5 The Cytoskeleton and Polarized Growth of Filamentous Fungi

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CONTENTS

I. Introduction

Polarized growth is the mechanism by which filamentous fungi extend their hyphae. Microtubules (MT) and filamentous actin (F-actin), in combination with their corresponding motor proteins, kinesin, dynein and myosin, play important roles in this process. Actin has an essential role for tip elongation and septation. It is required for vesicle secretion and cell wall extension, and possibly – together with the MT cytoskeleton – for the localization of so-called cell end marker or landmark proteins, which control growth directionality. The exact contribution of the MT cytoskeleton on polarized growth is less clear. Genetic, biochemical and cell biological approaches in *Aspergillus nidulans* and other fungi led to a modified view of many MT-related aspects within the past few years. There is increasing evidence that MT strings, which are visualized by immunostaining or GFP-tubulin fusion proteins, consist of several MTs and their dynamics appears to be different in fast-growing hyphal tips as compared with young germlings. Whereas the spindle pole bodies were considered as the only, or the main, microtubule organizing centres (MTOCs) in filamentous fungi, it appears that additional MTOCs outside the nuclei are responsible for the generation of the complex MT array. In addition to new insights into the MT network and its dynamics, the roles of several kinesins have been elucidated recently and their interplay with dynein investigated. It became clear that MT functions are interwoven with those of the actin cytoskeleton and that three main structures are required for polarized growth: the Spitzenkörper (vesicle supply centre), the polarisome and probably cell end markers at the cortex. We propose a model for polarized growth, where the MT cytoskeleton continuously provides the building material within vesicles to the Spitzenkörper and determines growth directionality by the delivery of cell end marker proteins and the actin cytoskeleton is crucial for the last step of vesicle secretion.

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II. The Hyphal Growth Form and the Spitzenkörper

One fascinating aspect of filamentous fungi is their continuous tip elongation. Whereas this phase of polarized growth only lasts a short time in the life cycle of the budding yeast *Saccharomyces cerevisiae*, it is the main growth form of filamentous fungi. Fungi are surrounded by a rigid cell wall and, in order to expand the hyphae, it is assumed that the walls need to be plasticized and new membrane has to be inserted. These two processes are linked because enzymes, which are required outside the cell, are transported towards the tip within vesicles. The process has been reviewed recently (Sietsma and

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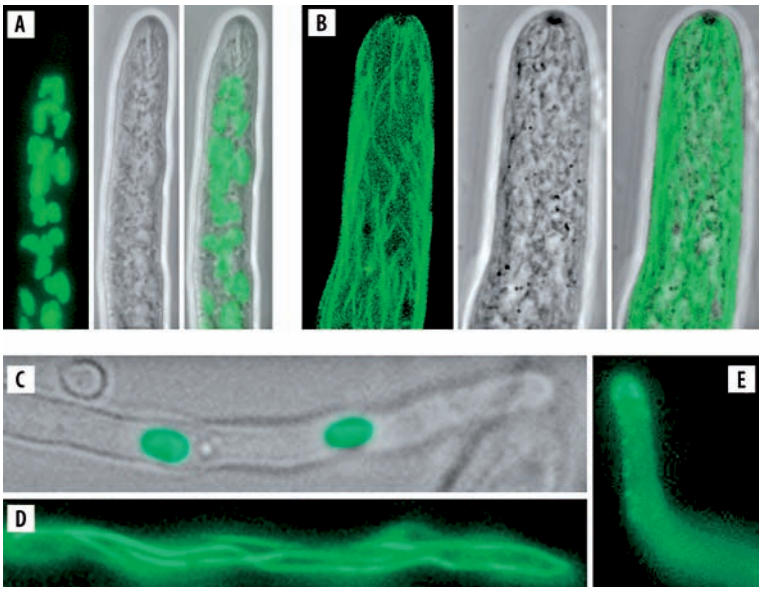

