ORIGINAL ARTICLE

Growth rate of *Aspergillus nidulans* hyphae is independent of a prominent array of microtubules

Michelle Hubbard · Susan Kaminskyj

Abstract Roles for the microtubule (MT) cytoskeleton in fungal growth include mitosis and nuclear migration but otherwise are less clearly understood. Confocal microscopy was used to quantify MT abundance and growth rate in hyphae of a haploid *Aspergillus nidulans* strain containing green fluorescent protein (GFP)-α-tubulin. There was no correlation between growth rate and MT abundance for 112 growing hyphae in an untreated population. However, 109 nongrowing hyphae from the same group had lower average MT abundance. Results for untreated cells were compared with cells treated for 30–120 min with the MT drugs benomyl and taxol, the actin drug latrunculin B, and with solvents used for the drug treatments. Compared with their respective controls, MT abundance was significantly increased by dimethyl sulfoxide (DMSO), significantly reduced by benomyl, and moderately increased by latrunculin, but was unaffected by ethanol. In the same cells, growth rates were significantly increased by ethanol and taxol, significantly reduced by latrunculin, and unaffected by DMSO. Average hyphal growth rate in the first 60 min following 1 µg/ml benomyl treatment was statistically similar to untreated cells, despite the absence of visible MTs after 2 min of treatment. However, growth rate was significantly reduced by 2.5 µg/ml benomyl over the same time period, implying additional effects at the higher concentration. For individual hyphae in each treatment, growth rates varied over short time periods; treatment with 0.1% ethanol substantially increased this variability. Growth rates of taxol-treated hyphae decreased following fluorescence observation, suggesting a possible application to cancer chemotherapy. Overall, there was no correlation between cytoplasmic MT abundance and *A. nidulans* growth rate within 2 h of cytoskeletal drug or solvent treatment.

Abbreviations

DIC   differential interference contrast
GFP   green fluorescent protein
MT   microtubule
TEM   transmission electron microscopy

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Introduction

The actin and microtubule (MT) cytoskeletons have many roles in polarized growth of filamentous fungi (Bartnicki-Garcia 2002; Heath 1990a, b, 1995; Heath et al. 2000). Filamentous actin arrays are concentrated in areas of active cell extension and cell wall deposition (Heath 1990b, 1995; Heath et al. 2000). In *Aspergillus nidulans,* cytoplasmic actin arrays have documented roles in hyphal extension and in morphogenesis (Harris et al. 1994; Torralba et al. 1998; Sampson and Heath 2005), septation (Momany and Hamer 1997), and mitochondrial motility (Suelmann and Fischer 2000a). A class I myosin is essential in *A. nidulans* and is enriched at hyphal tips (McGoldrick et al. 1995). The SEPA formin important in actin organization is enriched at *A. nidulans* hyphal tips (Sharpless and Harris 2002). A chitin synthase in *Ustilago maydis, mcs1* was shown to have an N-terminal myosin class-V-like domain (Weber et al. 2006), a novel link between the actin cytoskeleton and cell wall

deposition that might in future be related to chitin synthase localization and *Aspergillus* septum deposition (Ichinomiya et al. 2005). Taken together, Heath (1990b) and Heath et al. (2000) provide abundant evidence that actin is of primary importance in hyphal tip growth and morphogenesis, which is supported in *A. nidulans* by Harris et al. (1994), Sampson and Heath (2005), and Torralba et al. (1998).

There is abundant evidence that MTs are required for mitosis and for nuclear migration in filamentous fungi (Heath 1995; Morris and Enos 1992; Morris et al. 1995; Plamann et al. 1994; Suelmann and Fischer 2000b). However, unlike actin, roles for MTs specifically in hyphal growth are less consistent. Cytoplasmic MTs support rapid, long term, and/or morphologically wild-type growth in fungi, including *Aspergillus* (Ovechkina et al. 2003; Horio and Oakley 2005; Konzack et al. 2005; Sampson and Heath 2005), *Neurospora* (Mouriño-Pérez et al. 2006, Riquelme et al. 2002), and *Ustilago* (Fuchs et al. 2005, Schuchardt et al. 2005). However, *A. nidulans* conidia can germinate in the presence of the MT-depolymerizing agent benomyl (Oakley and Morris 1980). *A. nidulans* hyphal growth rate is maintained during mitosis, although most cytoplasmic MTs depolymerize (Riquelme et al. 2003; Trinci and Morris 1979); for contrary results, see Sampson and Heath (2005). Many fungi with tip-growing cells show dramatic growth-rate fluctuations within a few seconds (López-Franco et al. 1994; Sampson et al. 2003), whereas comparable quantitative changes in *A. nidulans* MT arrays require much longer times (Ovechkina et al. 2003; Sampson and Heath 2005).

The number and arrangement of cytoplasmic MTs vary significantly, even between filamentous ascomycetes, further complicating development of a general description of their role(s) in hyphal tip growth. For example, in *A. nidulans*, MTs are relatively few in number and are predominantly in the central cytoplasm (Meyer et al. 1987; Ovechkina et al. 2003; Sampson and Heath 2005), whereas in *N. crassa* MTs are relatively numerous and are found in both central and peripheral cytoplasm (Freitag et al. 2004; Mouriño-Pérez et al. 2006). *A. nidulans* MTs are mostly parallel to the hyphal axis (Meyer et al. 1987; Ovechkina et al. 2003; Sampson and Heath 2005), whereas *N. crassa* MTs are mostly parallel to the axis near the hyphal tip but more randomly oriented in basal compartments (Freitag et al. 2004).

