

The dynamic behaviour of microtubules and their contributions to hyphal tip growth in *Aspergillus nidulans*

Karina Sampson† and I. Brent Heath

Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

Correspondence

Karina Sampson
ksampson@bio.umass.edu

Creating and maintaining cell polarity are complex processes that are not fully understood. Fungal hyphal tip growth is a highly polarized and dynamic process involving both F-actin and microtubules (MTs), but the behaviour and roles of the latter are unclear. To address this issue, MT dynamics and subunit distribution were analysed in a strain of *Aspergillus nidulans* expressing GFP- α -tubulin. Apical MTs are the most dynamic, the bulk of which move tipwards from multiple subapical spindle pole bodies, the only clear region of microtubule nucleation detected. MTs populate the apex predominantly by elongation at rates about three times faster than tip extension. This polymerization was facilitated by the tipward migration of MT subunits, which generated a tip-high gradient. Subapical regions of apical cells showed variable tubulin subunit distributions, without tipward flow, while subapical cells showed even tubulin subunit distribution and low MT dynamics. Short MTs, of a similar size to those reported in axons, also occasionally slid into the apex. During mitosis in apical cells, MT populations at the tip varied. Cells with less distance between the tip and the first nucleus were more likely to lose normal MT populations and dynamics. Reduced MTs in the tip, during mitosis or after exposure to the MT inhibitor carbendazim (MBC), generally correlated with reduced, but continuing growth and near-normal tip morphology. In contrast, the actin-disrupting agent latrunculin B reduced growth rates much more severely and dramatically distorted tip morphology. These results suggest substantial independence between MTs and hyphal tip growth and a more essential role for F-actin. Among MT-dependent processes possibly contributing to tip growth is the transportation of vesicles. However, preliminary ultrastructural data indicated a lack of direct MT–organelle interactions. It is suggested that the population of dynamic apical MTs enhance migration of the 'cytomatrix', thus ensuring that organelles and proteins maintain proximity to the constantly elongating tip.

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INTRODUCTION

The integrated mechanisms involved in creating and maintaining cell polarity are highly complex. The cytoskeleton, in particular actin filaments (F-actin) and microtubules (MTs), has been linked to cell polarity in numerous cell types (Ahringer, 2003; Heath, 1994; Hepler *et al.*, 2001; Mata & Nurse, 1998; Sawin & Nurse, 1998). Tip growth, as seen in fungal hyphae, moss protonemata, pollen tubes and root

hairs, is a highly polarized process with extension occurring purely at the apex (Heath, 1990; Hepler *et al.*, 2001). F-actin is a vital component of tip growth, being concentrated at the apex and with growth disruption resulting from exposure to anti-actin drugs (Bachewich & Heath, 1998; Geitmann & Emons, 2000; Heath, 2000; Heath *et al.*, 2000; Ketelaar *et al.*, 2003; Srinivasan *et al.*, 1996; Vidali & Hepler, 2001).

In fungal hyphae, the roles of MTs are less clear (Heath, 1990; Hepler *et al.*, 2001; Srinivasan *et al.*, 1996). MTs are often seen at the apex (Han *et al.*, 2001; Hoch & Staples, 1985; McDaniel & Roberson, 1998; Minke *et al.*, 1999; Roberson & Fuller, 1988; Torralba *et al.*, 1998), but tip growth after MT depolymerization has been reported (deLucas *et al.*, 1993; Howard & Aist, 1980; Jochova *et al.*, 1993; Peterbauer *et al.*, 1992; Raudaskoski *et al.*, 1994; Rupes *et al.*, 1995; Temperli *et al.*, 1991; That *et al.*, 1988; Torralba *et al.*, 1996). However, anti-MT agents also cause reduced

†Present address: Department of Biology, University of Massachusetts, Amherst, MA 01003, USA.

Abbreviations: DIC, differential interference contrast; lat B, latrunculin B; MBC, methyl benzimidazole-2-yl carbamate (carbendazim); MT, microtubule; SPB, spindle pole body.

Micrographs showing no evidence of MT fluxing/treadmilling and graphs of cytoplasmic fluorescence intensity gradients are available as supplementary material with the online version of this paper.

growth rates (Akashi *et al.*, 1994; Jochova *et al.*, 1993; Niini & Raudaskoski, 1993; Pedregosa *et al.*, 1995; Temperli *et al.*, 1991; Torralba *et al.*, 1996), altered cell shape (Howard & Aist, 1980; Rupes *et al.*, 1995), tip swelling (Rupes *et al.*, 1995; Torralba *et al.*, 1998), uncharacteristic branching (Raudaskoski *et al.*, 1994; Rupes *et al.*, 1995; Torralba *et al.*, 1998), aberrant secretion of enzymes (deLucas *et al.*, 1993; Jochova *et al.*, 1993; Pedregosa *et al.*, 1995; Torralba *et al.*, 1996), the gradual disappearance of the Spitzenkörper (a region of densely packed vesicles; Howard & Aist, 1977) and abnormal vesicle distribution (Howard & Aist, 1980; Rupes *et al.*, 1995; Torralba *et al.*, 1998). However, as the above studies involved exposure for periods (2 h to 3 days) sufficient to affect the number and/or position of nuclei within apical cells, the effects could be a result of aberrant nuclear populations. Furthermore, the depolymerization of MTs has been reported to disrupt the actin network in *Candida albicans* (Akashi *et al.*, 1994), so indirect effects of MT disruption on actin-based systems are also possible. As fungal actin networks are notoriously hard to visualize (Heath, 2000) and monitor, this possibility may be difficult to verify. In addition, irrespective of the possible roles of apical MTs, there are very few data on the mechanisms by which their populations are generated and maintained in the dynamic growing apex.

Using confocal microscopy, we monitored MT dynamics in a strain of *Aspergillus nidulans* expressing GFP- α -tubulin. The aims were to determine how the apical population of MTs is generated and maintained and to what extent they contribute to tip growth.

METHODS

Fungal organisms, culture conditions and preparation for light microscopy. *A. nidulans* LO1052 (a strain expressing GFP- α -tubulin, a kind gift from Dr Berl Oakley, Ohio State University, OH, USA) was grown in liquid minimal medium [l^{-1} : 10 g glucose, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 g $NaNO_3$, 0.52 g KCl, 1.52 g KH_2PO_4 , 2.0 mg EDTA, 0.88 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $MnCl_2$, 0.064 mg $CoCl_2 \cdot 6H_2O$, 0.063 mg $CuSO_4 \cdot 5H_2O$, 0.044 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.29 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $FeSO_4 \cdot 7H_2O$] plus 1.0 mg *p*-aminobenzoic acid l^{-1} (personal communication, Elizabeth Oakley, Ohio State University, OH, USA). Small colonies were collected and mounted in the above medium on slides or in microchambers (Heath, 1988). Hyphal tips were observed at $\sim 22^\circ C$ after a recovery period of 10–30 min.