Fig. 5.1. Microtubule, actin and nuclear organization. **a** Hyphal tip of *N. crassa* with GFP-labelled nuclei: *left* GFP, *middle* phase contrast, *right* overlay. **b** GFP-labelled MTs in the tip of *N. crassa*. **c** GFP-tagged nuclei in a hyphal tip of *Asp. nidulans*. **d** GFP-labelled MTs in *Asp. nidulans*. **e** actin in *Asp. nidulans*. Images of *N. crassa* were kindly provided by Rosa R. Mouriño Pérez (Departamento de Microbiologia, Centro de Investigacion Cientifica y Educacion, Ensenada, Mexico). The actin–GFP construct was kindly provided by Miguel Peñalva (Cesic, Madrid, Spain)

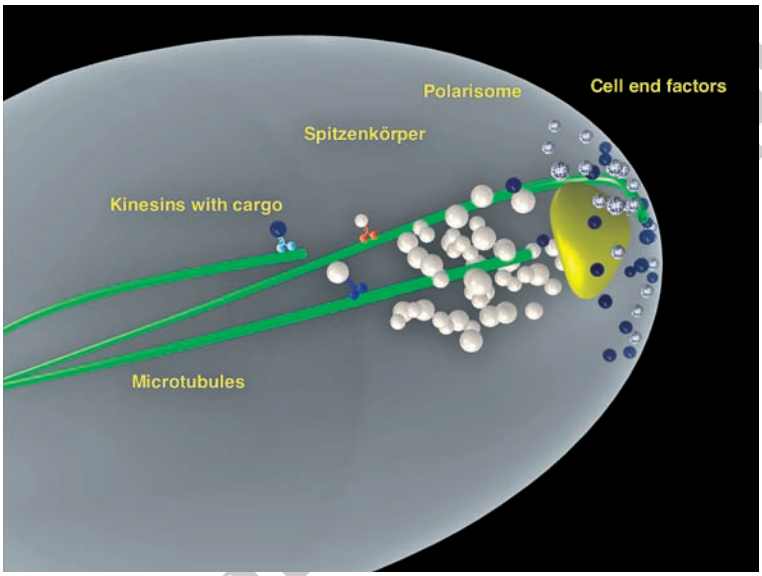

Fig. 5.2. Model of a growing hyphal tip with MTs, cargo-delivering kinesin motor proteins, the Spitzenkörper, the polarisome and cell end markers. Modified after Harris et al. (2005). See text for further explanation

Wessels 2006; Virag and Harris 2006a). The vesicles fuse with the cytoplasmic membrane and thus lead to expansion of the membrane and delivery of their contents (Bartnicki-Garcia et al. 1995). The place where vesicles are generated may be far behind the growing tip (see Chap. 1 in this volume). The involvement of vesicles for polarized growth was proposed many decades ago, when Brunswick observed an accumulation of vesicles in the apical dome of fungal hyphae, using phase contrast microscopy (Brunswick 1924; Girbardt 1957; Fig. 5.1). This structure was named with the German word "Spitzenkörper" (= apical body) or as the vesicle supply centre (VSC; Girbardt 1957; Bartnicki-Garcia et al. 1995). The latter name refers to its proposed function as a transit station for vesicles from the hyphal body to the plasma membrane. The position of the organelle determines growth direction (Riquelme et al. 1998). S. Bartnicki-Garcia (Riverside, Calif., USA) and C. Bracker (West Lafayette, Ind., USA) demonstrated fantastically the importance of this organelle for polarized growth.