Until recently, it was not possible to probe the dynamic relationship between MT arrays and tip growth rate, as MTs had to be visualized in fixed cells using immunofluorescence or electron microscopy. The development of *A. nidulans* strains with constitutive green fluorescent protein (GFP)-α-tubulin (Horio and Oakley 2005) allows investigation of MT dynamics in living hyphae. Horio and Oakley (2005) and Sampson and Heath (2005) provided evidence

that benomyl- and methyl 2-benzimidazole carbamate (MBC) (the primary metabolite of benomyl)-induced MT disassembly led to decreased growth rate. However, using an *AlcA*-regulated GFP-MT strain, Ovechkina et al. (2003) found that mitosis-induced MT disassembly in individual hyphae did not reduce growth rate. This lack of consistency indicates the need for further investigation into the roles of MTs in hyphal growth.

We used a haploid *A. nidulans* strain with constitutively tagged GFP-α-tubulin described in Horio and Oakley (2005) to study the quantitative relationship between the number of cytoplasmic MTs and growth rate in large numbers of individual hyphae. Our study differs from reports by Horio and Oakley (2005) and Sampson and Heath (2005) in several ways: (1) We used the haploid strain (LO1022) grown on nutrient-rich medium rather than a diploid strain (LO1052) grown on minimal medium. Most *A. nidulans* experimental strains are haploid, as are those isolated from nature. (2) We collected data on large numbers of hyphae from cultures at least 24-h old, chosen for their similar morphology, and then restricted analyses to hyphae that were shown to be growing. (3) We used both equivalent and lower concentrations of benomyl and latrunculin B and compared them statistically to solvent-only controls. (4) In addition, we estimated relative MT abundance using a quantitative index. (5) We present the first data on in vivo effect of taxol on *A. nidulans* MT abundance and growth rate.

Surprisingly, MT index and growth rate were not correlated for large numbers of individual untreated hyphae, although the average MT index for growing hyphae exceeded that of nongrowing ones. Similarly, when groups of hyphae were treated with the cytoskeleton-selective drugs benomyl, latrunculin B, and taxol or with comparable concentrations of carrier solvents, average growth rate and microtubule index varied independently following different treatments.

Materials and methods

Aspergillus nidulans growth conditions

A. nidulans strain LO1022 (GFP-α-tubulin; *pabaA1; wA2; cnxE16, sC12; veA1*) was maintained at 28°C on complete medium (CM; Kaminskyj 2001) supplemented with para-amino benzoic acid and methionine. LO1022 is ideal for this type of study, as it is haploid, like *A. nidulans* strains in nature and the majority of the strains studied experimentally, and can be grown on rich medium for optimal growth rate. For confocal microscopy, freshly harvested spores were inoculated onto sterile dialysis tubing overlying CM agar (Kaminskyj 2000) and grown for at least 24 h at 28°C.

The dialysis tubing and overlying hyphae were lifted from the CM agar, mounted in a microscope slide chamber (Heath 1988) in ~100 μl of liquid CM containing solvent and inhibitors as required, and allowed to recover for 30 min before observation. Mounting induced transient hyphal tip swelling that made a convenient marker for treatment initiation. Observations were terminated before 120 min.

Cytoskeletal inhibitors

Benomyl, paclitaxel (trade name, Taxol), and anhydrous dimethyl sulfoxide (DMSO) were obtained from Sigma http://www.sigmaaldrich.ca). Latrunculin B (hereafter, latrunculin) was obtained from Molecular Probes http://www.molecularprobes.com). All other chemicals were obtained from VWR http://www.vwrcanlab.ca). Inhibitors were diluted from stock solutions with room-temperature liquid CM immediately before use.

Benomyl was stored at 4°C as a 10 mg/ml stock in 100% ethanol and used at 1 μg/ml in 0.01% ethanol or at 2.5 μg/ml in 0.025% ethanol. Latrunculin B was stored as a 25 mg/ml stock in 100% ethanol at −20°C, and used at 5 μg/ml in 0.02% ethanol or 20 μg/ml in 0.08% ethanol. Taxol was stored at −20°C as a 2 mM stock in 100% DMSO and used at 50 μM in 0.25% DMSO. DMSO was purchased as 1 ml ampoules of dry solvent, and the stock was stored in aliquots over desiccant. The ethanol control concentration was 0.1%, at the top of the range used in preliminary experiments, and similar to previously published studies (Harris et al. 1994; Kaminskyj 2000), but higher than the one used for the benomyl and latrunculin data presented below. The inhibitor concentrations were similar to or lower than those used in the literature (benomyl and latrunculin) or were the lowest for which a response was detected (taxol).

Confocal microscopy

A. nidulans hyphae were imaged with a Zeiss META 510 laser scanning confocal microscope http://www.zeiss.com) using a Plan Apochromat 63 x, N.A. 1.2, multi-immersion objective equipped with differential interference contrast (DIC) optics. GFP-α-tubulin fluorescence was imaged with 488 nm excitation from an Argon laser, with emission controlled by a BP505-530 filter. Excitation intensity was 5–10% from a laser current of 5.9 amps. Eight or 16 scans per pixel at 0.6–2.5 μs/pixel were used to improve signal-to-noise ratio. Optical sections were 1.2-μm thick and chosen to be near-median focal level. Control experiments for taxol-treated cells used the HeNe2 laser, which has an emission of 634 nm, used at 10% of maximum.

Hyphae were chosen for analysis if they were located at the colony margin, had an even profile and a smoothly

tapered tip, and had grown out from the characteristic mounting-induced morphology, that is, a swollen tip or an abrupt change in growth direction. Hyphae that had not responded in this way to mounting were assumed to be nongrowing and were not selected for analysis. However, whether a particular hypha was actually growing, and at what rate, was not determined until after the data were collected. For each tip, 5–20 images were collected over 60–300 s. We did not use cells whose nuclei were in mitosis at any time during the analysis period. DIC images used to measure hyphal growth rates were captured simultaneously with the fluorescence images used to quantify MT profiles. Minor photobleaching was detected by the end of longer imaging sessions, but MT index values and hyphal growth rates were not noticeably affected.

