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Spindle Formation in *Aspergillus* Is Coupled to Tubulin Movement into the Nucleus[V]

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In many important organisms, including many algae and most fungi, the nuclear envelope does not disassemble during mitosis. This fact raises the possibility that mitotic onset and/or exit might be regulated, in part, by movement of important mitotic proteins into and out of the nucleoplasm. We have used two methods to determine whether tubulin levels in the nucleoplasm are regulated in the fungus *Aspergillus nidulans*. First, we have used benomyl to disassemble microtubules and create a pool of free tubulin that can be readily observed by immunofluorescence. We find that tubulin is substantially excluded from interphase nuclei, but is present in mitotic nuclei. Second, we have observed a green fluorescent protein/α-tubulin fusion in living cells by time-lapse spinning-disk confocal microscopy. We find that tubulin is excluded from interphase nuclei, enters the nucleus seconds before the mitotic spindle begins to form, and is removed from the nucleoplasm during the M-to-G_1 transition. Our data indicate that regulation of intranuclear tubulin levels plays an important, perhaps essential, role in the control of mitotic spindle formation in *A. nidulans.* They suggest that regulation of protein movement into the nucleoplasm may be important for regulating mitotic onset in organisms with intranuclear mitosis.

INTRODUCTION

Correct regulation of mitotic spindle formation is critical to successful cellular reproduction in eukaryotes. In animal and higher plant cells, spindle microtubules initially assemble in the cytoplasm, but spindle formation is not complete until the nuclear envelope breaks down, microtubules contact kinetochores and the spindle microtubules are organized into a mature, functional spindle. In contrast, the nuclear envelope is intact during mitosis in most fungi and in many protists (Heath, 1980). Microtubules assemble and are organized into a functional spindle within the nuclear envelope. Organisms with intranuclear mitosis make up much of the earth's biomass and include species that are enormously beneficial to humans and others that are serious human or agricultural pathogens. It is consequently important to understand mitotic spindle formation in these organisms.

In fungi such as *Aspergillus nidulans,* mitotic spindle formation is regulated very precisely (Oakley and Morris, 1983; Jung *et al.*, 1998, and earlier references therein). No microtubules are present in the nucleus in interphase, and the spindle assembles rapidly in the nucleus at the onset of mitosis. The nuclear envelope does not break down in mitosis (Robinow and Caten, 1969).

Experiments to date suggest two quite different but nonmutually exclusive models (Figure 1) for how spindle formation is initiated in organisms with closed spindles. One model (Figure 1, a–c) is suggested by work in *Schizosaccharomyces pombe* by Masuda *et al.* (1992) and Masuda and Shibata (1996). In this model, spindle assembly is controlled by a cell-cycle-regulated change in the ability of the fungal microtubule-organizing center, the spindle pole body (SPB), to nucleate microtubule assembly. Tubulin dimers would pass freely through the nuclear envelope, but there would be no microtubule assembly in interphase because there are no active microtubule nucleation sites within the nucleus. At

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Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; SPB, spindle pole body.

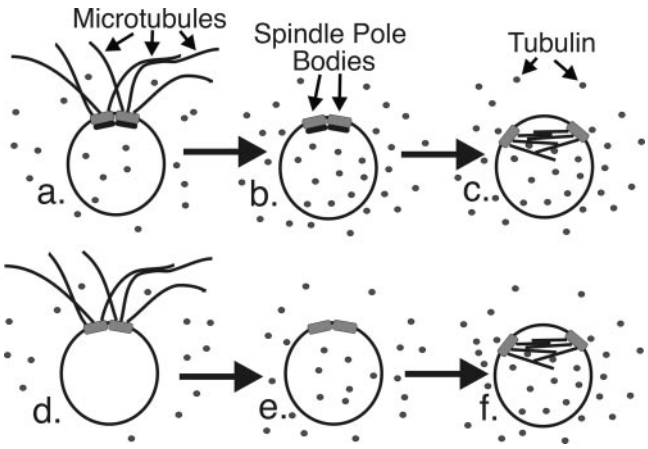

Figure 1. Two models for regulation of mitotic spindle formation in *Aspergillus*. In one model (a–c) tubulin moves freely through the nuclear envelope. The intranuclear microtubule nucleation sites are inactive. In this diagram the nucleation sites are shown as blocked (dark objects on the inside faces of the spindle pole bodies), but other mechanisms of inactivation and activation are possible. In interphase (a) cytoplasmic microtubules are in equilibrium with tubulin dimers that are present in the cytoplasm and in the nucleoplasm. As nuclei enter mitosis, cytoplasmic microtubules disassemble (b). The intranuclear microtubule nucleation sites are activated (c) allowing spindle formation between the separating spindle pole bodies. In the other model (d–f), the microtubule nucleation sites are always active, but tubulin is excluded from the nucleoplasm in interphase (d). As cytoplasmic microtubules disassemble and nuclei enter mitosis, tubulin moves into the nucleoplasm (e) allowing spindle formation (f).

the onset of mitosis the nucleation sites at the SPB are activated and spindle formation occurs. In *S. pombe*, this may be facilitated by the penetration of the SPB into a fenestra in the nuclear envelope at mitosis (Ding *et al.*, 1997).