Tip extension needs to be a well controlled process, because secretion of cell-wall lytic enzymes, required for loosening the cell wall prior to extension, as well as enzymes for cell wall synthe-

sis, may be deleterious for the cell. In addition, new places of polarized growth need to be established for every new branch formed (Riquelme and Bartnicki-Garcia 2004). This shows that tip elongation is likely to require the full equipment of the cellular toolbox. Several organisms are studied extensively to understand the phenomenon at the molecular level. Most importantly are *Sac. cerevisiae* and the closely related but filamentously growing *Ashbya gossypii*, the fission yeast *Schizosaccharomyces pombe*, the basidiomycete and plant pathogen dimorphic *Ustilago maydis*, the human pathogenic *Candida albicans* and several obligate filamentous fungi, such as *Neurospora crassa* and *Asp. nidulans* (Steinberg et al. 2001; Pruyne et al. 2004; Crampin et al. 2005; Harris et al. 2005; Martin and Chang 2005; Philippsen et al. 2005). Research on polarized growth currently has a very high impact, because it is considered as one major target for the development of antifungal drugs.

III. The Microtubule Cytoskeleton

A. Organization of the Microtubule Cytoskeleton

MTs are hollow tubes composed of 13 protofilaments, each of which is made up with the heterodimer αβ-tubulin as the building block. MTs have an inherent instability and continuously elongate at their plus end, where αβ-tubulin dimers are added. One parameter, which determines the elongation rate, is the concentration of dimers in the cell. Both tubulin subunits contain a bound GTP. The nucleotide-binding pocket is located at the interface between the α- and β-tubulin subunits and thus this GTP is rather stable. In contrast, GTP in the β-tubulin subunit is exposed and undergoes easily hydrolysis. Once β-tubulin contains GDP, the assembly is blocked and a catastrophic event occurs.

MTs are visible in fixed cells by immunolocalization light microscopy (Fischer and Timberlake 1995; Czymmek et al. 1996; Bourett et al. 1998) or by electron microscopy (Jung et al. 1998) but these methods do not allow the study of MT behaviour in living cells. This became possible after the advent of the green fluorescent protein (GFP). In *Sac. cerevisiae* interphase cells, short MTs are attached to nuclei and their growth towards the cortex and subsequent shrinkage causes short-distance movement of the nuclei. The situation changes once the yeast cell enters the division cycle. The nuclear spindle pole body divides, the two organelles move to opposite positions of the nucleus and polymerize the spindle MTs. In addition, the spindle pole bodies produce cytoplasmic MTs, which in turn mediate MT–cortex interactions (Hoepfner et al. 2000). In *Sch. pombe* interphase cells contain several cytoplasmic MTs, which span the entire cell. Because they serve as tracks to deliver so-called cell end markers, they determine growth directionality in this yeast (Tran et al. 2001).

In filamentous fungi, GFP-tagged MTs were studied in some detail in *N. crassa* and *Asp. nidulans* in X. Xiang's laboratory (Bethesda, Md., USA). MTs are quite inflexible structures and their orientation mainly depends on the shape of the fungal cell. Hence, they are mostly aligned parallel to the growth axis and they range in number over 3–6 (Fig. 5.1). *Asp. nidulans* MTs extend with a speed of about 14 µm/min, reach the cortex, pause for some time and undergo a catastrophic event. Subsequently, MTs shrink with a speed of about 30 µm/min and MTs either depolymerize all the way to the MTOC or rescue occurs before this, and MTs elongate again (Han et al. 2001). Slightly different values were recently obtained in the group of B. Heath (Sampson and Heath 2005). They also observed that short MT fragments were able to slide towards the hyphal tip. In *N. crassa* the MT network was first visualized by N. Read's group in Edinburgh (UK) and was analysed recently in more detail (Freitag et al. 2004; Mouriño-Pérez et al. 2006). From observations of the MT cytoskeleton in these two filamentous fungi it is obvious that the organization appears to be quite different. In *N. crassa* the MT cytoskeleton is far more complex than in *Asp. nidulans* and the number of nuclei in one compartment is also very different between the two fungi. Another big difference is the regulation of mitosis. Whereas nuclear division is synchronized in *Asp. nidulans* it is not in *N. crassa* (Suelmann et al. 1997; Freitag et al. 2004).