Microtubule quantification and microtubule index

MTs containing GFP-α-tubulin cannot be precisely counted using fluorescence microscopy, even with optimal confocal settings employed here. The theoretical resolution of our confocal system was 230 nm, whereas the width of a fluorescent MT is about 25 nm plus the GFP decoration. Fluorescent objects are self-luminous, which enhances detection but does not improve resolution. Williamson (1991) showed that fluorescence images of fixed MTs in plant cells underestimated number and overestimated continuity compared with serial reconstruction transmission electron microscopy (TEM). Sampson and Heath (2005) reported "approximate correlations" between numbers of MTs containing GFP-α-tubulin visualized by fluorescence and MTs in the same A. nidulans cells following chemical-fixed TEM. Precise correlations are simply not possible given the inevitable chemical fixation-induced cytoplasmic contractions (Kaminskyj et al. 1992). Other factors confounding MT quantification include the fact that an MT grazing the confocal optical volume may not be detected and GPF-tubulin dimers, protofilaments or MTs shorter than the resolution limit would contribute to background cytoplasmic fluorescence. Thus, our MT index values described below reflect relative rather than absolute abundance. Regardless, strains with constitutive GFP-α-tubulin are a major advance.

MT bundling in A. nidulans hyphae further complicates quantification. A freeze-substitution, cross section, serial reconstruction TEM analysis of an A. nidulans hypha revealed that about half of the cytoplasmic MTs were in bundles of two or three, typically at least one MT width apart (R. Roberson, personal communication). Confocal microscopy cannot resolve individual MTs within bundles nor unambiguously distinguish MT bundles from singletons, although occasionally we could infer bundling from abrupt changes in brightness, as did Mouriño-Pérez et al.

(2006) in *N. crassa*. Thus, some of the drug effects presented herein may be due to MT bundling/unbundling as well as polymerization/depolymerization.
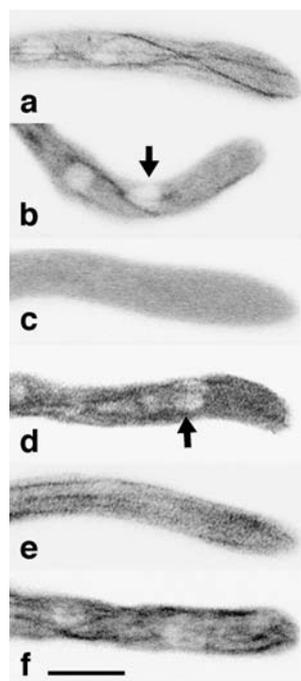
To compare relative MT abundance between hyphae or treatments, we developed the MT index using near-median confocal optical sections encompassing about 25% of the hyphal volume. The MT index for a hypha was defined as the sum of MT profile counts at 5 μm, 10 μm, 15 μm, and 20 μm from the hyphal tip. Counts for MT index used 12-bit Zeiss confocal images. Figure 1 shows Adobe Photoshop 8-bit images of typical cells, presented in reverse contrast to facilitate MT visualization.

To assess growth rate, the tip position imaged using DIC was marked at the beginning and at the end of the analysis period. Growth rate was the difference in length in hundredths of micrometers between the initial and final tip position divided by the difference in time seconds (both generated by the LSM510 software) and expressed as micrometers/min.

Statistical and graphical analysis

Data are expressed as the mean ± standard error of the mean. Statistical analyses used the 2000 version of Microsoft Excel with data analysis add-ins, or Statview SE+Graphics 1.01, both of which generate probability values. Statistical comparisons between treatments used one-way, single-factor analysis of variance (ANOVA), and post hoc comparisons used Fisher protected least significant difference (PLSD). Numerical data are presented using Cricket Graph

1.0. Images are presented using Adobe Photoshop 7.0 in reverse contrast.

Results

Cytoplasmic microtubules in *Aspergillus nidulans* hyphae

In untreated *A. nidulans* hyphae, cytoplasmic MTs are long and flexuous and run generally parallel to the long axis of the cell (Fig. 1a). Over seconds to minutes, cytoplasmic MT position varied, but MT index remained consistent. MT arrays were not affected by a low concentration of ethanol (Fig. 1b), whereas all detectable cytoplasmic MTs were lost within 2 min of treatment with benomyl/ethanol (Fig. 1c). MT array appearance was relatively unaffected by latrunculin/ethanol (Fig. 1d), consistent with the latrunculin cytoskeletal target being actin microfilaments rather than MTs. *A. nidulans* MT array appearance was relatively unaffected by a low concentration of DMSO (Fig. 1e), but cells treated with taxol/DMSO (Fig. 1f) had coarser arrays, perhaps due to MT bundling.

Growth rate and relative microtubule abundance in untreated *Aspergillus nidulans* hyphae

MT profile numbers were counted for 1.2-μm-thick near-median optical sections of 112 growing *A. nidulans* hyphae. The average number of MT profiles at 5 μm, 10 μm, 15 μm, and 20 μm behind the tip was 2.73±0.07, 3.06± 0.06, 3.09±0.07, and 3.07±0.06, respectively. There were significantly fewer MTs 5 μm from the tip than further back ($P=0.001$). In *A. nidulans*, cytoplasmic MTs are nucleated from nucleus-associated organelles (Oakley 2004), so this result is consistent with the typical position of the most apical nucleus (arrows in Fig. 1).

Tip growth rate and MT index (relative MT abundance) for 221 untreated *A. nidulans* hyphae are shown in Fig. 2. Many data points overlap for the 109 nongrowing hyphae. The average MT index for growing hyphae (11.75±0.73) was higher than for nongrowing hyphae (7.72±0.28) ($P< 0.0001$), but there was no significant correlation between MT index and growth rate among the population of growing hyphae ($r^2=−0.02$). Occasionally, even rapidly-growing hyphae had a low MT index: two cells with growth rates close to 1 μm/min had MT index values of 6.