Confocal microscopy and photobleaching

MT observation. GFP-tubulin was visualized with an Olympus Fluoview 300 confocal microscope, a 40 mW multiline argon ion laser and a 60 $\times$ oil-immersion (Plan Apo, 1.4 NA) objective. During image acquisition, laser power was set to 1–3% to minimize photobleaching of the fluorophore and photodamage to the cell. The confocal aperture was kept in position 3 to increase the depth of field. Images were captured using the Fluoview FV300 tiempo (version 4.1) software at a rate of 1 frame s^{-1} for a minimum of 160 s. MTs were also observed via the GFP-tubulin using epifluorescence optics (with a BI filter set: excitation 450–495 nm, dichroic mirror 510 nm, barrier >520 nm). All values given in the text are arithmetic means $\pm$ SD.

Photobleaching experiments. GFP-tubulin was visualized as above, for 2 s, and then a region of interest (mean area $17.74 \pm 9.55 \mu m^2$, using the Fluoview FV300 software) was bleached for 60 s using the laser at 100%. Each cell was only photobleached once and was followed for a minimum of 130 s after bleaching.

Confocal image analysis and processing

Image-Pro Plus software (Media Cybernetics) was used to measure MT length and calculate MT growth rates. MT catastrophe (transition from growth to shrinkage) and rescue (transition from shrinkage to growth) values were defined as the number of incidents recorded over the length of time for which the cell was observed. Each MT within a sequence was monitored and transitions from growth to shrinkage or shrinkage to growth noted. A filament was considered to be growing or shrinking if it showed an obvious change in length for more than 3 s. Image-Pro Plus software was used to make fluorescence intensity measurements.

Measurement of changes in cytoplasmic tubulin subunit gradient over time. The intensity line-plot function was used to measure changes in cytoplasmic tubulin subunit gradients over time following photobleaching. Ten parallel, longitudinal, regularly spaced line-plots spanning the bleached region were collected from several frames of a time series along the length of hyphae. These lines avoided regions occupied by MTs. The ten line-plots were averaged for each time period and their gradients were calculated using linear regression and then plotted over time.

The cytoplasmic GFP-tubulin gradients were also determined in a number of dividing cells. Several frames out of a single time series were processed as above, with the ten parallel line-plot functions being taken down the length of the hypha, between the tip and the nearest spindle.

Measurement of cytoplasmic fluorescence intensity near to nuclei. The mean fluorescence intensity of the area 1 μm from the spindle of the most apical dividing or interphase nucleus was measured using the mean of five adjacent parallel transverse line-plots taken from the region 1 μm tipward of the spindle or nucleus (see Fig. 6).

Control experiments for recognition of nuclei. Hyphae were fixed in 8% formaldehyde in PEM (60 mM PIPES/NaOH, 5 mM EGTA, 5 mM $MgCl_2$, pH 7.0) buffer for 40 min, rinsed in buffer alone for 30 min, stained for 5 min in 10 μg DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Sigma) ml^{-1} in PEM buffer, rinsed in buffer for 40 min and then mounted in buffer on a slide. Hyphae were observed using a Reichert Polyvar microscope with Nomarski differential interference contrast (DIC) and epifluorescence optics, a 100 $\times$, NA 1.32 objective, a 2 $\times$ intermediate lens and a 5 $\times$ camera tube lens. A U1 filter set (excitation 330–380 nm, dichroic mirror 420 nm, barrier >418 nm) was used for visualizing DAPI-stained nuclei. Images were captured using a Princeton Instruments MicroMax camera and WinView software.

Growth rate measurements. Hyphal tips were observed using the Polyvar DIC system, which gave a final magnification of 8500 $\times$ on the screen, a single pixel representing 0.03 μm . Images were collected at a rate of one frame every 5 min for 15–20 min (for drug effects) or one frame every 3 min for 6–12 min (for mitotic hyphae). Growth rates for each interval were calculated and then averaged.

Drug experiments

Drugs used. Carbendazim (also known as MBC, methyl benzimidazole-2-yl carbamate; Sigma-Aldrich) was dissolved in

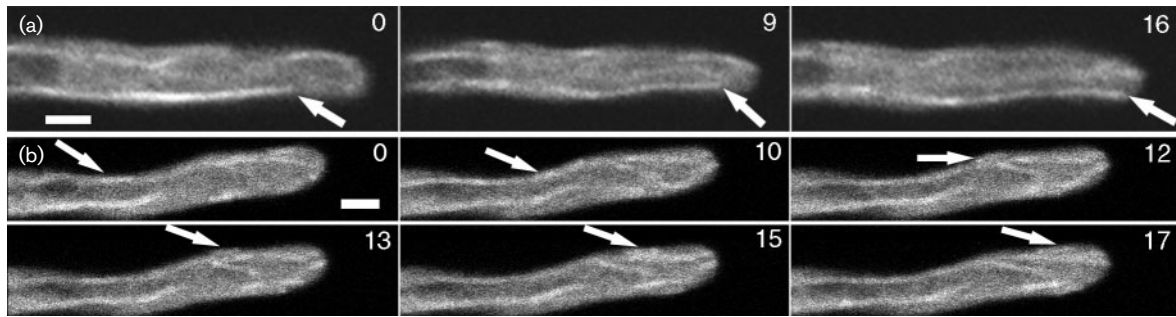


Fig. 1. MT growth and sliding in hyphal tips. (a) MT growth visualized via GFP-tubulin *in vivo*. Arrows point to an elongating MT. (b) MT sliding visualized via GFP-tubulin *in vivo*. Arrows point to an MT fragment that moves tipwards. Time (in seconds) is displayed in each frame. Bars, $\sim 2 \mu\text{m}$.

DMSO at 10 mg ml^{-1} and then stored at -20°C in the dark. This stock was diluted with culture medium to $2.5 \mu\text{g ml}^{-1}$ on the day of the experiment. Latrunculin B (lat B; CN Biosciences) was similarly used at 1 mg ml^{-1} (stock) and $20 \mu\text{g ml}^{-1}$ (working concentration).

Monitoring MT inhibition and recovery. Small colonies were mounted in medium in a microchamber, MTs were recorded and then MBC was added. MT dynamics were monitored for 15–20 min, the drug solution was then washed out with two to four times the chamber's volume of fresh medium and MT recovery was monitored.