Investigations of MT arrangements within a cell were done by immunofluorescence and recently by using fluorescently labelled tubulin (Fischer and Timberlake 1995; Ding et al. 1998; Han et al. 2001; Freitag et al. 2004; Czymmek et al. 2005). It appears that the structures, which can be seen after immunostaining or as GFP-labelled filaments, consist of several individual MTs. There is increasing evidence for this organization, especially coming from studies with *Sch. pombe*. Here it

was shown recently that the orientation of MTs can be opposite in one bundle and that a kinesin-like motor protein in combination with dynein is required for sliding of individual MTs within a bundle and maintenance of MT polarity (Carazo-Salas et al. 2005). For *Asp. nidulans* Konzack et al. (2005) reported that the fluorescence intensity of a MT varies dynamically and that the regions with low intensity do recover brightness after some time. Similarly, after bleaching of a given MT at one place, brightness returns quickly (Veith et al. 2005). In addition, thin MT filaments occasionally detach from a MT for some time before they merge again to form a thick MT (R. Fischer, unpublished data). These observations are in agreement with a model that MT filaments consist of a bundle, and that individual MTs within a bundle undergo individual behaviour and dynamics.

B. Origin of Microtubules

MTs cannot efficiently assemble *de novo* in a eukaryotic cell, but require an initiation point, the MTOC. This point is characterized by a protein complex, whose characteristic component is γ-tubulin (Pereira and Schiebel 1997; Job et al. 2003; Aldaz et al. 2005; Doxsey et al. 2005). Originally discovered in *Asp. nidulans*, γ-tubulin was found in all eukaryotes studied and the concept of γ-tubulin-mediated nucleation of MT polymerization is an accepted model (Oakley and Oakley 1989; Oakley et al. 1990; Oakley 1995, 2004). However, the exact mechanism is still under debate. It appears that γ-tubulin in higher eukaryotes forms a 2.2-MDa ring complex consisting of 12 or 13 (different numbers exist in the literature) γ-tubulin subunits associated with other proteins, the so-called γ-tubulin ring complex (?TuRC; Aldaz et al. 2005). The γ-TuRC acts as an initiator complex, where 13 tubulin protofilaments emanate. It has been known for a long time that fungal spindle pole bodies (SPBs) are very active MTOCs (Jaspersen and Winey 2004). The SPB is embedded into the nuclear envelope, divides prior to mitosis and, by definition, localizes at the poles of the mitotic spindle. SPBs consist in *Sac. cerevisiae* of an inner and an outer plaque and they are able to polymerize MTs on both sides of the nuclear envelope. During mitosis the outer MTs are called astral MTs, but also in interphase SPBs act as active MTOCs in *Sac. cerevisiae* as well as in filamentous fungi (Heath 1981). Whether the protein composition of the SPB in *Sac. cerevisiae* and in filamentous fungi is largely conserved or more diverse remains to be determined.

It seems that the SPBs are the only places from which the yeast *Sac. cerevisiae* polymerizes MTs (see movies accompanying Hoepfner et al. 2000). However it must be noted that cytoplasmic MTs appear not to play many important roles in *Sac. cerevisiae*, besides the positioning of the nucleus prior to mitosis (Maekawa and Schiebel 2004). The cytoplasmic MT array is not very pronounced and is usually limited to a few MTs growing out of the SPB into the cytoplasm. In contrast, filamentous fungi employ MTs for their fast, polarized growth during interphase (Riquelme et al. 2003; Horio and Oakley 2005; Fig. 5.1). Nevertheless, it was assumed for a long time that SPBs are the only place for MT initiation (Oakley 2004; Czymmek et al. 2005; Sampson and Heath 2005). This assumption was based on the finding that the intracellular αβ-tubulin pool is used for the assembly of spindle MTs as well as for cytoplasmic MTs. Indeed, cytoplasmic MTs are generally disassembled prior to mitosis and regenerate thereafter (Ovechkina et al. 2003; Sampson and Heath 2005). In order to determine the origin of new MTs, re-growth of MTs was observed in *Sch. pombe* after depolymerization of MTs by drugs (Mata and Nurse 1997). These studies revealed that, in fission yeast, MTs are generated from the SPB and other MTOCs around the nucleus and in the cytoplasm. During cell division an equatorial MTOC (EMTOC) becomes very important (Hagan 1998; Sawin et al. 2004; Venkatram et al. 2005). The origin of MTs from the cell centre leads to an orientation with their plus ends towards the growing ends. Recently, another tool was used to determine the origin of MTs. Using MT plus end-localizing proteins, such as homologues of the mammalian EB1, MT initiation was analysed in the plant pathogenic basidiomycete *Ustilago maydis*. It was found that MT nucleation occurs at three places: at dispersed cytoplasmic sites, at a polar MTOC and at the SPB (Straube et al. 2003).