Effect of cytoskeleton-selective drugs on relative microtubule abundance and hyphal growth rate

Our preliminary data from untreated *A. nidulans* hyphae (Fig. 2) showed that amongst growing cells, growth rate was not correlated with MT index. However, there was a
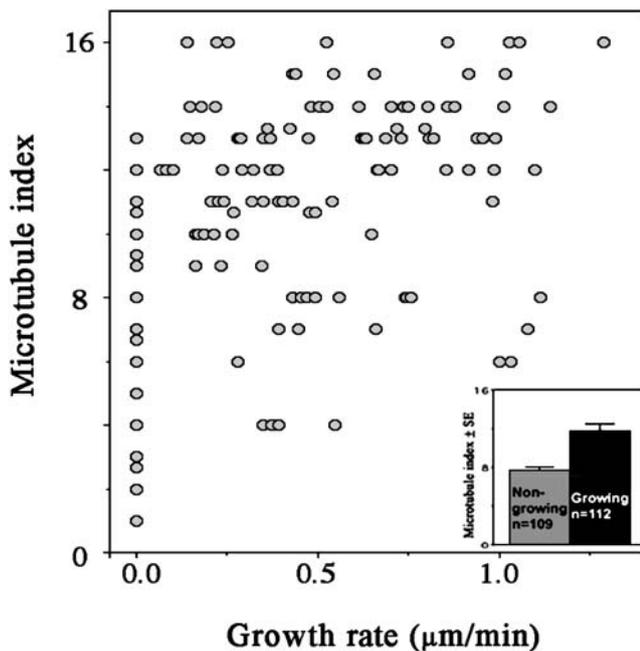
Fig. 1 Representative images of *Aspergillus nidulans* hyphae containing green fluorescent protein (GFP)-α-tubulin visualized using confocal epifluorescence microscopy of single near-median sections. Images are shown with inverted contrast so microtubules are seen as *dark lines*. Hyphae were (**a**) untreated or treated ~30 min with (**b**) 0.1% ethanol, (**c**) 1 μg/ml benomyl in 0.01% ethanol, (**d**) 5 μg/ml latrunculin B in 0.02% ethanol, (**e**) 0.25% dimethyl sulfoxide (DMSO), (**f**) 50 μM taxol in 0.25% DMSO. Some treatments, including taxol, give the impression of inducing microtubule bundling, but individual microtubules cannot be resolved with fluorescence microscopy. *Arrows* in **b** and **d** indicate the position of the most-apical nucleus. *Bar* in **f**= 5 μm, for all images

Fig. 2 The correlation between microtubule index (an estimate of relative microtubule abundance) and growth rate for untreated *Aspergillus nidulans* hyphae containing constitutively tagged green fluorescent protein (GFP)-α-tubulin. See "Materials and methods" for calculations of microtubule index and growth rate. Some data points overlap for the growing cells; most data points overlap for the group of 109 nongrowing cells. *Inset*: Average microtubule index values and standard errors for the 109 nongrowing and 112 growing hyphae

difference between the average MT indexes of 112 growing vs. 109 nongrowing hyphae (Fig. 2, inset). To explore the relationship between growth rate and MT abundance, we used well-characterized drugs, benomyl and latrunculin B, known to affect hyphal tip growth in *A. nidulans* (Horio and Oakley 2005; Sampson and Heath 2005) by targeting MTs and filamentous actin, respectively. In addition, taxol has been shown to induce polymerization of *A. nidulans* MTs in vitro (Yoon and Oakley 1995) but to our knowledge had not been studied in vivo. If there were treatment-related differences between groups of hyphae, we might yet discern broad relationships between relative MT abundance and hyphal growth rate.

Cytoskeleton drugs are sparingly soluble in aqueous solutions and are used at low concentrations, so they are diluted into growth medium from stocks prepared in ethanol or DMSO (e.g., Harris et al. 1994; Kaminskyj 2000). *A. nidulans* grows well in medium containing ≥1% ethanol as a sole carbon source (Fernández-Ábalos et al. 1998; Palmer et al. 2004), and it tolerates 1% DMSO without noticeable morphological effects. Average MT index and growth rate for populations of untreated, 0.1% ethanol-treated, and 0.25% DMSO-treated *A. nidulans* hyphae are shown in Fig. 3a. The 0.1% ethanol treatment significantly increased hyphal growth rate ($P=0.0001$) but

did not affect MT index ($P>0.1$). Most of the ethanol-treated hyphae were growing compared with half of the untreated cells (Fig. 3a). In contrast, 0.25% DMSO treatment significantly increased the average MT index ($P=0.0001$), but not the hyphal growth rate ($P>0.05$). Half of the DMSO-treated hyphae were growing, similar to untreated hyphae. Thus, low solvent concentrations caused significant effects on *A. nidulans* cytoplasmic MT abundance or on hyphal growth rate but not both.

Treatments to depolymerize cytoplasmic MTs were 1 µg/ml and 2.5 µg/ml benomyl dissolved in 0.01% and 0.025% ethanol, respectively. Both concentrations were effective (Fig. 3b): one MT was found in 76 hyphae after 1 µg/ml benomyl, and none in 27 hyphae after 2.5 µg/ml benomyl. Benomyl blocks *A. nidulans* nuclei at metaphase (Oakley and Morris 1981) and the mitotic cycle is about 100 min. By 2 h in 1 µg/ml benomyl, all nuclei had metaphase spindles (Oakley and Morris 1981); clearly, factors involving cytoplasmic MT and mitotic spindle polymerization differ in their response to benomyl treatment. Due to the dramatic reduction in cytoplasmic MT abundance during mitosis, we did not consider cells with mitotic nuclei for growth-rate studies, so data collection for benomyl treatments ended about 60 min. Unexpectedly, about a third of the hyphae in each benomyl-treated group continued to grow at least during the first hour following benomyl application (Fig. 3b). For 1 µg/ml benomyl, the average growth rate was statistically similar to the ethanol control treatment (Fig. 3b), whereas 2.5 µg/ml benomyl caused a significant growth-rate reduction ($P<0.001$). Overnight treatment with 1 µg/ml benomyl induced the formation of multiple apical branches (data not shown) comparable to Riquelme et al. (1998). Thus, in addition to the rapid loss of cytoplasmic MTs following 1 µg/ml benomyl application, higher benomyl concentrations, or longer treatment times had additional deleterious consequences on hyphal growth. We were unable to abolish hyphal tip growth by eliminating cytoplasmic MTs, implying that the actin cytoskeleton was delivering growth-related materials to the hyphal tip, potentially over substantial distances.