Drug effects on growth rates. Small colonies were mounted in medium in a microchamber and growth was measured for 15 min each before, during and after drug administration. Controls substituted medium for drug solution.

RESULTS

In order to understand the way in which MTs populate growing hyphae and contribute to the growth processes, we analysed MT dynamics and subunit pools in functionally different regions of the hyphae, exploiting the natural modulation of MT populations accompanying mitosis and experimentally manipulating them with MBC.

Hyphal tips are populated by highly dynamic MTs

Approximate correlations between the numbers of MTs seen via fluorescence microscopy and TEM serial sectioning (unpublished observations) indicate that all MTs were revealed by fluorescence microscopy. Image acquisition did

Table 1. Characteristics of MT dynamics for different regions within the hypha

Values are means $\pm$ SD. The number of hyphae observed is given in parentheses; if there are two numbers in parentheses, the first is the total number of growing MT observed and the second is the number of hyphae observed. Unless specified, cells used in counts were in interphase. NO, Never observed.

Characteristic	Tip	Region of a hyphal branch	Between 1st and 2nd nuclei in the apical cell	Between nuclei of subapical cells	Tip of dividing cells*
New MT initiations min^{-1} (<i>n</i>)	3.19 ± 0.66 (20)	1.84 ± 0.43 (10)	1.96 ± 0.66 (15)	1.30 ± 0.50 (6)	0.85 ± 0.48 (10)
MT dynamics (%) directed:					
Tipwards	98.0 ± 4.0 (20)	94.0 ± 19.8 (10)	84.5 ± 17.2 (15)	54.1 ± 34.3 (6)	98.0 ± 7.00 (10)
Away from the tip	2.0 ± 4.0 (20)	6.0 ± 19.8 (10)	15.5 ± 17.2 (15)	45.9 ± 34.3 (6)	2.0 ± 7.00 (10)
MT tipwards dynamics (%) represented by:					
MT growth	69.7 ± 16.3 (20)	63.7 ± 25.4 (10)	51.3 ± 23.1 (15)	55.8 ± 24.1 (6)	90.0 ± 16.0 (10)
MT sliding	9.6 ± 12.5 (20)	3.1 ± 8.6 (10)	9.1 ± 9.4 (15)	4.1 ± 5.9 (6)	0.0 ± 0.0 (10)
Indiscernible	20.7 ± 21.1 (20)	28.4 ± 22.3 (10)	39.5 ± 21.9 (15)	34.4 ± 17.6 (6)	10.0 ± 16.0 (10)
Rates ($\mu\text{m min}^{-1}$) of:					
MT growth	12.21 ± 2.37 (28, 13)	10.65 ± 2.73 (18, 10)	11.04 ± 3.26 (21, 10)	13.15 ± 2.70 (8, 5)	10.73 ± 1.88 (17, 11)
MT sliding	14.93 ± 3.15 (7, 4)	27.59 ± 2.58 (3, 2)	14.35 ± 2.43 (3, 2)	NO	NO

*Only cells with medium-MT dynamics were included.

not detectably affect cells; scanned hyphae continued to grow at rates comparable to control hyphae and mitosis proceeded normally. Interphase MTs parallel the hyphal axis, extend to the hyphal tip and are often close to the cell cortex (Fig. 1). Hyphal tips contain 6.4 ± 1.5 ($n=7$) MTs, with 4.1 ± 0.4 ($n=110$, from 11 cells) seen in a typical focal plane. Compared with TEM data, the bright fluorescent lines represent single MTs.

Hyphal tip MTs are highly dynamic, elongating predominantly towards the apices (Table 1). MTs reach the tip by elongating tipwards (70%; Table 1 and Fig. 1a) and by sliding of short MTs (mean length 2.09 ± 0.48 μm , $n=24$) (Fig. 1b).

Upon reaching the apex, MTs typically vanish within the 1 s interframe interval; however, less rapid depolymerization was observed, allowing the calculation of shrinkage (MT depolymerization, 52.81 ± 17.78 $\mu\text{m min}^{-1}$), catastrophe (transition from growth to shrinkage, 0.021 events s^{-1}) and rescue (0.003 events s^{-1}) rates.

Rearward MT movements were rare ($<2\%$; Table 1) and consisted primarily of MT sliding; only one of the 448 MTs observed grew rearwards. Rearward and tipward sliding rates were similar [13.1 ± 4.4 $\mu\text{m min}^{-1}$ ($n=3$) versus 14.9 ± 3.2 $\mu\text{m min}^{-1}$ ($n=7$)]. Thus, the majority of MTs populating the tip appear to have been nucleated in sub-apical regions and not at the tip.

The dominant tipward movements of MTs and their rapid depolymerization there predict a tip-high gradient of tubulin subunits. Cytoplasmic fluorescence indicates cytoplasmic tubulin; no autofluorescence was seen in strains of *Aspergillus* not expressing GFP-tubulin (with identical microscope settings; data not shown). The cytoplasm between the tip and the first interphase nucleus displayed a tip-high fluorescence gradient (Fig. 2 and Table 2). However, rearward flow of subunits was not detected; following photobleaching, the residual gradient in the bleached region was primarily tip-low (Fig. 2 and Table 2), most consistent with influx of unbleached subunits from the subapical region. During recovery, the