In filamentous fungi, our knowledge of MT organization is restricted to a few species, such as the chytridiomycete *Allomyces macrogynus*, the basidiomycete *U. maydis*, and the ascomycete *Asp. nidulans* which is one of the best studied examples. Whereas Sampson and Heath (2005) reported that MTs emanate only from SPBs, Konzack et al. (2005) demonstrated that MTOCs exist apart from the SPBs. This discrepancy may be due to the dif-

ferent methods used. In the first study, the authors observed GFP-labelled MTs and the location of nuclei was determined by the absence of cytoplasmic fluorescence. The authors of the second study used simultaneous labelling of nuclei with a red fluorescent protein and GFP-labelled tubulin. In addition, a plus end-tracking protein, KipA, was used to determine the origin of MTs. MTOCs were found at the SPBs but also in the cytoplasm and at septa of *Asp. nidulans* (Fig. 5.3). This organization recently received further evidence through the characterization of a novel MTOC-associated protein, ApsB (Veith et al. 2005). Here, the authors demonstrated that MTOCs at septa are important for the production of the interphase cytoplasmic MT array (Fig. 5.3). These findings are in good agreement with the results obtained in *Sch. pombe* and *U. maydis*.

It is still an open question whether there are MTOCs at hyphal tips of filamentous fungi. Whereas γ-tubulin can be visualized at the tips of *All. macrogynus* hyphae and thus MTs polymerize from the tip to the back (McDaniel and Roberson 1998), γ-tubulin has not yet been detected at that place in e.g. *Asp. nidulans*. Nevertheless, using the kinesin motor KipA, Konzack et al. (2005) found that sometimes MTs do also polymerize from the tip. It has to be considered that a MT occasionally does not depolymerize upon contact with the cortex but bends along the cortex towards the rear of the hypha. If this MT would continue growth, it could explain the observed comets from the tip to the back of the hypha. In *N. crassa* the situation appears to be far more complicated because of the higher number of MTs and nuclei (Freitag et al. 2004; Mouriño-Pérez et al. 2006). Detailed studies of MT origin have not yet been performed.

C. The Microtubule Plus End

MTs grow and shrink in a treadmilling manner if they are polymerized in vitro. In comparison, in vivo MTs are rather stable at the minus end and dynamics occur mainly at the MT plus end. It is well accepted that this MT end consists of a large protein complex, which is involved in the regulation of MT dynamics as well as in the regulation of interactions with cortical actin, membrane proteins or proteins associated with the kinetochore of chromosomes (Schuyler and Pellman 2001b; Hestermann et al. 2002; Akhmanova and Hoogenraad 2005). Given

