutant, the region covering the first four ATP-binding sites of the heavy

chain is replaced by the *pyrG*-selective marker gene and the heavy chain protein is not detectable (Xiang *et al.*, 1995a). Interestingly, in most of the $\Delta nudA$ cells, the GFP-NUDM dynactin comets were no longer observed near the hyphal tip (Figure 2). Because microtubules in the $\Delta nudA$ mutant reach the hyphal tip (Figure 2), our result suggests that cytoplasmic dynein is required for dynactin's plus-end accumulation. Similar to our previous observation that an Arp1 mutation abolishes GFP-NUDA localization (Xiang *et al.*, 2000), herein we found that the *nudM116* mutation of p150 dynactin also abolishes GFP-NUDA localization (Figure 2), further supporting dynactin's role in dynein localization. Together, these results indicate that dynein and dynactin are dependent upon each other for their accumulation at microtubule plus ends.

Presence of Cytoplasmic Dynein Is Important for Localization of NUDF

We have noticed that the signal intensities of GFP-NUDF comets are relatively lower in various loss-of-function mutants of cytoplasmic dynein (*nudA2* in the heavy chain, *nudI416* in the IC) and dynactin (*nudK317* in Arp1, *nudM116* in the p150 dynactin). The decrease in intensity is most obvious in the dynein heavy chain deletion mutant $\Delta nudA$ (Figure 3). Because NUDF/LIS1 interacts directly with the dynein heavy chain (Sasaki *et al.*, 2000; Hoffmann *et al.*, 2001; Tai *et al.*, 2002), we tested whether the NUDF protein level is decreased in the absence of the dynein heavy chain. Our Western blot and densitometry analyses showed that the GFP-NUDF protein level was not significantly decreased in $\Delta nudA$ (Figure 3; our unpublished data). Together, these results suggest that the presence of cytoplasmic dynein may enhance NUDF's association with microtubule plus ends. This is consistent with previous observations in mammalian cells that the signal intensity of the kinetochore-localized LIS1 decreases in dynein-defective mitotic cells (Coquelle *et al.*, 2002; Tai *et al.*, 2002).

Figure 2. Images of GFP-NUDM dynactin in wild-type (GFP-NUDM) and the $\Delta nudA$ mutant (GFP-NUDM/ $\Delta nudA$) cells, GFP-TUBA (α tubulin) in the $\Delta nudA$ mutant (GFP-TUBA/ $\Delta nudA$), and GFP-NUDA dynein heavy chain in wild-type (GFP-NUDA) and the *nudM116* mutant (GFP-NUDA/*nudM116*) cells.

Figure 3. (A) Images of GFP-NUDF (LIS1-like protein) in wild-type and the $\Delta nudA$ mutant cells. (B) Western blot analysis on the level of GFP-NUDF in protein extract from the wild-type and the $\Delta nudA$ mutant cells. A monoclonal anti-GFP antibody (BD Biosciences Clontech) was used at 1/500. The level of protein loading was shown by Ponceau S staining of the membrane.

nudF Deletion Mutant Increases Signal Intensity of GFP-Cytoplasmic Dynein and Dynactin Comet-like Structures

NUDF is apparently not required for cytoplasmic dynein heavy chain targeting to the microtubule plus ends because bright GFP-NUDA comets are obvious at the hyphal tip in

the background of a *nudF* deletion mutant (Zhang *et al.*, 2002; Figure 4). Herein, we show that the GFP-NUDI and GFP-NUDM comets are also bright in the *nudF* deletion mutant, suggesting that NUDF is also not required for the plus end localization of dynein IC as well as the p150 dynactin (Figure 4). During our observation of many cells, we have had the impression that the GFP-NUDA, GFP-NUDI, and GFP-NUDM comets are brighter in the *nudF* deletion mutant background compared with that in the wild-type cells (Figure 5). To show the differences in intensity quantitatively, we measured the fluorescence intensity of individual comets close to the hyphal tip of each cell. Our measurements showed that the sum of fluorescence of individual GFP-NUDA, GFP-NUDI and GFP-NUDM comets in the *nudF* deletion cells is significantly higher than that in wild-type cells (Figure 5). These results suggest that although NUDF does not play a role in dynein/dynactin targeting to microtubule plus ends, it could be required for the dynamic departure of dynein/dynactin from the microtubule plus ends. In some $\Delta nudF$ cells, a cloud of background fluorescence around the hyphal tip can be observed (Figure 4). Although we do not have a good interpretation of this result, it is possible that overaccumulation of the proteins causes them to fall off the microtubule ends.