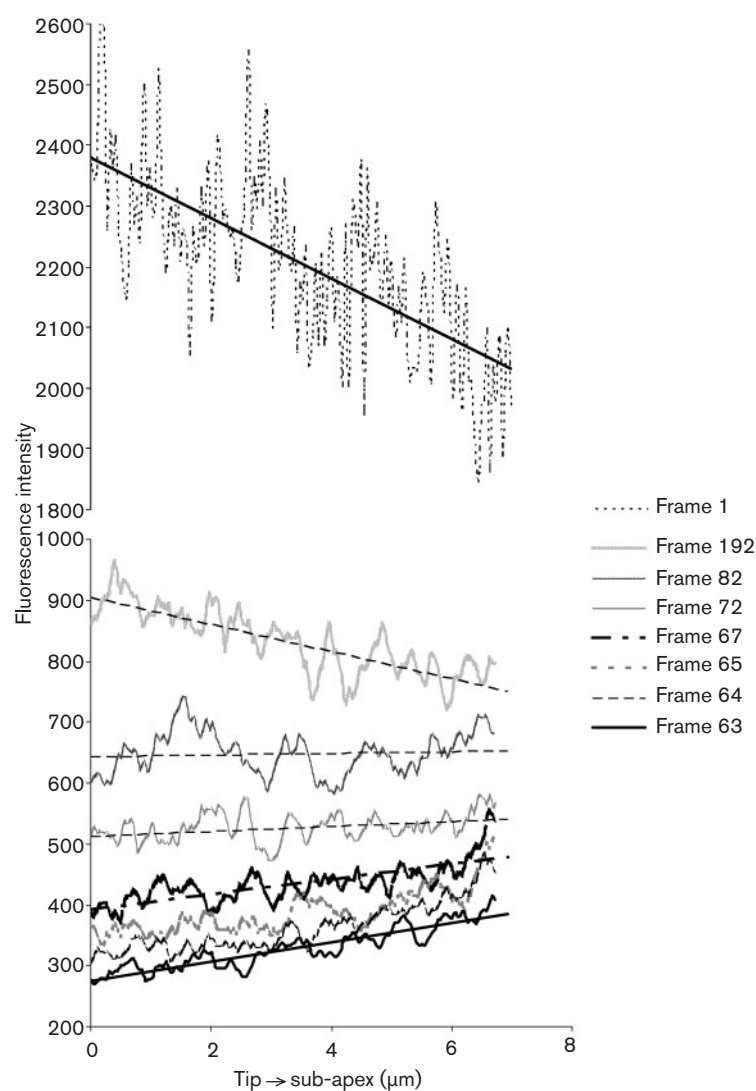


Fig. 2. Representative line plots of cytoplasmic fluorescence intensity over the length of a hyphal tip, prior to and following photobleaching. Line numbers correspond to the time (in seconds) after recording commenced (sampling rate of 1 frame s^{-1}). Prior to bleaching (1 s), fluorescence was intense and the gradient was tip-high; bleaching was between 2 and 62 s, after which the gradients reversed and intensity dropped, but later both intensity and original orientation recovered (e.g. 192 s).

Table 2. Changes in cytoplasmic fluorescence intensity gradients following photobleaching for different regions within hyphae

Two examples each of growing tips, branch tips and between nuclei within a subapical cell (Subap.) and four examples of gradients within the region between the most apical and second nucleus of apical cells (Internuc.) are included. In all four tips, the gradients reverted to normal tip-high within the observation period. However, in other locations the gradient displayed no particular disposition prior to or following bleaching. Positive gradients are tip-low and negative ones tip-high. Italic entries are estimated from graphed data. Times are approximate. Bold entries highlight negative gradients.

Location	Before photobleaching	Time after photobleaching (s)					
		1	5	10	20	70	130
Tip 1	-50	+16	+13	+5	+3	-7	-21
Tip 2	-18	+10	+12	+10	+7	+4	-5
Branch 1	-34	+11	+2	+7	-7	-12	-16
Branch 2	-22	+30	+8	+20	-14	-15	-12
Means 1–4	-31.0	+16.8	+8.8	+10.5	-2.8	-7.5	-13.5
Internuc. 1	-9	-5	0	-8	-5	-20	-26
Internuc. 2	+3	-2	+3	-2	-4	+1	-4
Internuc. 3	-5	+5	+3	+14	+8	-2	+3
Internuc. 4	-24	+8	0	+9	+9	+6	+6
Subap. 1	+4	0	0	0	+1	0	+2
Subap. 2	-6	0	0	+1	+4	-2	+1
Means 5–10	-6.2	+1.0	+1.0	+2.3	+2.2	-2.8	-3.0

gradient returned to tip-high (Fig. 2 and Table 2), presumably due to renewed input of subunits from apically depolymerizing microtubules. Photobleaching should also reveal MT treadmilling. There was no evidence for MT treadmilling (i.e. translocation of the photobleached region within an MT; see Supplementary Fig. S1 available with the online version of this paper), thus indicating that all MT elongation was due to apical polymerization.

The positions of hyphal nuclei were identified as approximately spherical areas lacking cytoplasmic fluorescence, confirmed by DAPI staining. The region between the most apical and second nucleus initiated fewer MTs and showed more rearward movements relative to the tips, but rates were comparable (Table 1). However, unique to this region, MTs commonly moved laterally and/or buckled, there was no consistent tip-high cytoplasmic tubulin gradient and the direction of recovery after photobleaching was variable (Table 2).

The behaviour of MTs and their subunits in developing branches was similar to that of growing tips (Tables 1 and 2) except that branches had fewer MT initiations (Table 1) and possibly a higher rate of MT sliding.

MT dynamics of subapical cells differ from apical cells

The region between nuclei in subapical cells displayed fewer MT initiations than apical cells and little bias of movements towards the tips (Table 1). Unlike hyphal tips, subapical cells rarely recovered cytoplasmic fluorescence after photobleaching (Fig. 3). On the contrary, photobleaching was commonly accompanied by a loss of

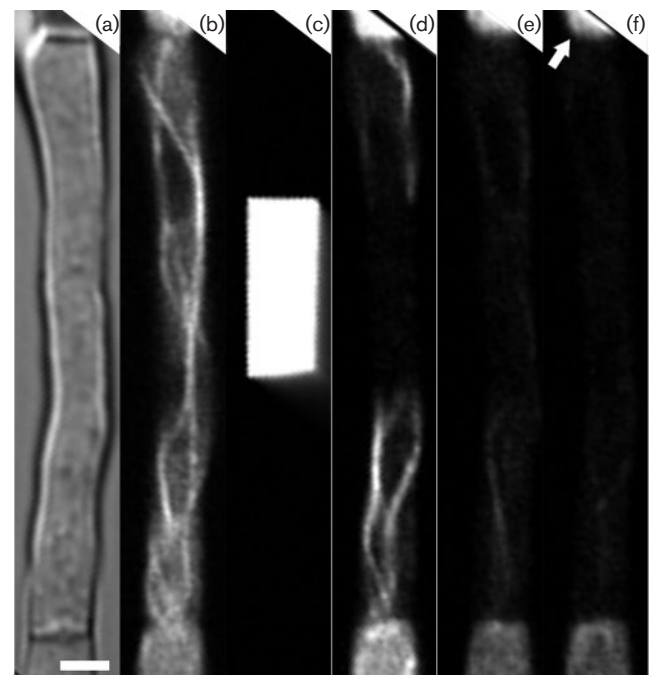


Fig. 3. A subapical cell fails to recover fluorescence after photobleaching. (a) DIC image. (b)–(f) MTs visualized via GFP-tubulin: (a) and (b) prior to photobleaching; (c) during bleaching, showing bleached region; (d)–(f) 1, 60 and 129 s after bleaching, respectively. Neighbouring cells do not contribute to fluorescence recovery. Arrow points to apical cell. Bar, ~2 μ m.

fluorescence intensity throughout the cell (Fig. 3). Interestingly, even though cells supposedly maintain a cytoplasmic continuum through septa (Alexopoulos *et al.*, 1996), the apical cell apparently did not contribute unbleached subunits to the subapical cell's recovery (Fig. 3).