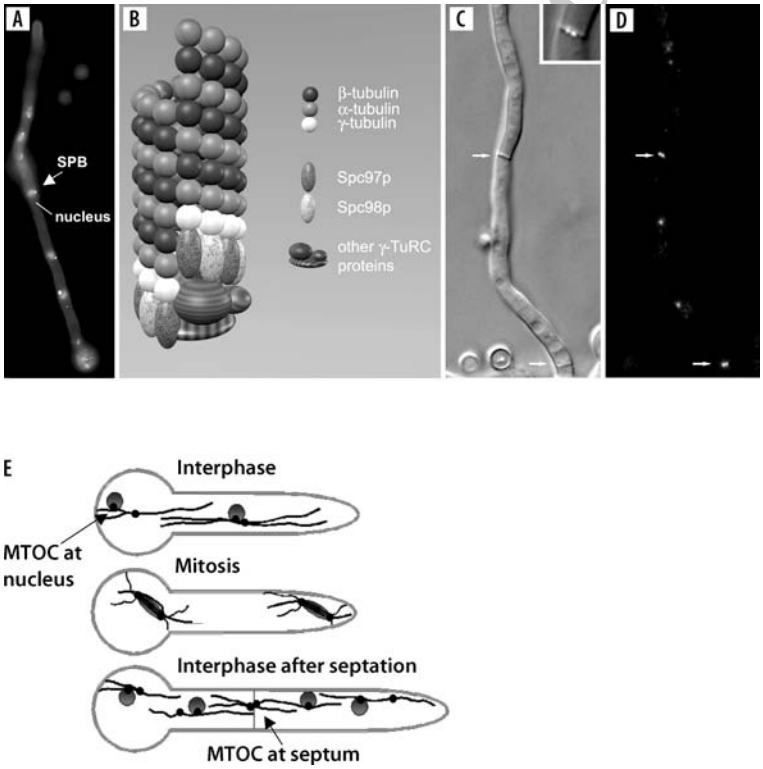

Fig. 5.3. MTOCs in *Asp. nidulans*. **a** Hypha with DAPI-stained nuclei and GFP-labelled spindle-pole body (SPB)-associated ApsB. Nuclei are evenly spaced and at each nucleus a SPB is visible. **b** Scheme of a MTOC with γ-tubulin and other proteins described in *Sac. cerevisiae*. Adapted from Pereira and Schiebel (1997) and Oakley (2000). **c**, **d** MTOCs visualized by GFP-ApsB fusion, at septa: *left* phase contrast, *right* same hypha under fluorescent conditions. *Inset* in **c** Enlargement of the septum and overlay of phase contrast and fluorescent images. **e** MTOCs are found at the nuclei, in the cytoplasm and at septa. Taken from Konzack et al. (2005), with permission

the diversity of interacting partners, it is obvious that the protein complex composition may vary depending on the function of the MT and is likely to be a highly controlled and organized structure. There are three different ways of how proteins can reach the MT plus end and remain associated with it as the MT is growing (Howard and Hyman 2003; Al-Bassam et al. 2006).

In fungi the best studied organisms with respect to the MT plus end are *Sac. cerevisiae* and *Sch. pombe*. In *Sac. cerevisiae* MT–cortex interactions play important roles for the positioning of the mitotic spindle and nuclear migration (Schuyler and Pellman 2001a). One of the most prominent examples of a MT plus end-associated protein is dynein (Fig. 5.4). It localizes to the MT tip and hitchhikes with the growing filament to the cell periphery. Once at the cortex, dynein is activated and pulls the attached MT towards the cortex. This leads to translocation of the nucleus (Schuyler and Pellman 2001a; Maekawa et al. 2003; Sheeman et al. 2003; Maekawa and Schiebel 2004). The kinesin motor protein Kip2 appears to be responsible for the plus end localization of several proteins, e.g. the CLIP170-like protein Bik1 (Carvalho et al. 2004). Similar to the situation in *Sac. cerevisiae*, the CLIP170-like protein of *Sch. pombe*, Tip1, localized to MT plus ends. The responsible motor for this localization was Tea2 (Busch et al. 2004). However MTs do not play such important roles for polarized growth in yeasts, in comparison with filamentous fungi. Only some components have been found which localize at MT plus ends, among them are subunits of the dynein motor complex (Zhang et al. 2002). Interestingly conventional kinesin, KinA, is required for their MT tip localization (Zhang et al. 2003; Fig. 5.4). The CLIP170-like protein, ClipA, in *Asp. nidulans* does also accumulate at MT plus ends and its localization is also dependent on the Tea2/Kip2 homologue KipA (Efimov et al. 2006).