Deletion of kinA Kinesin Gene Diminishes Plus-End Comets of Both Cytoplasmic Dynein and Dynactin, but Not NUDF

Because cytoplasmic dynein is a minus-end-directed microtubule motor, its *in vivo* localization to the plus ends of microtubules may depend on the transport function of a plus-end motor such as kinesin. So far, at least seven kinesins have been found in the *A. nidulans* genome (Fischer, unpublished data; Liu, personal communication), and KINA is the only identified conventional kinesin. Although the *kinA* deletion mutant grows better than the dynein mutants, it does exhibit a partial defect in nuclear positioning (Requena *et al.*, 2001). In addition, although microtubules look normal in the *kinA* deletion mutant, genetic evidence suggests that they are more stable than in wild-type cells, which is also similar to what has been described for the cytoplasmic dynein mutants (Willins *et al.*, 1995; Han *et al.*, 2001; Requena *et al.*, 2001). To test whether KINA is involved in

Figure 4. Images of GFP-NUDA (dynein HC), GFP-NUDI (dynein IC), and GFP-NUDM (p150 dynactin) in wild-type and the $\Delta nudF$ mutant cells.

Figure 5. Graphic presentation of the fluorescence intensities of GFP comets near the hyphal tip. Values of mean and SD were generated based on 12 individual comets of each strain.

cytoplasmic dynein localization to the plus ends *in vivo*, we crossed a *kinA* deletion strain ($\Delta kinA$) to the GFP-*nudA* (cytoplasmic dynein HC) strain and the GFP-*nudI* strain (cytoplasmic dynein IC), and observed GFP-NUDA as well as GFP-NUDI in the $\Delta kinA$ background. Interestingly, cyto-

plasmic dynein comet-like structures near the hyphal tip are much less obvious in the $\Delta kinA$ mutant (Figure 6). These results demonstrated that KINA is important for cytoplasmic dynein accumulation to the plus ends of microtubules.

Because dynactin and NUDE/LIS1 also accumulate at the plus ends of microtubules, it was of interest to test whether their localization is also KINA dependent. To do this, we crossed $\Delta kinA$ to the GFP-*nudM* (p150 dynactin) strain and to the GFP-*nudF* strain and observed GFP-NUDM as well as GFP-NUDF in the $\Delta kinA$ background. Our results show that similar to GFP-NUDA, GFP-NUDM comet structures near the hyphal tip are hardly visible in the $\Delta kinA$ mutant (Figure 6), indicating that KINA is also important for the plus-end accumulation of dynactin. However, GFP-NUDF comets were easily observed near the hyphal tip (Figure 6), suggesting that the plus-end localization of NUDE does not require the KINA kinesin. However, consistent with the notion that KINA is required for dynein/dynactin localization and that the presence of dynein/dynactin may enhance NUDE's association with microtubule plus ends, we found that the signal intensity of many GFP-NUDF comets was lower.

To address whether KINA functions only in the cytoplasmic dynein genetic pathway, a previous study has analyzed the double mutant containing a temperature-sensitive *nudA* allele, *nudA1*, and the $\Delta kinA$ allele (Requena *et al.*, 2001). Although the nuclear migration phenotype of the double mutant resembles that of the *nudA1* single mutant, the double mutant grows more poorly than the *nudA1* single mutant (Requena *et al.*, 2001). In this study, we compared an *alcA*-promoter-based *nudA* conditional null mutant (GFP-*nudA*)

Figure 6. Images of GFP-NUDA (dynein HC), GFP-NUDI (dynein IC), GFP-NUDM (p150 dynactin), and GFP-NUDF (LIS1-like protein) in wild-type and the $\Delta kinA$ mutant cells.

Figure 7. NudA/kinA double mutant analyses. (A) Growth of the wild-type, $\Delta kinA$, GFP-*nudA*, and $\Delta kinA$ /GFP-*nudA* (JZ4) strains on YUU that represses the expression of the *alcA*-driven GFP-*nudA* fusion gene, the only intact version of *nudA* in the genome. The plate was incubated at 32°C for 2 d. (B) 4,6-Diamidino-2-phenylindole staining of the wild-type, $\Delta kinA$, GFP-*nudA*, and $\Delta kinA$ /GFP-*nudA* (JZ4) cells that were grown in YUU at 32°C for 7.5 h.

with the corresponding double null mutant (GFP-*nudA*/Δ*kinA*). On a glucose-containing medium that shuts off the *alcA* promoter, the double null mutant exhibited a more severe growth defect than that of the *nudA* or the *kinA* single null mutant (Figure 7A). However, the nuclear migration phenotype of the double null mutant is similar to that exhibited by the *nudA* single null mutant when germlings of similar lengths are compared (Figure 7B). These results suggest that, in addition to being involved in dynein-related functions such as nuclear migration, KINA also participates in pathway(s) that regulate other cellular processes.