Latrunculin targets filamentous actin (Sampson and Heath 2005), whose functions in hyphal tip growth are well established (Bartnicki-Garcia 2002; Harris et al. 1994; Heath 1990b, 1995; Torralba et al. 1998). Latrunculin does not affect MTs (Sampson and Heath 2005). Our latrunculin concentrations were chosen to significantly reduce tip growth rate (5 µg/ml) or to match the concentration (20 µg/ml) used previously on *A. nidulans* (Sampson and Heath 2005). Both latrunculin concentrations significantly reduced average growth rate ($P<0.01$) with a clear dose relationship but did not affect MT index values (Fig. 3c). By 120 min, latrunculin treatment caused apical swelling and/or branch initiation (not shown). All these effects were
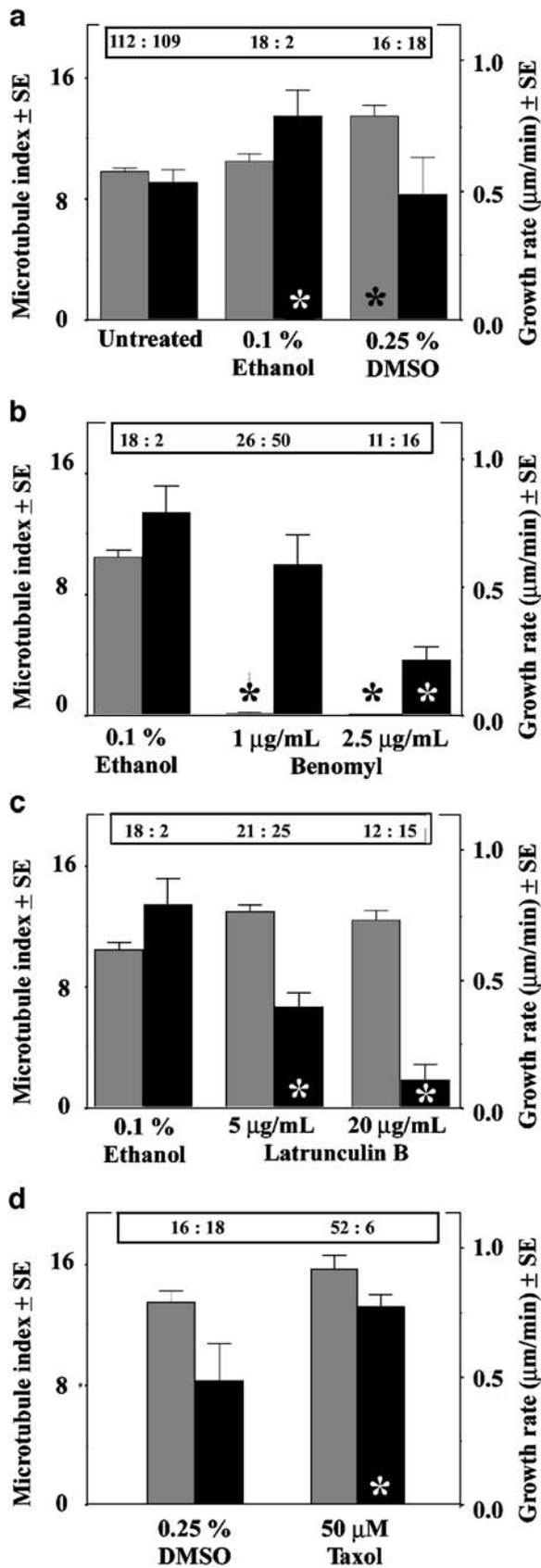
◀ Fig. 3 Effect of solvents and cytoskeleton-selective drugs on microtubule index (an estimate of relative microtubule abundance described in "Materials and methods") and growth rates of *Aspergillus nidulans* hyphae. Microtubule index (*light bars*) and growth-rate (*dark bars*) data are shown for growing hyphae. Growth and treatment methods and calculation of microtubule index and growth rate are described in "Materials and methods". The *boxed numbers* indicate the number of growing and nongrowing cells for each treatment. Observations were made between 30–120 min after treatment application (30–60 min for benomyl due to its effect on nuclei; see "Results"). *Error bars* indicate standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$) with respect to the corresponding control. **a–d** Results for untreated and solvent-treated hyphae, ethanol vs. benomyl/ethanol treatment, ethanol vs. latrunculin/ethanol treatment, and dimethyl sulfoxide (DMSO) vs. taxol/ DMSO treatment. **a** Microtubule index and growth rate for untreated, 0.1% ethanol-treated, and 0.25% DMSO-treated hyphae. The ethanol concentration is consistent with Kaminskyj (2000) and early experiments in this study (not shown) but is higher than used for the benomyl and latrunculin B treatment results. Ethanol treatment significantly increased growth rate but not microtubule abundance; the converse was found for DMSO. **b** Effect of benomyl dissolved in low concentrations of ethanol. Both concentrations of benomyl depolymerized microtubules within 2 min; growth rates measurements began 30 min after treatment and ended about 60 min, as by then, most nuclei had metaphase spindles. Growth rates for the 1 μg/ml-treated cells were statistically similar to ethanol control cells, but fewer cells in the benomyl-treated population were growing. Average growth rate was lower following 2.5 μg/ml benomyl. **c** Effect of latrunculin B dissolved in low concentrations of ethanol. Latrunculin reduced the number of growing cells in the treated population and the growth rate of the growing cells. Latrunculin treatment was associated with a higher microtubule index, but the difference was not statistically significant ($P = 0.08$). **d** Effect of taxol dissolved in 0.25% DMSO. Taxol significantly increased growth rate but not microtubule index compared with DMSO, and substantially more cells in the taxol-treated population were growing than in the control

expected. Latrunculin also reduced the proportion of growing cells compared with the ethanol control (Fig. 3c): about half the latrunculin-treated cells continued to grow during the analysis period.