Another model is shown in Figure 1, d–f. In this model, the SPB is capable of nucleating microtubule assembly throughout the cell cycle. Spindle formation is regulated by the concentration of tubulin in the nucleus, which is, in turn, regulated at the level of tubulin movement across the nuclear envelope. Tubulin is excluded from the nucleus at interphase, allowed to enter at the onset of mitosis and removed as the daughter nuclei transit from M to G_1. To date, there is no direct evidence for this model, but Wu *et al.* (1998) found that in *A. nidulans* the activity of the NIMA kinase is required for the translocation of the cdc2/cyclin B complex into the nucleus at the onset of mitosis and, thus for initiation of mitotic entry. These data suggest that mitotic onset could be triggered by transport of key proteins into the nucleus.

As mentioned, these models are not mutually exclusive. Both mechanisms might operate redundantly. It is also important to note that each of these mechanisms would be affected by cytoplasmic microtubule dynamics. In *A. nidulans*, for example, the rapid disassembly of cytoplasmic microtubules, which occurs as cells approach mitosis, should greatly increase the size of the tubulin pool available for incorporation into the assembling spindle.

To evaluate the model shown in Figure 1, d–f, we have attempted to determine whether tubulin is excluded from interphase nuclei and enters at the onset of mitosis. This attempt was complicated by the facts that 1) in interphase the cytoplasmic microtubule array is extensive and it is normally difficult to observe free tubulin in the cytoplasm, and 2) the G_2-to-M transition is very rapid in *A. nidulans* and it is difficult to get an accurate picture of the process from static immunofluorescence images.

We have circumvented these difficulties in two ways. First, we have used benomyl to disassemble cytoplasmic microtubules at interphase. This creates a large pool of free tubulin that can be readily observed by immunofluorescence. We find that tubulin is substantially excluded from interphase nuclei, but is present in mitotic nuclei. Second, we have observed a green fluorescent protein (GFP) α-tubulin fusion by spinning-disk confocal microscopy. Tubulin levels are low in interphase nuclei and there is a rapid movement of tubulin into the nucleoplasm seconds before spindle formation begins. Tubulin is removed from the nucleoplasm at the end of mitosis. These data provide a strong indication that regulation of intranuclear tubulin levels plays an important, perhaps essential, role in the regulation of mitotic spindle formation.

MATERIALS AND METHODS

Strains and Growth Conditions

A. nidulans strain FGSC4 (Glasgow wild-type) was used for immunofluorescence microscopy. Growth and preparation for immunofluorescence microscopy was as described previously (Ovechkina *et al.*, 1999).

Three strains were used for GFP-tubulin observations: GFP-tub7, LO716, and L0770. GFP-tub7 carries *pyro*A4, *w*A2, *pyr*G89, and the green fluorescent protein fused to the *tub*A (α-tubulin) gene (GFP-*tub*A) (Han *et al.*, 2001). GFP-*tub*A is under the control of the regulatable *alc*A promoter. LO716 is a diploid formed by fusion of GFP-tub7 with strain R21 (*y*A2, *paba*A1). It, thus, carries an inducible GFP-*tub*A fusion as well as a wild-type *tub*A. LO770 was created by crossing GFP-tub7 with a strain that carries *nim*T23. Its genotype is *w*A2, *nim*T23, *pyr*G89 and it carries the GFP-*tub*A insertion at the *tub*A gene.

For observations of GFP fluorescence, strains were grown in two ways. In some experiments they were grown in four-well Lab-Tek-chambered cover glasses with covers (Nalge Nunc, Naperville, IL). Each well contained 6×10^4 conidia (spores) inoculated into 750 μl of induction medium. Induction medium consisted of minimal medium (Pontecorvo *et al.*, 1953) with 50 mM fructose substituted for *d*-glucose and with appropriate supplements for nutritional markers. Threonine (6.25 mM) was used to induce GFP-*tubA* expression in most experiments, but 1.56, 3.13, or 12.5 mM was used in some experiments. Growth was robust under these conditions and nuclei were observed to complete mitosis normally. In most experiments, including all experiments in which GFP-*tubA* levels were quantified, strains were grown as follows. Conidia were inoculated into 1% low-melting temperature agarose containing minimal medium with 50 mM fructose and 1 mM threonine at 42°C to a concentration of 1×10^7/ml. (For reasons that are not clear, the optimum threonine concentration was lower under these conditions than in liquid cultures.) Two pieces of Scotch Magic tape were placed ~35 mm apart on a slide, and 5 μl of the molten spore suspension was placed on the slide between the pieces of tape. A 24- $\times$ 40-mm coverslip was quickly placed on the spore suspension and pressed down gently such that it rested on the tape. The agarose spread into a thin layer that did not contact the tape. This construction was placed in a Petri dish with moist paper and chilled at 4°C for 15 min to allow the agarose to solidify. It was then incubated at 30°C overnight (in the