DISCUSSION

In this study, we have provided the genetic evidence that KINA, a conventional kinesin, is involved in the localization of both cytoplasmic dynein and dynactin to the plus ends of microtubules in *A. nidulans*. In the deletion mutant of *kinA*, the microtubule plus-end accumulation of both cytoplasmic dynein (the NUDA heavy chain and the NUDI IC) and dynactin (the NUDM p150 dynactin) are significantly diminished. Although it is possible that KINA may transport cytoplasmic dynein and dynactin toward the microtubule plus end, other possibilities also exist. For example, KINA itself may be targeted to the plus ends, and its presence there is required for dynein/dynactin to associate to the plus ends. However, a previous study has shown that KINA itself is not a plus-end-associated protein. Although in many cells the GFP-KINA fusion protein does not show specific localization, it does light up microtubule-like structures in some cells (Requena *et al.*, 2001). Similarly, a homologous conventional kinesin in *Ustilago maydis* is also a cytoplasmic protein,

which exhibits localization to microtubule-like structures only when cells are treated to deplete ATP (Lehmle *et al.*, 1997). At this point, we have not detected any physical interaction between KINA and cytoplasmic dynein. Therefore, it is also possible that the effect of *kinA* deletion on dynein and dynactin localization is indirect. For example, the deletion of *kinA* may change the microtubule ends in such a way that dynein and dynactin can no longer be associated. In fact, genetic evidence has shown that the *kinA* deletion mutant slows down microtubule dynamics (Requena *et al.*, 2001). However, because dynein and dynactin are clearly accumulated at the plus ends of microtubules in the *nudF* mutant, in which microtubules are also less dynamic (Han *et al.*, 2001), it is unlikely that dynein/dynactin's failure to accumulate at the microtubule plus ends in the *kinA* mutant is caused by microtubules being too stable.

Interestingly, the KINA-dependent mechanism may not be the only mechanism for dynein/dynactin's accumulation to the plus ends of microtubules. In some *kinA* deletion cells, faint dynein and dynactin comets moving toward the hyphal tip can still be observed. This suggests the participation of a redundant player. The fact that the *kinA* deletion mutant exhibits a nuclear distribution defect, which is less severe than that of a dynein heavy chain null mutant, is also consistent with KINA being a player in the dynein pathway whose function is partially redundant with other protein(s) yet to be identified.

It would be interesting to know whether dynein/dynactin's localization to the microtubule plus ends in higher eukaryotic cells also depends on conventional kinesin. Several studies have suggested that cytoplasmic dynein may be present on cargoes that are transported by kinesin (Hirokawa *et al.*, 1990; reviewed by Goldstein and Yang, 2000; Ma and Chisholm, 2002; Gross *et al.*, 2002). A very recent study in *Drosophila* has also demonstrated the role of kinesin in dynein localization (Brendza *et al.*, 2002). Whether other +TIPs use kinesins to get to the plus ends is also an interesting question. APC, a tumor suppressor protein that associates with another plus-end protein EB1, physically interacts with KAP3, a KIF3A-KIF3B kinesin superfamily-associated protein, and this interaction is important for APC's accumulation to the membrane protrusions (Jimbo *et al.*, 2002). Our results show that NUDF's localization is not dependent upon the conventional kinesin KINA, suggesting that +TIPs may use different mechanisms for their microtubule plus-end targeting. The CLIP170 protein that physically interacts with LIS1 may be targeted to the microtubule plus end by copolymerization with tubulins (Diamantopoulos *et al.*, 1999; Perez *et al.*, 1999; Coquelle *et al.*, 2002). However, its localization to the plus end rather than along the length of a microtubule would require its dissociation from the early-polymerized segments, and how that may be achieved is not clear. The growing end distinguishes itself from the rest of the microtubule in both its structure and the presence of a GTP cap (reviewed by Desai and Mitchison, 1997). Although the size of the GTP cap is estimated to be very small (Panda *et al.*, 2002), such a cap combined with the structure feature of a growing end may be sufficient to prevent the proteins from falling off the end.

In this study, we have also found that cytoplasmic dynein is required for the plus-end localization of dynactin. This

Figure 8. Model providing an explanation for the observation that accumulation of dynein/dynactin at the microtubule plus ends is increased in the *nudF* deletion mutant. NUDF/LIS1 may facilitate dynein-mediated cargo departure from the plus end toward the minus end of the microtubule. Failure of such departure is expected to cause a more intense plus-end accumulation of dynein/dynactin, which is what has been found in absence of NUDF.

result seems contradictory to the observation made in mammalian cells that only dynactin but not dynein accumulates at the microtubule plus end under physiological conditions (Vaughan *et al.*, 1999; Habermann *et al.*, 2001). However, it is possible that cytoplasmic dynein is required for the initial targeting but not for maintaining the association of dynactin to the microtubule plus end. In contrast to *A. nidulans* in which the plus-end localization of cytoplasmic dynein has been observed under physiological temperatures (Xiang *et al.*, 2000; Han *et al.*, 2001), the plus-end localization of cytoplasmic dynein in mammalian cells has only been observed upon a shift to a lower temperature (Vaughan *et al.*, 1999). This may indicate that mammalian cytoplasmic dynein but not dynactin leaves the microtubule plus end frequently under physiological conditions.