The onset of mitosis is accompanied by reduced apical MTs and hyphal growth

Mitosis provided a natural modulation of cytoplasmic MTs. *Aspergillus* tip cells contain 8 or 16 nuclei (Fiddy & Trinci, 1976), with mitosis initiating sequentially from one end of a cell. All mitotic apical cells reduced their population of cytoplasmic MTs as they entered mitosis, but there were two populations, defined as those which retained more (medium MTs; 46 %, $n=26$) or fewer (low MTs) than two MTs per focal plane between prophase and aster formation. These MT numbers were less labile (Table 1; MT initiations) than those of interphase cells, so that the number identified in the focal plane containing most MTs in each cell at the start of mitosis (the number used to categorize each hypha) changed little during the observation period. The medium-MT cells contained 3.6 ± 1.0 (range 2–5, $n=9$) MTs per focal plane, while the low-MT cells contained 0.6 ± 0.5 (range 0–1, $n=7$). Ten of the original 26 cells observed could be categorized as medium or low but were not adequate for accurate counts, or started aster formation too soon to permit normal observation periods of >2 min.

These changes in MT populations related to the mean distance between the tip and the most apical nucleus, with the distance in medium-MT mitotic hyphae being greater than that in low-MT ones (16.11 ± 3.33 μm , $n=14$, versus 10.80 ± 5.09 μm , $n=12$; $P<0.006$, Student's *t*-test). Furthermore, the cytoplasmic MTs between subapical nuclei

were rarely maintained during mitosis, prior to aster formation (Fig. 4).

The reduction in cytoplasmic MTs in mitotic hyphae correlated with reduced hyphal growth rates, with medium-MT ones averaging 29 % of interphase ($P<0.00$, Student's *t*-test) and low-MT ones only 8 % ($P<0.00$, Student's *t*-test) (Fig. 5).

Cytoplasmic tubulin concentration and distribution change with the onset of mitosis

At the onset of mitosis in *Aspergillus* there is a rapid influx of tubulin into the nucleoplasm (Ovechkina *et al.*, 2003). This was reflected in comparative tubulin concentrations in regions of cytoplasm 1 μm tipward of mitotic nuclei, which were lower than similar regions adjacent to interphase nuclei ($P<0.001$, Student's *t*-test; Fig. 6). However, there was no statistical difference between the tubulin concentrations of these regions in medium- and low-MT mitotic cells, probably due to measurement sensitivity limitations. In addition to the decline in cytoplasmic tubulin concentrations in mitotic cells, the tubulin gradients were also typically lost or reversed. Among nine cells measured, four were low-MT cells and five were medium-MT ones. Only two retained tip-high gradients during the observation period; the others either became tip-low or wavered between tip-high and tip-low, with no relationship to their MT contents (Supplementary Fig. S2).

The anti-MT drug MBC depolymerizes tip MTs and reduces hyphal growth rate

To monitor tip growth in the absence of MTs, the anti-MT drug MBC was used. After a 10 min exposure, MTs had depolymerized at the tip but persisted around the nucleus and, by 15 min, all MTs were depolymerized. On washout,

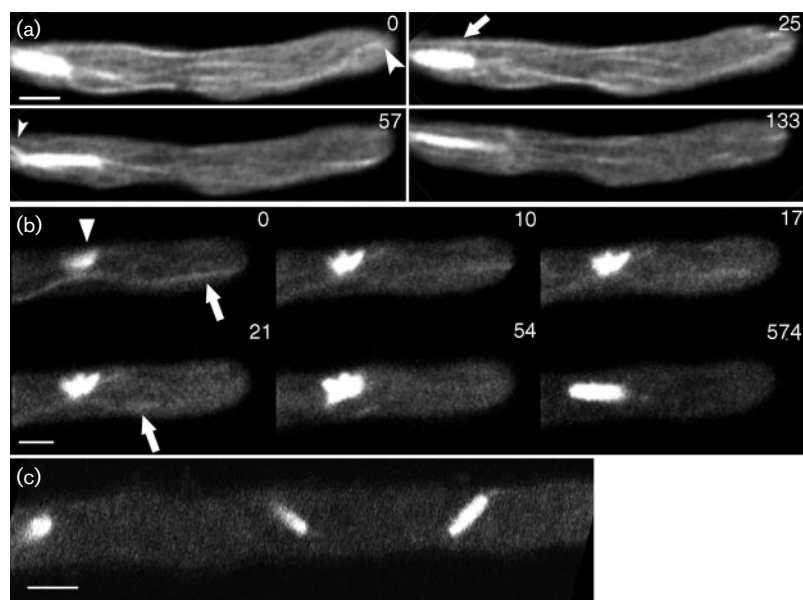


Fig. 4. MT dynamics in mitotic apical cells visualized via GFP-tubulin *in vivo*. (a) Medium-MT cell. Large arrowhead identifies an MT at the tip. Arrow points to an MT that extends past the nucleus. Small arrowhead points out the formation of astral MTs. (b) Low-MT cell. Arrowhead indicates forming spindle. Arrows point to an MT that is breaking down. (c) Subapical mitotic nuclei. Time in seconds in each frame. Bars, ~ 2 μm .

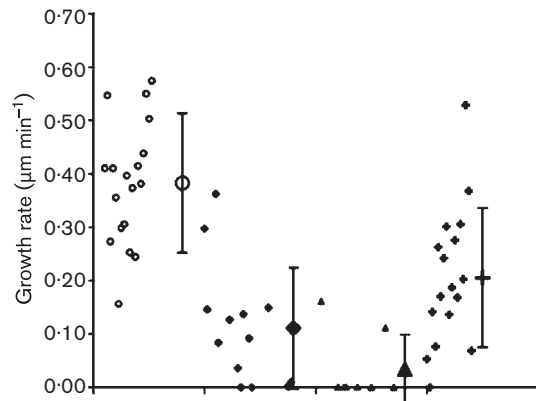


Fig. 5. Scatter plot of mean growth rates in medium-MT (◆) and low-MT (▲) dividing cells, interphase cells (○) and MBC-treated cells (+). Larger symbols represent the group mean and error bars indicate the SD.