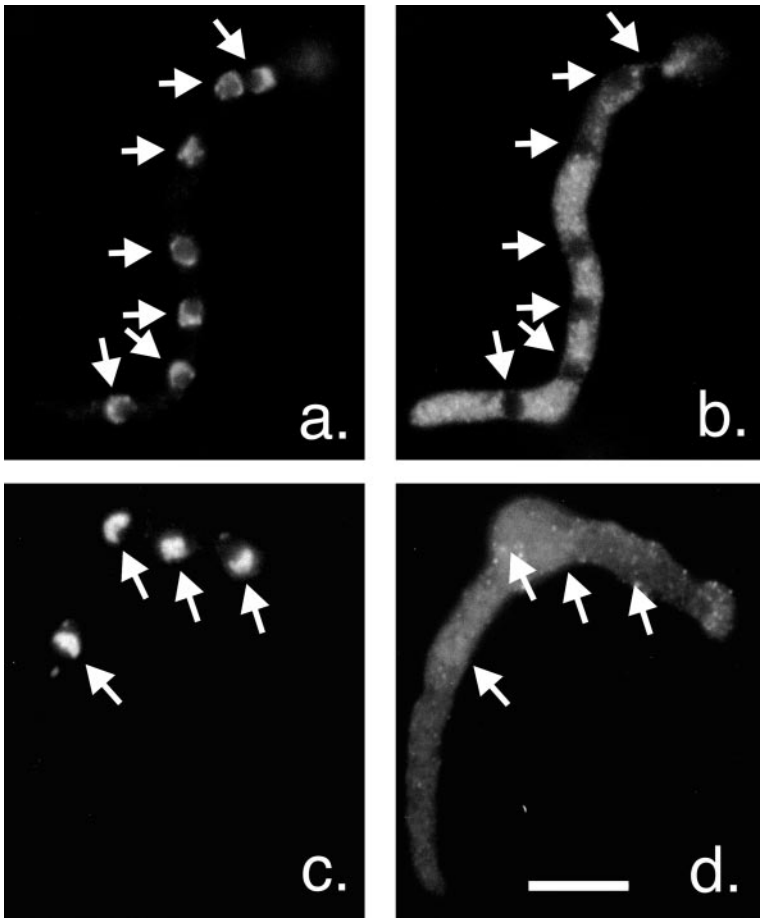

Figure 2. Interphase and mitotic benomyl-treated germlings. (a) Interphase nuclei (arrows) stained with DAPI. (b) The same germling stained with an anti-β-tubulin antibody. Benomyl has caused the cytoplasmic microtubules to disassemble, but the tubulin is visible as granular staining in the cytoplasm. Tubulin is excluded from the nucleoplasm (arrows), however. (c) DAPI staining of a mitotic germling. The chromatin in each nucleus (arrows) is condensed. (d) shows anti-β-tubulin staining. Tubulin is present in the nucleoplasm (arrows). The slightly fainter tubulin staining near the ends of the germling is due to the ends of the germlings being slightly out of the focal plane. All figures are the same magnification. Bar (d), 10 μm.

same moist chamber) before observation. Conidia near the edge of the agarose germinated and grew robustly.

Microscopy

Immunofluorescence micrographs were taken on a standard microscope (Carl Zeiss, Thornwood, NY) with a 100× Neofluor objective (1.30 numerical aperture) by using T-MAX 400 film (Eastman Kodak, Rochester, NY). The film was developed in T-MAX developer and negatives were scanned into Adobe Photoshop with a Polaroid SprintScan 35 Plus scanner. Time-lapse GFP images were taken with a 100× Plan Apochromatic objective (1.40 numerical aperture) on an Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with an Ultraview spinning-disk confocal system (PerkinElmer Life Sciences, Boston, MA) controlled by Ultraview software. Time-lapse series were saved as 16-bit tiff files. For quantitation of GFP-*tub*A fluorescence, the tiff files were imported into MetaMorph (Image 1). Composite figures were prepared using CorelDraw 8 (Macintosh).

RESULTS

Ovechkina *et al.* (1999) have shown that benomyl at 2.4 μg/ml causes a rapid disassembly of microtubules in *A. nidulans*. We have observed that the tubulin from benomyl-disassembled microtubules distributes through the cytoplasm and can be seen easily by immunofluorescence microscopy with the anti β-tubulin antibody Tu27B. Observations of material stained with 4,6-diamidino-2-phenylindole (DAPI) and Tu27B revealed that in interphase germlings there were voids in the β-tubulin staining that precisely coincided with the DAPI-stained nuclei (Figure 2, a and b). In *A. nidulans*, chromosome condensation occurs during mitosis and can be observed by DAPI staining (Jung *et al.* 1998, and earlier references therein). We observed that in benomyl-treated germlings with condensed chromosomes the tubulin was not excluded from the nucleoplasm (Figure 2, c and d). Rather, the tubulin staining through and around the chromosomes was indistinguishable from that of the remainder of the germlings. These results suggest that tubulin is substantially excluded from interphase nuclei, but not from mitotic nuclei.

To determine whether these initial observations were statistically significant, we treated germlings for 30 min with benomyl and then fixed and stained them with Tu27B and DAPI. We then scored germlings for chromosome condensation and for whether there was a tubulin-deficient void corresponding to the nucleoplasm. We scored a total of 1252 germlings in three separate experiments (Table 1). At the incubation temperature (37°C), the cell cycle for the strain used is <90 min, and 30 min of treatment with benomyl should result in ~38% of germlings being blocked in mitosis

Table 1. Correlation of condensed chromatin with tubulin inside of the nucleoplasm in germlings treated with benomyl

	Mitotic with high nuclear tubulin	Mitotic with low nuclear tubulin	Interphase with high nuclear tubulin	Interphase with low nuclear tubulin	Intermediate nuclei with intermediate tubulin
Number	472	0	0	756	24
Mean (%) ± SD	37.6 ± 4.93	0	0	60.5 ± 5.58	1.9 ± 0.65

Nuclei in germlings were scored as mitotic if the chromatin was condensed as detected by DAPI staining. Intermediate nuclei had chromatin that was partially condensed. Tubulin was visualized by immunofluorescence with an anti-β-tubulin antibody. A total of 1252 germlings were scored in three experiments. The total number of germlings with nuclei in each category is given in row two (sum of the three experiments), and percentage of total (mean and sample standard deviation) is given in the third row.