The question is which role do the plus end-localized proteins play for polarized growth. As mentioned above, MT–cortical interactions are necessary for dynein-dependent nuclear positioning prior to mitosis in *Sac. cerevisiae* (Carminati and Stearns 1997). In *Asp. nidulans* dynein is also required for nuclear positioning and migration; and recently Veith et al. showed that the interaction of MT plus ends with the cortex contributes to the dynamics of mitotic spindles (Xiang et al. 1994; Xiang and Fischer 2004; Veith et al. 2005). Whether interphase nuclei are pulled through similar MT–cortex interactions is not clear yet.

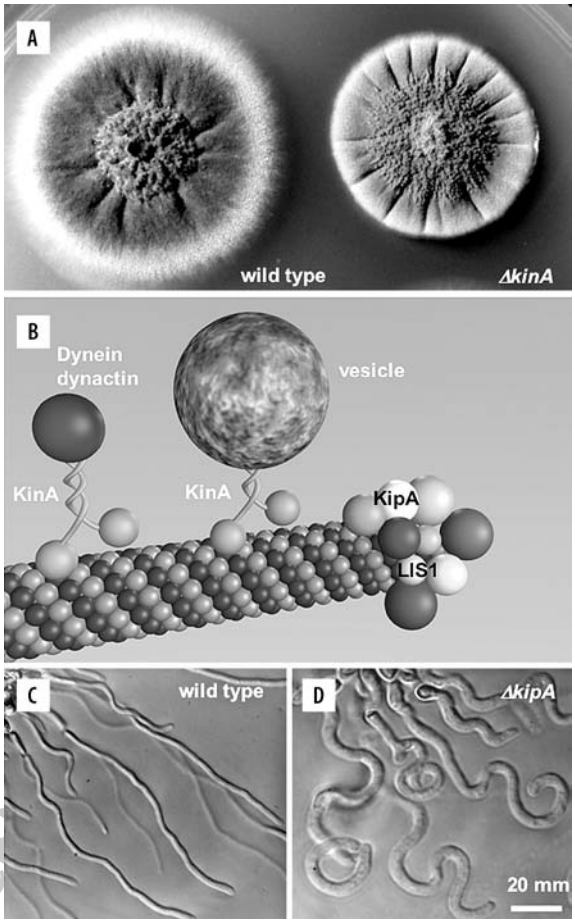

Fig. 5.4. The role of conventional KinA and Kip2 family kinesin KipA. **a** Comparison of a wild type with a conventional kinesin deletion mutant (taken from Requena et al. 2001). **b** Scheme of a MT with the MT plus end complex. This protein complex consists of several proteins, e.g. KipA or LIS1, conventional kinesin transports vesicles and components of the plus end complex, for instance dynein (Zhang et al. 2003). A direct interaction between KinA and dynein or dynactin has not yet been verified. Modified after Hestermann et al. (2002) **c**, **d** When KipA, which is suggested to be involved in the delivery of cell end markers, is missing, hyphae lose directionality. Images taken from Konzack et al. (2005), with permission