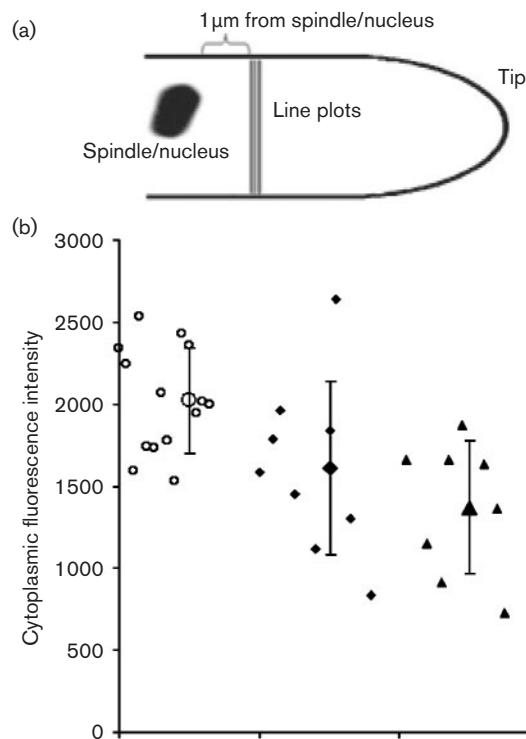


Fig. 6. Mean cytoplasmic fluorescence intensity in the region of the nucleus. (a) Diagrammatic representation of the region measured. (b) Scatter plot of mean cytoplasmic fluorescence intensity of the area 1 μm from the spindle of dividing cells (◆, medium-MT; ▲, low-MT) or the nucleus of interphase cells (○), on the tipwards side for the nucleus closest to the tip. Larger symbols represent the group mean and error bars indicate the SD.

MTs returned first to the area surrounding the nucleus and then to the region of the hyphal tip (Fig. 7), with 95 % of cells displaying MTs in both regions within 10 min. In no instance did MT polymerization initiate at the tip, although bright spots were occasionally seen there. MT nucleation occurred close to the nuclei, most probably at the spindle pole bodies (SPBs).

MBC-induced MT depolymerization reduced growth by 50 % (Figs 5 and 8) but only slightly increased hyphal diameter in 33 % of cells ($n=18$) (Fig. 8c), the majority retaining their normal shape. Fifteen minutes of recovery produced 82 % of cells ($n=17$) with clear signs of morphological recovery and 50 % of these had completely recovered.

Control experiments substituted drug-free media for the MBC, with no adverse effects; on the contrary, tip growth rates increased slightly following addition of fresh medium (Fig. 8; Table 3).

Lat B disrupts hyphal growth more severely than MBC

In *C. albicans*, depolymerization of MTs disrupts actin (Akashi *et al.*, 1994). To assess whether the above observations resulted from an indirect effect on actin, experiments were performed with actin-selective lat B. On average, hyphal growth was reduced to 10 % of controls (Fig. 8; Table 3) and eliminated in 42 % ($n=12$) of cells. Moreover hyphal tip swelling occurred in 75 % of cells, with 33 % developing bulbous tips, something never seen in MBC-treated cells.

Lat B-treated cells did not recover as well as MBC-treated cells. On washout, 58 % of cells showed signs of recovery within 15 min and only 17 % fully recovered after 30 min. Unlike MBC-treated cells, 75 % of cells recovered with the formation of lateral lumps or swellings (Fig. 8d), which often developed into branches (50 %). This was never observed in MBC-treated cells.

DISCUSSION

How do MTs populate hyphal tips?

The way in which MTs keep pace with the continuously elongating hyphal tip and maintain the characteristic apical population is unknown. MTs may populate the hypha from the tip backwards, presumably involving apical nucleation and rearward polymerization or transport, or they may originate subapically, with subapical nucleation and tipward movement. Our results support the latter option, with both tipward polymerization and transport of released MTs.

Subapical MT nucleation

SPBs are MT organizing centres (MTOCs) (Heath, 1981). While the location of MT nucleation may change during the

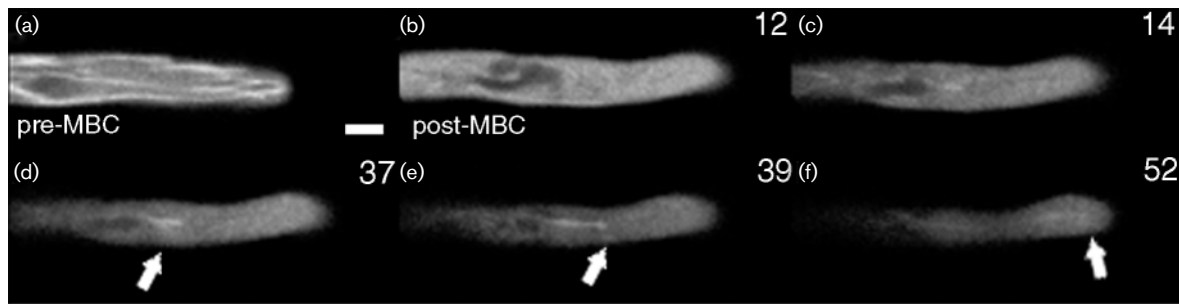


Fig. 7. MT depolymerization on MBC treatment and recovery on washout. MTs visualized via GFP–tubulin *in vivo*. (a) Prior to MBC treatment. MTs extend to the apex. (b)–(f) Following MBC treatment; time (in seconds) after washout in top corners. (c)–(f) MTs seen in the vicinity of the nucleus, but not at the tip. Arrows point to an elongating MT, extending tipwards. Bar, ~2 μm.

cell cycle (Heitz *et al.*, 2001; Straube *et al.*, 2003), our results indicate that the SPBs are the only MTOCs within *Aspergillus*; certainly those responsible for MT production in growing hyphae. MTs always originated close to nuclei and the majority of MT movement was tipward. On MBC treatment, the perinuclear region was the last to lose MTs and the first to recover them on washout. MTs that have

their ‘–’ ends capped by an MTOC are more stable than free MTs (Keating *et al.*, 1997) and therefore would be more resistant to anti-MT drugs. Our results are consistent with the localization of γ -tubulin (which is MTOC-associated; Murphy & Stearns, 1996; Oakley, 2000) to SPBs and not hyphal tips in *Aspergillus* (Oakley *et al.*, 1990). They contrast with reported apical MTOC activity in a basidiomycete