(5% already in mitosis when the benomyl was added plus 33% that enter mitosis in one-third of the cell cycle). We found that, as expected, ~38% of the germlings had nuclei with condensed chromatin. As Table 1 shows, there was a remarkable correlation between chromosomal condensation and the presence of tubulin in the nucleoplasm. In all 472 of the germlings with nuclei with condensed chromosomes, tubulin was present in the nucleoplasm at levels at least as high as that in the surrounding cytoplasm. In all 756 of the germlings with interphase nuclei (noncondensed chromatin), tubulin fluorescence in the nucleoplasm was visibly much lower than that in the surrounding cytoplasm. In 24 of the germlings, chromatin was partially condensed and there was an intermediate level of tubulin in the nucleoplasm. Because the benomyl block was in place for much less than a single cell cycle, we can infer with confidence that the nuclei with partially condensed chromatin were entering mitosis rather than exiting. Because intermediate tubulin levels correlate with partially condensed chromatin, we can also deduce that tubulin movement into the nucleoplasm occurs at the same time that condensation occurs, in prophase.

To confirm and expand these experiments we observed a GFP α-tubulin fusion protein in living hyphae. For brevity, we will refer to tubulin dimers containing the GFP α-tubulin fusion protein as GFP-tubulin. The brightness of the GFP-tubulin fluorescence varied to some extent among hyphae, but microtubule arrays were clear. There was also a general GFP-tubulin fluorescence in the cytoplasm. Because tubulin dimers are known to exist in equilibrium with microtubule polymer, we are confident that the general GFP-tubulin fluorescence is due to tubulin dimers in the cytoplasm. In these hyphae, nuclei could be distinguished as volumes with reduced GFP-tubulin fluorescence. These volumes could be reliably identified as nuclei because of their sizes, shape, number, and location and the fact that, in many cases, cytoplasmic microtubules extended from a point on the nuclear surface (the SPB) (our unpublished data).

We observed nuclei entering mitosis in two ways. For most experiments, we took advantage of the facts that there are multiple nuclei in each cell (or, more properly, hyphal segment) in *A. nidulans* and that nuclei in a hyphal segment enter mitosis at almost, but not quite, the same time. The nucleus at one end of the hyphal segment generally goes into mitosis first, the one next to it goes in second, and the other nuclei enter sequentially until all nuclei are eventually in mitosis. We searched for hyphae in which nuclei at one end of a hyphal segment were in the early stages of mitosis and in which nuclei at the other end were still in G_2. We then took time-lapse images of the G_2 nuclei entering mitosis (Figure 3) by using a spinning-disk confocal microscope. The spinning-disk confocal microscope was particularly useful for these studies because it removed most of the signal from GFP-tubulin above and below the nucleus, and it allowed us to capture images rapidly and with little bleaching.

In a second approach, we used a temperature-sensitive mutation to synchronize cells. We prepared a strain (LO770) carrying *nim*T23 and the GFP α-tubulin fusion. *nim*T23 is a temperature-sensitive mutation in the phosphatase that regulates $p34^{cdc2}$ activity. It blocks the cell cycle in late G_2 at 42°C and is rapidly reversible, allowing entry into mitosis shortly after a shift to a permissive temperature (O'Connell *et al.*, 1992; Martin *et al.*, 1997). To observe entry into mitosis, we grew germlings overnight at 32°C and then blocked cells in G_2 by incubating at 43°C for 3 h. We released the block by placing the chambers containing the germlings on the microscope stage at room temperature (~24°C). We then took time-lapse images of cells entering and carrying out mitosis. The two approaches gave similar results, but the first approach allowed us to acquire data more rapidly.

As mentioned, interphase nuclei were visible as GFP-tubulin–deficient volumes (Figure 3a, nuclei 3 and 4, for example). They became particularly obvious as cytoplasmic microtubules began to disassemble before mitosis increasing the pool of cytoplasmic tubulin (Figure 3a, nuclei 3 and 4). As nuclei approached mitosis, there was a sudden movement of tubulin into the nucleoplasm (Figure 3, and accompanying movie Ovetne.mov) followed shortly by the initiation of mitotic spindle formation (Figure 3, f and k, arrows).

To estimate the timing and extent of tubulin movement into the nucleus, we quantified GFP-tubulin fluorescence in 10 nuclei in eight hyphae and measured the changes in these values over time. We initially considered measuring GFP-tubulin intensity in the nucleus relative to an area identical in size and shape in the cytoplasm. We noted, however, that GFP-tubulin intensity was not uniform through the cytoplasm (Figures 3 and 4). We presume that the lack of uniformity is due, at least in part, to organelles such as mitochondria and vesicles that exclude tubulin. Because the cytoplasmic GFP-tubulin intensity depended on the region of the cytoplasm chosen, this approach did not provide an objective measure of cytoplasmic GFP-tubulin levels. We also noted that variation in GFP-tubulin intensity among hyphae and differences in exposure conditions among ex-