Taxol has been shown to induce polymerization of *A. nidulans* MTs in vitro (Yoon and Oakley 1995), but to our knowledge, this is the first in vivo study in this species. We used 50 μM taxol in DMSO, which Yoon and Oakley (1995) had found effective in vitro for MT polymerization. We were seeking to avoid toxic side effects, especially as DMSO had already been shown to significantly increase MT index values over untreated controls (Fig. 3a). Taxol treatment did not increase the MT index significantly (Fig. 3d). Ovechkina et al. (2003) showed that mitotic spindle MTs form from the same subunits as interphase cytoplasmic MTs, so there appears to be a limited pool of unpolymerized tubulin in *A. nidulans*. However, unexpectedly, taxol-treated cells had a significantly higher average hyphal growth rate ($P < 0.01$) than the DMSO control (Fig. 3d), and a higher proportion of taxol-treated cells were growing than DMSO control cells (Fig. 3d).

Hyphal growth-rate variability

Hyphal growth rates vary over short time intervals (López-Franco et al. 1994; Sampson et al. 2003), although the underlying mechanism(s) are not fully understood. In *N. crassa*, growth-rate variations are temporally correlated with de novo generation and fusion of satellite Spitzenkörper (López-Franco et al. 1995), but this phenomenon has not been reported for *A. nidulans*. Having data in hand for large numbers of hyphae that we knew had grown during 60–300 s data collection intervals, we examined their growth rates for 15- to 30-s intervals. Confocal imaging requires fluorescence irradiation, thus energy absorption and the potential for damage. We wished to minimize this, so used longer intervals than previous studies of this type. Results for four to seven hyphae per treatment are shown in Fig. 4 to demonstrate the range of growth-rate variation without compromising visual clarity. For a larger number of hyphae than we have shown graphically, Fig. 4 also reports the number of time intervals in which hyphae did or did not grow.

Growth rates of untreated hyphae varied considerably (Fig. 4a), with rate changes up to 1 µm/min within 60 s. As expected from Fig. 2, there were substantial differences in average growth rates between untreated hyphae, but almost all intervals had measurable growth (Fig. 4a). DMSO treatment (Fig. 4b) did not affect the growth-rate variation compared

with untreated cells, but fewer intervals had measurable growth (Fig. 4b). Ethanol treatment (Fig. 4d) dramatically increased hyphal growth-rate variability, with rate changes exceeding 2 µm/min in 30 s. However, compared with untreated cells, ethanol-treated hyphae had fewer intervals with measurable growth (Fig. 4d).

Benomyl treatment depolymerized cytoplasmic MTs but did not abolish tip growth (Fig. 3b). Compared with the ethanol control (Fig. 4d), there were fewer intervals with measurable growth following benomyl treatment (Fig. 4e). Latrunculin treatment (Fig. 4f) suppressed hyphal growth-rate variability, consistent with its effect on reducing average hyphal growth rate (Fig. 3c), but the proportion of intervals with measurable growth was similar to the ethanol control (Fig. 4d).

Hyphal growth rate was not affected by repeated imaging for most of the treatment populations (Fig. 4a,b, d–f). Consequently, its effect on taxol-treated cells was notable: their average growth rate decreased significantly following imaging (Fig. 4c). Growth rates for 12 taxol-treated cells including the six shown in Fig. 4c were grouped by time from the beginning of observation (0–39.9 s, 40–79.9 s, 80–119.9 s, 120–159.9 s). The average growth rate for the 0- to 39.9-s interval (1.24±0.15 µm/min) was significantly higher than for all subsequent periods (0.78±0.11 µm/min, 0.71±0.10 µm/min, 0.51±0.08 µm/min, respectively) (P=0.001, ANOVA), with a trend for further
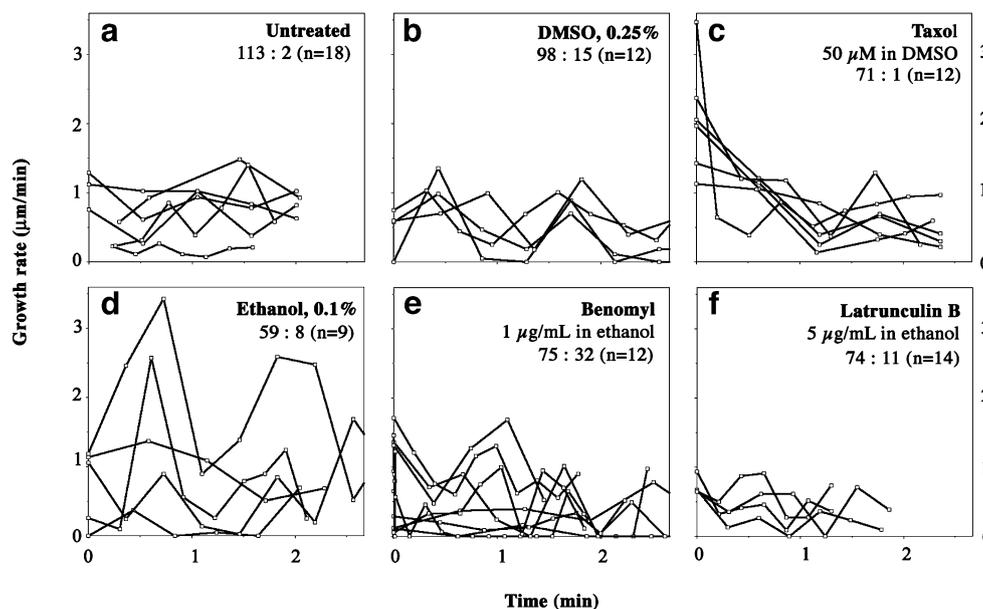