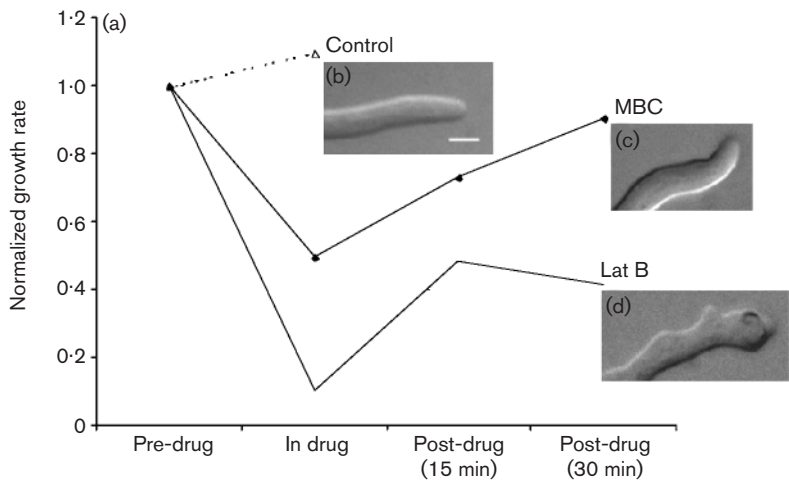


Fig. 8. Effects of MBC and lat B on hyphal growth. (a) Line plots of mean hyphal growth rates prior to, during and following drug treatment. The data for each hypha were normalized to the growth rate displayed by that hypha prior to drug treatment. The normalized readings were then averaged. Insets (b), (c) and (d) are DIC images of hyphae all taken at the same magnification. (b) Hyphal tip 15 min after medium exchange (control). Bar, ~2 μm. (c) Hyphal tip, displaying slight swelling, following 15 min exposure to MBC. (d) Hyphal tip displaying unusual swelling and the formation of lateral lumps following a 20 min recovery from exposure to lat B.

Table 3. Normalized hyphal growth rates after treatment with MBC or lat B

The highest and lowest rates observed in each group are shown. Rates were calculated from the data summarized in Fig. 8(a).

Treatment	Extreme	Pre-drug	In drug	Post-drug (15 min)	Post-drug (30 min)
MBC (<i>n</i> = 18)	High	1	0.83	1.53	1.35
	Low	1	0.00	0.00	0.35
Lat B (<i>n</i> = 12)	High	1	0.33	1.67	0.77
	Low	1	0.00	0.00	0.00
Control (<i>n</i> = 10)	High	1	1.77	–	–
	Low	1	0.86	–	–

(Hoch & Staples, 1985) and a chytridiomycete (McDaniel & Roberson, 1998), suggesting taxon-specific diversity in MT organization. However, subapical MTOCs in fungi and other tip-growing organisms are more consistent with obligatory tip-high cytoplasmic Ca^{2+} gradients (Hepler *et al.*, 2001; Hyde & Heath, 1997; Jackson & Heath, 1993), because high concentrations of Ca^{2+} typically induce MT depolymerization (Dustin, 1984).

Approximately 30 % of the MTs between the tip and its closest nucleus had both ends free in the cytoplasm (free MTs; data not shown) and MTs often extended or slid past the most apical nucleus en route to the apex. This indicates that the apical MTs are nucleated by the SPBs of numerous nuclei and then exported tipwards. MT release from centrosomes (SPB equivalents) has been reported (Keating *et al.*, 1997). This could explain how MTs are maintained at the tip in mitotic cells when the SPBs of the most apical nucleus are engaged in mitosis; other nuclei at different mitotic stages (mitosis is asynchronous) would produce MTs for the tip. Supporting this, MTs between nuclei often buckled, presumably as a result of oppositely polarized MTs, emanating from subapical and apical nuclei meeting ‘+’-end on. MT buckling was never observed between the tip and its closest nucleus.

Cytoplasmic nucleation of MTs may also occur, as Akashi *et al.* (1997) reported that 30 % of γ -tubulin in *Aspergillus* is in the cytoplasm and Martin *et al.* (1997) found that γ -tubulin is not essential for cytoplasmic MT assembly. We saw no evidence of cytoplasmic or septal nucleation. Septal nucleation has been reported in *Aspergillus*, but only for germlings (Konzack *et al.*, 2005).

Apical MT dynamics

The dominant form of MT production in *Aspergillus* is elongation at rates similar to those reported previously (Table 1; Han *et al.*, 2001). The frequency of MT catastrophe was lower than reported previously, and this is probably underestimated, as those that apparently disappeared on reaching the tip probably depolymerized faster than our range of detection. The mean depolymerization rate is the highest reported and is similarly likely to be underestimated. It is almost double that reported for *Aspergillus* by Han *et al.* (2001) but, while both studies sampled at the same frequency, they worked at 42 °C versus 22 °C. Temperature-dependent changes in MT dynamics have been observed in *Aspergillus* (Requena *et al.*, 2001). Furthermore, Han *et al.* (2001) also used a different strain of *Aspergillus*. Dynein and a number of kinesins influence MT dynamics (Han *et al.*, 2001; Konzack *et al.*, 2005; Requena *et al.*, 2001; Rischitor *et al.*, 2004); the two strains, also of differing ploidy, may differ in motor protein expression levels. Alternatively, if the current strain is faster growing than that used by Han *et al.* (2001) (not specified in their report), it would have higher cytoplasmic $[\text{Ca}^{2+}]$ at its hyphal tips, since higher growth rates correlate with higher

apical cytoplasmic $[\text{Ca}^{2+}]$ (Hyde & Heath, 1997). Elevated $[\text{Ca}^{2+}]$ accelerates MT depolymerization (above).

MT subunit transport

The tip-high gradient of tubulin demonstrated by cytoplasmic fluorescence in apical cells is probably the result of both MT depolymerization at the apex (possibly generated by the tip-high cytoplasmic $[\text{Ca}^{2+}]$) and the tipward flow of subunits shown by photobleaching. In contrast, subapical regions of apical cells, especially between nuclei, lacked tipward flow of tubulin and the distribution of subunits varied. Thus, the unknown tubulin subunit distribution mechanism is only active in the tips, with variable subapical distribution possibly being due simply to the level of transcriptional activity of the nearest nuclei.

Subapical cells generally displayed an even distribution of tubulin, with the lack of fluorescence recovery after photobleaching indicating an inactive distribution system. Septal pores supposedly permit cytoplasmic continuity (Alexopoulos *et al.*, 1996), but the absence of tubulin influx into photobleached subapical cells suggests that osmophilic material within the pore (data not shown) selectively limits molecular flux. Consistent with this, entry of nuclei into mitosis in apical cells appears to be coordinated by a diffusible factor (data not shown) which does not trigger mitosis in the subapical cells, suggesting that the trigger molecule(s) does not traverse the septal pores. Photobleaching may have closed the pores; cell damage can do so (Collinge & Markham, 1985; Trinci & Collinge, 1973), but this is unlikely since no harmful effects were noted and mitotic cells completed division normally.