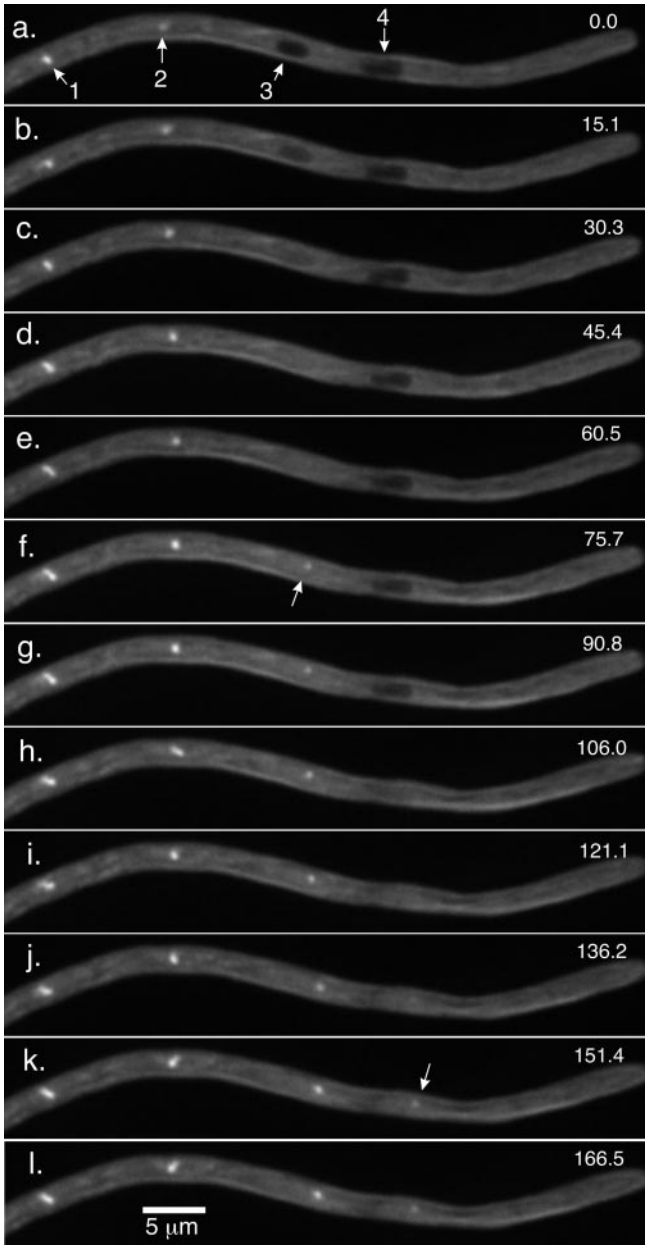

Figure 3. GFP-tubulin movement into nuclei at the onset of mitosis. These images are from a time-lapse series taken with a spinning-disk confocal microscope. Time (in seconds) is shown at the upper right of each panel. The images are of a portion of a growing hyphal tip cell and a wave of mitosis is proceeding from left to right (i.e., toward the hyphal tip). Most cytoplasmic microtubules have disassembled as the cell entered mitosis. Four nuclei are present. (a) Spindles are present in nuclei 1 and 2, whereas nuclei 3 and 4 are in interphase. The tip grew slightly toward the right during the time course of the experiment and nuclei 3 and 4 moved slightly toward the growing hyphal tip. Nucleus 3 is visible as a tubulin-deficient region in a. It fills with GFP-tubulin in b–e and a spindle is visible in f (arrow). Nucleus 4 fills with GFP-tubulin in g–j and a spindle is visible in k (arrow). The spindles in nuclei 3 and 4 form at the anterior (tip side) of the nucleus, a fact that is consistent with our observations that spindle pole bodies are normally located on the leading sides of migrating nuclei.

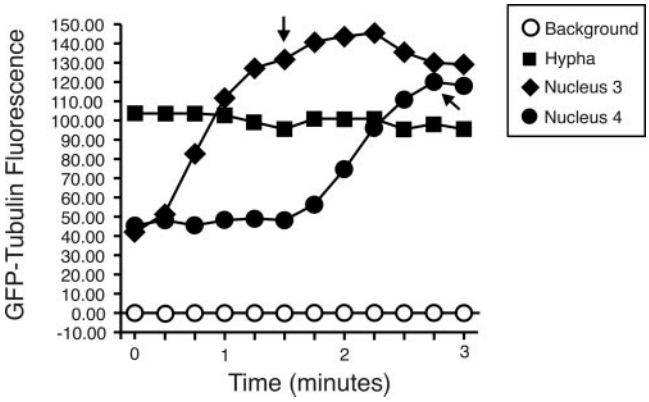

Figure 4. Quantitation of GFP-tubulin movement into nuclei at the onset of mitosis. This figure quantifies the images shown in Figure 3. As discussed in the text, the average GFP-tubulin fluorescence signal intensity for the hypha was assigned the value 100, and the average signal intensity of an identically shaped background area away from the hypha was assigned the value 0. Hyphal GFP-fluorescence faded very slightly during the course of the experiment, and there was almost no change in background fluorescence. The GFP-tubulin fluorescence in the two nuclei was <50% of the hyphal average before they entered mitosis. Nucleus 3 entered mitosis first. It took ~1 min for the nucleus to fill with GFP-tubulin and a spindle was visible at the next time point (arrow). Nucleus 4 entered mitosis later but the timing of GFP-tubulin entry into the nucleus and spindle formation was similar to that of nucleus 3. In both nuclei, the GFP-tubulin fluorescence reached a value greater than the hyphal average before spindle formation was observed.

periments affected the absolute GFP fluorescence values. To circumvent these potential problems, we determined the GFP-tubulin fluorescence of the nuclei relative to the background outside the hypha and to the average GFP-tubulin fluorescence of the entire hypha under consideration. We first determined the average GFP-tubulin signal intensity for the hypha over a period starting before the tubulin began to move into the nucleus and ending with spindle formation. This average was assigned the value 100. (In instances in which two nuclei entered mitosis, the end point was the formation of the second spindle.) We next determined the average signal intensity over the same period for a background region identical in size and shape to the hypha. The signal intensity of the background was remarkably uniform so the positioning of the background region did not alter the average intensity significantly. The average background intensity was assigned the value 0. A region was then selected within each nucleus and the average intensity of the region was measured at each time point. This approach provides an objective measurement of the GFP-tubulin levels in the nucleus relative to the hyphal GFP-tubulin levels. One point that needs to be noted, however, is that because tubulin is almost certainly excluded from mitochondria, vesicles, and nuclei, the cytosolic GFP-tubulin is confined to a volume somewhat smaller than the entire hypha and the cytosolic GFP-tubulin levels are higher than the hyphal average. If, for example, the organelles that exclude GFP-tubulin make up 20% of the hyphal volume, all the GFP-tubulin will be in the remaining 80% of the hyphal volume. The true cytosolic