Fig. 4 Effect of solvents and cytoskeleton-selective drug-solvent solutions on growth rates of *Aspergillus nidulans* hyphae calculated for 15- to 30-s intervals. All graphs use the same axis scales. Each *line* represents a different hypha; only a representative subset from each treatment is shown for visual clarity. The *upper panel* compares untreated (**a**), DMSO-treated (**b**) and taxol in DMSO-treated cells (**c**); the *lower panel* compares ethanol treated (**d**), benomyl in ethanol-treated (**e**), and latrunculin in ethanol-treated cells (**f**). A tally of growing: non-growing intervals from a larger number of cells (n) is shown for each treatment population. The drug treatments used the same (DMSO) or lower (ethanol) solvent concentrations than the solvent controls

growth rate to depression with continued fluorescence imaging. However, almost all intervals had measurable growth. For each hypha shown in Fig. 4c, the first fluorescence image was taken after about 90 min of taxol treatment, again suggesting that growth-rate depression was fluorescence-imaging related. As a control, the growth rates of 12 taxol-treated cells were assessed as before but using irradiation with 634 nm light, which does not excite GFP. There was no change in average growth rate of the control cells with repeated imaging.

Discussion

Cytoplasm is permeated by a meshwork of cytoskeletal filaments and associated proteins that provide structure and permit function. Apart from very small molecules, little moves by diffusion through a living cell (Bray 1992), and even diffusion can be constrained by membranes. Consequently, energy must be expended for motility of organelles within the cytoplasm (Bray 1992) and for apical migration of bulk cytoplasm with respect to cortical cytoplasm (Kaminskyj and Heath 1996). Motor proteins associated with MTs and with actin have been characterized in *A. nidulans* (reviewed in Bartnicki-Garcia 2002; Konzack et al. 2005; Suelmann and Fischer 2000a, b), but the mechanics underlying wall-vesicle motility remain unclear (Bartnicki-Garcia 2002).

As well as MTs, *A. nidulans* hyphal tips contain filamentous actin arrays that have been imaged using electron tomography (Hohmann-Marriott et al. 2006), complementing whole-cell studies using actin immunofluorescence (Harris et al. 1994; Torralba et al. 1998). *A. nidulans* actin is concentrated in the apical 5 μm of growing hyphae; its abundance in more basal regions is much lower. Conversely, *A. nidulans* MTs are more abundant in basal regions than near the apex. As the metabolic resources of a considerable length of hypha (the hyphal growth unit: Trinci 1973) are needed to support tip growth, it is intuitively attractive to ascribe long-distance transport of hyphal growth materials to MTs and near-apical transport to actin. If this notion is correct, then even if individual hyphal growth rates fluctuated over short time periods (López-Franco et al. 1995; Sampson and Heath 2005), average growth rates for groups of hyphae should correlate with average relative MT abundance.

This is the first study to examine the quantitative relationship between relative MT abundance, expressed as MT index, and hyphal growth rate in large numbers of *A. nidulans* hyphae. Contrary to our initial expectation, there was no correlation between growth rate and MT index for 112 growing, untreated hyphae. Untreated hyphae that failed to grow could not be predicted from their morphol-ogy prior to data collection, or from their MT index. The only correlation we could find between growth rate and cytoplasmic MT abundance for untreated hyphae was that a large group of nongrowing hyphae had relatively fewer MTs than an equally large group of growing hyphae.

Effect of cytoskeleton-selective drugs on relative microtubule abundance and hyphal growth rate

Our early studies on untreated hyphae showed MT-index-related differences between growing vs. nongrowing cells. Our subsequent growth-rate comparisons were between growing cells (only) from different treatment groups so that our data could not be confounded by treatment-induced changes in proportion of growing cells. We compared groups of hyphae treated with cytoskeleton-selective drugs known to be effective in *A. nidulans*; we also considered the effects of low levels of solvents in which the drugs would be dissolved.

Previous work, including Kaminskyj (2000), had shown that 0.1% ethanol or DMSO had a negligible growth effect on *A. nidulans*, but that study considered a longer time period and a different cellular event. Compared with untreated cells, low concentrations of ethanol or DMSO each had significant effect: ethanol increased growth rate but not MT index, and the converse was true for DMSO. Thus, group MT index and growth-rate responses to these solvents did not change in a coordinate manner.

Benomyl at 1 μg/ml rapidly abolished essentially all visible MTs (one MT in 76 hyphae in the treated population compared with an expected 893 MTs estimated for 76 ethanol control hyphae) but did not significantly reduce the average growth rate. However, 2.5 μg/ml benomyl significantly reduced hyphal growth rate compared with the control and to the 1 μg/ml dose. It seems unlikely that there were invisible but functional MTs, whereas secondary drug effects cannot be discounted. Based on our data, we recommend use of the lowest drug concentrations possible and cautious interpretation.