Relationship between MT dynamics and nuclear cycle

As apical nuclei entered mitosis, cytoplasmic MTs were reduced or lost; the rate of new MT formation was reduced and these changes were most marked in smaller cells, indicated by shorter nucleus to tip distances. This relationship is similar to the observations of Horio & Oakley (2005) of MT loss at mitosis, but only in germlings. Both observations are consistent with larger cells maintaining larger total tubulin pools (the concentrations of tubulin were similar in mitotic cells whether MTs persisted or not) and thus being able to sustain both mitotic and cytoplasmic MTs. In cells retaining tip MTs, they often extended past the first mitotic nucleus (arrow, Fig. 4a), indicating that, although the nucleus influences cytoplasmic MTs, it does not simply generate a zone of depolymerization.

Our observations differ from those of Horio & Oakley (2005), who reported greater retention of tip MTs in mitotic hyphae (but not germlings) and no growth rate change. The studies differ in culture temperatures and media, which may cause the different results, but their combined germling and hyphal data are consistent with the independence

between cytoplasmic MT regulation, mitosis and hyphal tip growth discussed below.

MT functions in hyphal growth

Our results indicate that normal cytoplasmic MTs are not essential for hyphal tip growth, as witnessed by slowed but morphologically normal growth in the absence of apical MTs in some mitotic cells and all MBC-treated hyphae. Horio & Oakley (2005) likewise reduced (more than we observed, but the variables cited above and a different carrier for the MBC may explain the differences), but did not eliminate, tip growth with MBC. All these data clearly indicate that, while MTs contribute to the tip growth process, they are not obligatory for either growth or normal tip shape. Similar conclusions have been reached previously (e.g. Heath, 1994; Heath *et al.*, 2000, 2003; Oakley & Rinehart, 1985; Torralba & Heath, 2001). Possible MT contributions to tip growth are discussed below.

Organelle positioning

MT-dependent processes that, if disrupted, are likely to affect the multicomponent tip growth process include the transportation and organization of vesicles (Heath & Kaminskyj, 1989; Lehmler *et al.*, 1997; Seiler *et al.*, 1997; Steinberg & Schliwa, 1993, 1995; Steinberg *et al.*, 1998; Wedlich-Soldner *et al.*, 2000; Wu *et al.*, 1998), nuclei (Heath & Kaminskyj, 1989; Herr & Heath, 1982; Kaminskyj *et al.*, 1989; McKerracher & Heath, 1986; Meyer *et al.*, 1988; Oakley & Morris, 1980; Oakley & Rinehart, 1985; Steinberg & Schliwa, 1993; Wedlich-Soldner *et al.*, 2000; Wu *et al.*, 1998), vacuoles (Allaway *et al.*, 1997; Herr & Heath, 1982; Hyde *et al.*, 1999; Shepherd *et al.*, 1993; Steinberg *et al.*, 1998), mitochondria (Heath & Kaminskyj, 1989; Herr & Heath, 1982; Steinberg & Schliwa, 1993; Wu *et al.*, 1998) and the endoplasmic reticulum (Wedlich-Soldner *et al.*, 2002). However, direct structural evidence of MT and organelle interactions is rare (Heath, 1994). Instead organelle trafficking may involve MTs interacting with other cytoskeletal components such as actin (Heath, 1990, 1994, 2000; Kaminskyj *et al.*, 1989). Our limited ultrastructural data (not shown) support MT-actin interactions in *Aspergillus*; MTs were in contact with actin-like filaments and not organelles or vesicles. The only direct interaction with an organelle involved a mitochondrion (data not shown). Previous tubulin mutant studies in *Aspergillus* (Oakley & Rinehart, 1985) indicated that MTs do not participate in mitochondrial transportation, suggesting that our observed association may be the result of chance. A statistical analysis such as that showing a non-random association between mitochondria and MTs in *Uromyces* (Heath & Heath, 1978) would resolve this.

There need be no direct link between MTs and organelles for MTs to influence organelle distribution. The cytoplasm consists of a concentrated mixture of interacting proteins (Heuser, 2003; Luby-Phelps, 1993; McNiven, 2003) termed the cytomatrix (McNiven, 2003). Thus, the dynamics of

MTs could affect the position of other proteins and organelles without direct contact. MT activity at the hyphal tip could nudge the cytomatrix, thus ensuring organelles and proteins maintain their proximity to the elongating tip. This model is similar to the 'polar ejection force' model for mitosis, in which Rieder *et al.* (1986) proposed that MTs growing away from the spindle poles generated movement by impacting upon components of the cytoplasm. This model could explain how the distribution of vesicles which are not in contact with MTs can be affected by MT disruption (Herr & Heath, 1982).

Cytoplasm migration and subapical vacuolation

Cytoplasm migrates as the hypha extends to maintain its position relative to the tip. Cytoplasmic migration is an active process comparable to amoeboid movement (Bachewich & Heath, 1999; Heath & Steinberg, 1999; Kaminskyj & Heath, 1996). This migration can be accompanied by coordinated vacuolation of subapical regions (Bachewich & Heath, 1999; Heath & Steinberg, 1999) to minimize the energy expenditure of hyphal growth (Heath & Steinberg, 1999; Kaminskyj & Heath, 1996). MTs are involved in vacuolation and vacuolar transport (Allaway *et al.*, 1997; Bachewich & Heath, 1999; Herr & Heath, 1982; Hyde *et al.*, 1999; Shepherd *et al.*, 1993; Steinberg *et al.*, 1998), but the mechanisms that drive cytoplasmic migration are unknown. The high level of MT activity at the apex may help to drive the cytoplasm tipwards, but they are not obligatory, as hyphae grown in the absence of MTs do not display abnormal apical vacuolation (current study; Bachewich & Heath, 1999). Bachewich & Heath (1999) suggested that both actin and MTs contribute to cytoplasmic migration, as the bulk migration of cytoplasm seen on recovery from intracellular acidification was sensitive to both MT and actin disruption.

Conclusion

In this study, MTs proved not to be obligatory for growth. However, the level of MT activity at the tip indicates considerable energy commitment, and reduced MTs reduce growth rate. Thus, MTs maximize growth rates but are not essential for either continued growth or normal tip morphogenesis, and their regulation is independent of tip growth. MTs populate the tip via nucleation in subapical regions and both elongation and transport tipwards. Elongation is facilitated by the tipward migration of MT subunits, a process lacking in subapical cells.

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