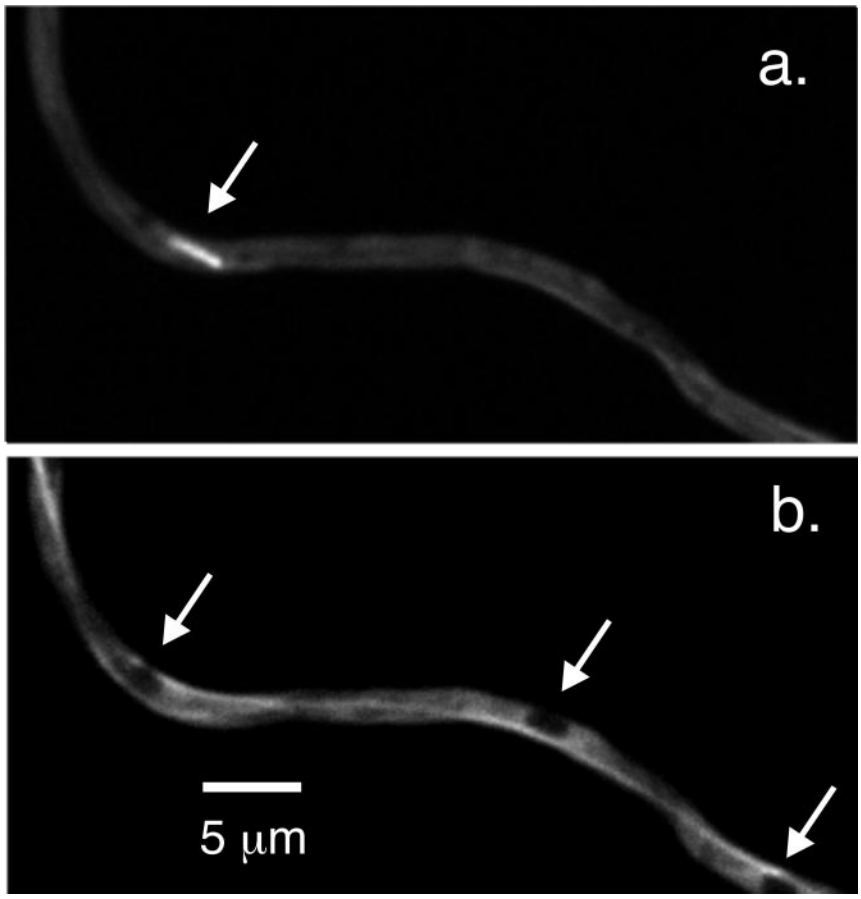

Figure 5. Tubulin removal from nuclei at the M-to-G_1 transition. Images are from a time-lapse series showing GFP-tubulin taken with a spinning disk confocal microscope. (a) A mitotic hypha. One mitotic spindle is visible (arrow) and a second spindle is out of the plane of focus. (b) The same hypha ~11.5 min later. Three G_1 nuclei are visible (arrows) and clearly have reduced GFP-tubulin fluorescence relative to the surrounding cytoplasm. Tubulin has clearly been removed from nuclei as they enter G_1. Cytoplasmic microtubules are also visible. The background GFP tubulin fluorescence seems to be more intense in b, but this is due to the fact that these are 12-bit images scaled to 8 bits for publication. The spindle in panel a is ~50 times brighter than the cytoplasmic microtubules in b so when the images are scaled to 8 bits the cytoplasmic GFP-tubulin fluorescence in a seems less intense.

GFP-tubulin levels would thus be 1.00/0.80 = 1.25 or 125% of the hyphal average. The approach we have used, thus, provides an accurate way of measuring the relative concentrations of intranuclear GFP-tubulin in interphase and mitotic nuclei and the changes in these concentrations as nuclei enter and exit mitosis. This approach does not, however, provide an accurate estimate of nuclear GFP-tubulin relative to the surrounding cytosol. Rather, the values we obtain will tend to overstate nuclear GFP-tubulin concentrations (mitotic and interphase) to some extent. Thus, if the GFP-tubulin level in an interphase nucleus was 50% of the hyphal average, it might actually be 30% of the level in the surrounding cytosol.

The results for the nuclei shown in Figure 3 are quantified in Figure 4. In this example, the GFP-tubulin fluorescence level in the interphase nuclei was <50% of the level of the average hyphal intensity. As each nucleus entered mitosis, there was a rapid increase in the GFP-tubulin fluorescence. In less than one minute the nuclear GFP-tubulin fluorescence exceeded that of the hyphal average and the mitotic spindle became visible in the next image which was taken ~15 s later.

In the 10 nuclei in which GFP-tubulin fluorescence was quantified, the interphase nuclear GFP-tubulin fluorescence averaged 42.8 ± 8.0% (mean ± SD) of the hyphal average. Because the spinning-disk confocal microscope removes most, but not all, out of focus fluorescence, this value probably represents an overestimate of the amount of GFP-tubulin in the nucleus. The maximum GFP-tubulin fluorescence in the nuclei reached 126 ± 16.1% of the average hyphal intensity before spindles became visible. The intranuclear tubulin level thus increases by a factor of three at mitotic onset. The time from interphase until the nuclei filled with GFP-tubulin was 1.68 ± 0.99 min. The time from interphase until spindle formation was observed was 2.25 ± 1.25 min. The time from the nuclei being filled until visible spindle formation was 0.58 ± 0.43 min (34.5 ± 25.5 s). Because some spindles probably began to form out of the plane of focus, and were not immediately visible, this value is probably an overestimate.