Interestingly, NUDF/LIS1 may be required for the departure of dynein and dynactin from the microtubule plus end. The fluorescence intensities of the dynein and dynactin comets near the hyphal tip are significantly higher in the absence of NUDF. Because cytoplasmic dynein is involved in retrograde vesicle transport in filamentous fungi (Seiler *et al.*, 1999), the plus-end dynein/dynactin in filamentous fungi may also represent a cargo-loading site as has been suggested and recently demonstrated in mammalian cells (Vaughan *et al.*, 1999, 2002; Valetti *et al.*, 1999; Habermann *et al.*, 2001). Because LIS1 physically interacts with both the cargo-binding region and the first AAA (ATPases associated with cellular activities) repeat of the dynein heavy chain, it was proposed that LIS1 may be involved in the coordination between motor activity and cargo binding in vivo (Tai *et al.*, 2002). Thus, it is possible that in the absence of NUDF, dynein and dynactin fail to depart from the microtubule plus ends toward the minus end, which results in an overaccumulation of dynein and dynactin at the plus end (Figure 8).

The function of the plus-end cytoplasmic dynein may be multifold. Recent work has shown that in the budding yeast *Saccharomyces cerevisiae*, cytoplasmic dynein IC is also localized to the microtubule plus end (Beach and Bloom, personal communication). The microtubules and their motors in *S. cerevisiae* are used only for spindle function and nuclear migration (Cottingham *et al.*, 1999), and thus the plus-end dynein may be involved in regulating microtubule-cortex interaction and microtubule dynamics, which are important

for nuclear migration and spindle orientation in fungi (Oakley and Morris, 1981; Willins *et al.*, 1995; Carminati and Stearns, 1997; Shaw *et al.*, 1997; Cottingham *et al.*, 1999; Adames and Cooper, 2000; Han *et al.*, 2001; Requena *et al.*, 2001; Tran *et al.*, 2001). Our previous observation that microtubules are less dynamic in both the *nudF* and dynein mutant cells indicates that NUDF may also regulate microtubule dynamics (Han *et al.*, 2001). In some $\Delta nudF$ cells, disorientated dynein/dynactin comets can be observed, which is also consistent with some microtubules curling around and growing back from the hyphal tip in the *nudF* and dynein mutant cells (see movie GFP-NUDI/ $\Delta nudF$ in Supplementary Materials; Han *et al.*, 2001).

Note added in proof. Since the acceptance of this article, the articles listed below, covering two related topics, have been published.

Topic A: *A. nidulans* and *S. cerevisiae* dynein localization:

Efimov, V.P. (2003). Roles of NUDE and NUDF proteins of *Aspergillus nidulans*: insights from intracellular localization and overexpression effects. *Mol. Biol. Cell.* 14, 871–888.

Lee, W.L., Oberle, J.R., and Cooper, J.A. (2003). The role of the lissencephaly protein Pac1 during nuclear migration in budding yeast. *J. Cell Biol.* 160, 355–364.

Liu, B., Xiang, X., Lee, Y.R. (2003). The requirement of the LC8 dynein light chain for nuclear migration and septum positioning is temperature dependent in *Aspergillus nidulans*. *Mol. Microbiol.* 47, 291–301.

Sheeman, B., Carvalho, P., Sagot, I., Geiser, J., Kho, D., Hoyt, M.A., and Pellman, D. (2003). Determinants of *S. cerevisiae* dynein localization and activation. Implications for the mechanism of spindle positioning. *Curr Biol.* 13, 364–372.

Xiang, X. (2003). LIS1 at the microtubule plus end and its role in dynein-mediated nuclear migration. *J. Cell Biol.* 160, 289–290.

Topic B: The kinesin-dynein relationship in *Drosophila*

Duncan, J.E., Warrior, R. (2002). The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the *Drosophila* oocyte. *Curr Biol.* 12, 1982–1991.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J.A., Lopez-Schier, H., Johnston, D.S., Brand, A.H., Roth, S., and Guichet, A. (2002). Polar transport in the *Drosophila* oocyte requires dynein and kinesin I cooperation. *Curr Biol.* 12, 1971–1981.

Palacios, I.M., and Johnston, D.S. (2002). Kinesin light chain-independent function of the kinesin heavy chain in cytoplasmic streaming and posterior localisation in the *Drosophila* oocyte. *Development.* 129, 5473–5485.

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