Growth at 0.5 μm per min (Fig. 3b) of a hypha averaging about 3-μm wide (Fig. 1) requires addition of 4.7 μm² of cell surface per minute. Wall-forming vesicles are ~50 nm in diameter (Hohmann-Marriott et al. 2006) and have a membrane surface of 7850 nm². At least 600 vesicles this size must fuse at the tip each minute in order to generate sufficient new cell membrane. Generating these vesicles and their contents likely requires much of the resources of the hyphal growth unit. Wall vesicles are unlikely to move significant distances by diffusion, and for tip growth, wall-vesicle motility must be predominantly apex directed, whereas diffusion is random. If long-distance vesicle transport in *A. nidulans* were MT based, associations between the relatively few cytoplasmic MTs and

many vesicles should be abundant and close range. Hohmann-Marriott et al. (2006) and R. Roberson (personal communication) showed that putative wall-forming vesicles were about ~50 nm from cytoplasmic MTs, whereas cytoplasmic dynein is about 25-nm long, suggesting MTs are not the direct scaffold for vesicle transport. Furthermore, wall-vesicle transport in benomyl treated cells must use a different mechanism, presumably involving the actin cytoskeleton, as they lack MTs. Taken together, the actin cytoskeleton appears to be responsible for much of the long-distance vesicle transport in benomyl-treated as well as in untreated cells of *A. nidulans*.

Actin and MTs have been shown to interact in vitro (Pollard et al. 1984) and in vivo in *Schizosaccharomyces* (Chang et al. 2005) and *Ustilago* (Schuchardt et al. 2005). The growth-rate reduction in *A. nidulans* caused by 2.5 μg/ml benomyl, despite complete MT depolymerization from 1 μg/ml benomyl, implies there could be an as-yet uncharacterized effect of benomyl on actin-based transport, the other cytoskeleton-based motor system in fungal cells. Latrunculin treatments reduced growth rate and caused morphological abnormalities, as shown previously (Sampson and Heath 2005), as well as increased MT index values. Prolonged treatment with sublethal benomyl or cytochalasin A (another actin poison) concentrations induced multiple apical branch formation in *N. crassa* (Riquelme et al. 1998) and in *A. nidulans* (Kaminskyj and Hubbard, data not shown), so both MTs and actin appear to have complementary roles in Spitzenkörper stability. The cytoplasm tensegrity model proposed for *Saprolegnia* hyphae (Kaminskyj and Heath 1996) suggests that MTs may be important for cytoplasm cohesion. Thus, our data support results from other systems showing that actin and MT systems interact in vivo.

Hyphal growth-rate variability

Growth rates in many species vary over periods as short as 1–5 s (López-Franco et al. 1994; Sampson et al. 2003), related in part to dynamic cytoplasmic ion gradients (Torralba and Heath 2000); likely, there are multiple feedback loops. The erratic growth-rate variation we observed for untreated cells over 15- to 30-s intervals is consistent with previous reports, and as with Sampson et al. (2003), we found that growth rate varied between hyphae as well as over time. Treatment with 0.1% ethanol greatly increased growth-rate variability and increased average growth rate, perhaps by affecting membrane permeability and ion homeostasis.

Most notable regarding growth-rate variability was the effect of fluorescence imaging on taxol-treated *A. nidulans* cells. Taxol treatment induced rapid growth (Fig. 3d), but repeated imaging over time, even for minimal duration and using low intensity, tended to decrease growth rate of individual taxol-treated hyphae. All hyphae had been in taxol for a similar time prior to fluorescence imaging, and repeated imaging did not reduce average growth rates following other treatments. The average growth rate for taxol-treated *A. nidulans* hyphae in the first 40 s of imaging was significantly faster than for all subsequent intervals, although growth was not halted during the observation period. Growth rates were not diminished for hyphae irradiated with 634 nm light, where growth could be measured but GFP was not excited, suggesting that this effect is related to fluorescence excitation.

MT–taxol interactions are of considerable interest due to the use of taxol in cancer chemotherapy. The taxol binding site does not impinge on tubulin polymerization (Horwitz 1992; Ross and Fygenson 2003) and so its mechanism of action requires further clarification. Binding studies sometimes employ fluorescent taxol derivatives (Li et al. 2000). Visible light irradiation may enhance the efficacy of taxol chemotherapy given the use of fluorescent taxol derivatives. This would require that the effect of fluorescence imaging of taxol-treated GFP-α-tubulin in *A. nidulans* was comparable with fluorescent-taxol-treated cancer cell MTs. Treatment could be achieved by treating tissue with a fluorescent taxol derivative followed by appropriate wavelength visible light irradiation.

What roles do MTs appear to play in hyphal tip growth?

Our results show that relative MT abundance (MT index) and hyphal growth rate were not correlated for individual cells or for groups of cells following solvent or drug treatments. Solvent control treatments significantly affected growth rate or MT index but not both. A 1 μg/ml benomyl treatment depolymerized all cytoplasmic MTs but did not significantly affect growth rate; however, hyphal growth rates were significantly reduced by 2.5 μg/ml benomyl, suggesting that some short-term benomyl effects may be ancillary to MT depolymerization. *A. nidulans* mitosis is associated with loss of cytoplasmic MTs (Ovechkina et al. 2003; Riquelme et al. 2003), and benomyl impairs exit from metaphase (Oakley and Morris 1981). However, even when we excluded hyphae containing mitotic nuclei from our analysis and considered only hyphae that had been shown to be growing during our analysis period, we could not demonstrate a correlation between relative cytoplasmic MT abundance and tip growth rate. Conversely, weakening the actin cytoskeleton with latrunculin, which reduced growth rates, was associated with more abundant MTs.

There was a correlation between treatment effects on average growth rate and proportion of growing cells. Treatments that increased average growth rate also increased proportion of growing cells (ethanol compared with untreated; taxol compared with DMSO) and vice versa

(latrunculin compared to ethanol). With the exception of benomyl-treated cells, the average MT index in nongrowing cells was similar to or lower than in growing cells for each treatment.

Taken together, our results show the most likely role of MTs in tip growth per se is in overall regulation of average tip growth rates, perhaps mediated by the Spitzenkörper, but our understanding remains incomplete. The cell can respond to actin-selective damage by increased MT polymerization, but additional MTs do not appear to compensate for loss of actin-specific functions. Both tip growth rate and dynamics are influenced by cytoplasmic ion homeostasis.

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