At the end of mitosis, it was apparent that GFP-tubulin was removed from nuclei (Figure 5). G_1 nuclei are smaller than G_2 nuclei, and move rapidly around the hyphae and in and out of the plane of focus so it was not possible to track telophase/G_1 nuclei well enough to determine a precise time course for GFP-tubulin removal. Nuclei were clearly visible, however, as tubulin-deficient volumes by 4 min after mitotic spindle breakdown.

DISCUSSION

Successful cell replication depends on a precise control of the assembly of the mitotic apparatus by the cell cycle reg-

ulatory machinery. This control is of obvious importance but is incompletely understood. Our data demonstrate that, in *A. nidulans*, tubulin levels are low in interphase nuclei. At mitotic onset, tubulin moves rapidly into the nucleus reaching a level 3 times greater than in interphase and the spindle begins to assemble seconds later. Tubulin is removed rapidly at the end of mitosis after spindle disassembly. These data raise the obvious possibility that regulation of tubulin movement into the nucleoplasm is important for the regulation of mitotic spindle formation. If tubulin in interphase nuclei is below the critical concentration for microtubule assembly, spindle microtubules could not assemble even if SPBs were capable of nucleating microtubule assembly. The influx of tubulin at the onset of mitosis would increase the concentration such that microtubules could assemble. Two additional points are worth making in this regard. First, it seems unlikely that mechanisms for controlling tubulin movement into and/or out of the nucleus would have evolved if they were not functionally significant. Second, as mentioned, it is quite possible that spindle formation is regulated both by tubulin movement in and out of the nucleus and by changes in the ability of the SPB to nucleate microtubule assembly. This would provide redundant mechanisms for preventing spindle formation except at the right point in the cell cycle.

Two general models for the mechanism of movement of tubulin into and out of the nucleoplasm have occurred to us. One is that there is an active transport mechanism that moves tubulin into or out of the nucleus. For example, tubulin could be moved into the nucleus by an active transport mechanism that would be inactive in interphase and would be activated at the G_2-to-M transition. A search (Columbia University Bioinformatics Center, http://cubic.bioc.columbia.edu/) revealed no nuclear localization sequences in any of the *A. nidulans* α- and β-tubulin sequences. Any active transport mechanism would, thus, require that tubulin form a complex with one or more proteins that have nuclear localization signals. This has been shown to occur for γ-tubulin (Pereira *et al.*, 1998). A variant of this model is that tubulin is actively removed from interphase nuclei by a nuclear export mechanism that is inactivated at mitotic onset and reactivated at the G_2-to-M transition.

A second model is that the nuclear envelope becomes more permeable at the G_2-to-M transition. Tubulin and other proteins would, thus, be free to diffuse into the nucleoplasm during mitosis. Although electron microscopy demonstrates that the nuclear envelope does not break down in mitosis in *A. nidulans*, it is certainly possible that its permeability characteristics could be altered at the onset of mitosis.

The notion that the nuclear envelope becomes transiently permeable in mitosis is attractive because it provides an economical mitotic regulatory mechanism. Tubulin, the cdc2/cyclin B complex (see below) and other proteins required for mitosis might be excluded from interphase nuclei. At mitotic onset a single event, the alteration of nuclear envelope permeability, would allow these proteins to flood into the nucleoplasm while mitotic inhibitor proteins might diffuse out. At the end of mitosis the nuclear envelope would again become impermeable and proteins involved in mitosis would be removed. This model suggests that mitosis in fungi is more similar to that of higher organisms than previously thought and that the presence of a morphologically intact nuclear envelope in mitosis may be less significant than previously thought.

At present, there is insufficient data to determine which of these models is correct. Our GFP-tubulin quantitation data might seem to suggest that the movement into the nucleus is active. In all 10 nuclei examined, the GFP-tubulin in the nucleus reached a level higher than that of the remainder of the hypha before spindle formation (mean value 126 $\pm$ 16.1% of the average hyphal value). If tubulin were diffusing passively into the nucleoplasm, one would not expect the GFP-tubulin concentration in the nucleoplasm to exceed that of the cytoplasm. As mentioned, however, the cytosolic GFP-tubulin concentration must be higher than the hyphal average because GFP-tubulin is excluded from many organelles and the hyphal average would include the cytosolic GFP-tubulin as well as the regions from which it is excluded. It is, thus, quite possible that the GFP-tubulin level in the nucleus is simply reaching the cytosolic level, not exceeding it, and that the movement is passive.

Information on whether the nuclear envelope becomes permeable at mitotic onset is limited at present. An obvious experiment would be to determine whether GFP or other fluorescent proteins (not coupled to tubulin or any other protein) enter the nucleus at mitotic onset. GFP is not excluded from the nucleoplasm of interphase nuclei of *A. nidulans* (Fernandez-Abalos *et al.*, 1998), however, and neither is DS red fluorescent protein (our unpublished data). In the fungi *Fusarium ventricilloides* and *Magneporthe grisea*, however, another fluorescent protein, ZsGreen, is excluded from interphase nuclei and enters at mitosis (Bourett *et al.*, 2002). In *A. nidulans*, a GFP fusion to a portion of the putative transcription factor *stuA* that carries a nuclear localization signal localizes to the nucleoplasm in interphase, and at mitosis it leaves the nucleoplasm (Suelmann *et al.*, 1997). Finally, interphase nuclei of living *A. nidulans* hyphae are visible by phase contrast microscopy, but they become nearly invisible in mitosis (Robinow and Caten, 1969). This indicates that the refractive index of the nucleoplasm changes upon mitotic entry to match that of the surrounding cytoplasm. Although limited, these data are all consistent with the possibility that the nuclear envelope becomes permeable in mitosis.

Regardless of the mechanism of tubulin movement into the nucleus, it seems that there must be a rapid, active mechanism to remove tubulin from the nucleoplasm at the end of mitosis. It is difficult to see how it could be removed by passive diffusion. The removal mechanism could involve proteolytic degradation of tubulin or export back to the cytoplasm.

Because movement of tubulin into and out of the nucleoplasm is timed so precisely with respect to the cell cycle, we can infer that it is controlled by the cell cycle regulatory machinery. At present, we know, from our observations with GFP-tubulin, that it occurs downstream of cdc2 activation. *nim*T is required for cdc2 activation and at the *nim*T23 block point cdc2 is inactive and tubulin is excluded from the nucleoplasm. When the block is released by a shift to permissive temperature, tubulin moves into the nucleoplasm and the spindle assembles shortly afterward.

Could the changes in intranuclear tubulin levels be artifactual? Our evidence suggests strongly that this is not the case. First, two quite different procedures, immunofluores-

cence with an antibody against β-tubulin and time-lapse imaging of GFP-tubulin, provide mutually supportive evidence that there is a rapid influx of tubulin into the nucleus at the onset of mitosis and removal at the M-to-G_1 transition. Second, the apparent exclusion of tubulin from interphase nuclei is not simply due to the fact that a large fraction of the tubulin dimers is tied up in cytoplasmic microtubules because even when cytoplasmic microtubules are disassembled completely by benomyl, tubulin levels in interphase nuclei are low. Third, the exclusion is not simply a displacement of tubulin by chromatin. Our observations with immunofluorescence reveal that tubulin is uniformly distributed through mitotic nuclei and is not displaced by mitotic chromatin, which is more condensed than interphase chromatin. In addition, when GFP-tubulin moves into nuclei, there is a brief period before spindle formation when it is distributed uniformly through the nucleoplasm and is clearly not displaced by chromatin. Finally, GFP alone is not excluded from interphase nuclei (Fernandez-Abalos *et al.*, 1998), so tubulin is responsible for the behavior of the GFP-tubulin, not the GFP moiety.

It is important to note that three proteins or protein complexes important to mitosis, the tubulin dimer, the cdc2/cyclin B complex (Wu *et al.*, 1998), and the NIMA kinase (De Souza *et al.*, 2000; De Souza and Osmani, unpublished data) have now been shown to enter the nucleoplasm at the G_2-to-M transition. It is tempting to speculate that this may be true for other proteins required for mitosis and that regulation of protein movement across the nuclear envelope may be a general regulatory mechanism for the G_2-to-M and M-to-G_1 transitions in *A. nidulans* and other organisms with intranuclear mitosis.

Could similar mechanisms operate in other organisms? In many fungi and protists, mitosis is intranuclear and microtubules are only present in the nucleus for the short period required for mitosis (mitosis in protists reviewed by Heath, 1980). In these organisms it is quite possible that regulation of the movement of tubulin and other proteins into and out of the nucleus is an important switch for regulating the progression into and out of mitosis. In other fungi, including *Saccharomyces cerevisiae*, some microtubules are present in the nucleus for most of the cell cycle (Byers and Goetsch, 1975; Heath, 1994). There is, thus, no sudden switching on of tubulin movement into the nucleus. In these fungi, however, there is an increase in microtubule number and length as nuclei pass through the cell cycle, and this could be regulated by a more gradual change of tubulin levels in the nucleus.

Our findings may establish an evolutionary context for recent findings on the roles of importins and the GTPase Ran in spindle formation. Importins are involved in transport of proteins into the nucleus (Mattaj and Englmeier, 1998; Weis, 1998; Gorlich and Kutay, 1999). They form complexes with cargo proteins in the cytoplasm and transport them through nuclear pores. Once in the nucleus the cargo proteins are released by interaction of importins with RanGTP. It has been known for some time that in many animal cells chromatin can stabilize microtubules (Zhang and Nicklas, 1995, and earlier references therein). More recently it has been shown that RanGTP promotes spindle formation (Nakamura *et al.*, 1998; Ohba *et al.*, 1999; Wilde and Zheng, 1999). The likely mechanism is that proteins that are required for spindle formation, including those that stabilize microtubules, are bound to importins, such that they are inactive. RanGTP causes their release and this activates them allowing them to promote spindle formation (Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001). The RanGTP concentration is highest near chromatin because its guanine nucleotide exchange factor, RCC1, is associated with chromatin (Ohtsubo *et al.*, 1989). An obvious question raised by these findings is why the nuclear transport machinery is used in the regulation of spindle formation. Because mitosis is intranuclear in many protists, it is likely that it was intranuclear in ancestral eukaryotes. One possibility raised by our finding is that tubulin is actively transported into the nucleus in *A. nidulans* by nuclear transport proteins. If nuclear transport is important to the regulation of spindle formation in organisms with intranuclear spindles, it follows that when nuclear envelope breakdown evolved, nuclear transport proteins retained an important role in the regulation of spindle formation.

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