Whereas the role of the MT plus end protein complex is quite obvious for force generation to translocate organelles, a role for polarized growth is less obvious. Some new ideas came from observations in growing tips of *Asp. nidulans* and visualization of MTs. Konzack et al. (2005) described that MTs merge into one point in the apex. Given that vesicles constantly travel towards the vesicle supply centre, the position of MT ends determines the vesicle supply centre location. In the *kipA* (*tea2/kip2*) mutant, MTs did not merge into one point and

hyphae grew in meandering curves rather than straight. This was explained by the lack of proteins (normally transported by KipA) at the plus end which mediate cortical contact. There is good evidence for such a situation in *Sch. pombe*. It was shown that the cortex protein Tea1 is transported by Tea2 (Browning et al. 2003; Martin and Chang 2003; Sawin and Snaith 2004). If either of the two genes is deleted, *Sch. pombe* cells appear curved or T-shaped (Snell and Nurse 1994; Browning et al. 2000). Hence Tea1 and other proteins were named cell polarity determinants or cell end marker proteins. However, to prove such a model in *Asp. nidulans*, cargoes of KipA have to be identified and characterized. Another crucial piece in the puzzle is the identification of cortex proteins. Whereas cortical contacts of MTs involved in nuclear migration require the cortical protein ApsA in *Asp. nidulans* (Num1 in *Sac. cerevisiae*; Veith et al. 2005), this interaction appears not to be necessary for polarized growth (Fischer and Timberlake 1995). In *Sch. pombe* a new protein, Mod5, was described as a membrane anchor for the polarized growth machinery (Snaith and Sawin 2003). However, in filamentous fungi, a protein with significant sequence similarity has not yet been identified.

D. The MT Lattice

MT function and dynamics are not only determined by the plus and minus ends, but also by the filament lattice, which in higher eukaryotes can be decorated with a number of different microtubule-associated proteins (MAPs), which in turn may control the activity of associated motor proteins (Baas et al. 1994; Cassimeris and Spittle 2001; Baas and Qiang 2005; Fig. 5.4). Despite the abundance of those proteins in higher eukaryotes, it is not clear yet whether proteins like Tau exist in filamentous fungi. Proteins such as katanin and spastin could be conserved proteins, because sequences with high similarity can be found in the *Asp. nidulans* and *Asp. fumigatus* genomes (Konzack, unpublished data). Experimental data for the role of this class of MT-associated proteins are not yet available for filamentous fungi.

E. MT-Dependent Motor Proteins

MTs and their dynamics are in principle sufficient to create force and transport cargoes (attached to the growing end) in a cell. However, two classes of motor proteins have evolved which guarantee fast MT-dependent movement in the cell, the minus end-directed dynein and the plus end-directed kinesins (Fig. 5.4). Some kinesins also move in the opposite direction. Both motor classes are characterized by a motor domain in which ATP is hydrolysed (Hirokawa 1998). Within the protein the location of the motor domain can be N- or C-terminal as well as in the middle region. How chemical energy is converted into conformational changes and how force is generated is best understood for conventional kinesin. Interested readers should refer to several recent reviews (Woehlke and Schliwa 2000; Schliwa and Woehlke 2003; Yildiz and Selvin 2005; Adio et al. 2006).

Whereas all fungi employ a single dynein for their transport processes, their genomes usually contain several kinesin-encoding genes. For instance, *Asp. nidulans* harbours 11 and *N. crassa* harbours ten different kinesins (Rischitor et al. 2004; Fuchs and Westermann 2005). BimC was the first kinesin discovered in *Asp. nidulans* and defines the entire class of BimC-like kinesins (Enos and Morris 1990). The gene was discovered in a screen for temperature-sensitive *Asp. nidulans* mutants with defects in mitosis (bim = block in mitosis). BimC is a C-terminal motor which forms a tetramer with two motor domains opposite to each other. Because every head domain binds to a MT, this arrangement allows cross-linking of adjacent MTs. This feature is very important during mitosis, where mitotic spindle MTs slide along each other to distribute chromosomes (Kapitein et al. 2005). Whereas BimC was discovered in a genetic screen (Morris 1976), four other kinesins were isolated in reverse genetic approaches.

A second motor with functions in mitosis is the C-terminal kinesin-like protein KlpA with similarity to *Sac. cerevisiae* Kar3 (Prigozhina et al. 2001). The gene was isolated through a PCR approach and subsequently characterized. Deletion of *klpA* alone did not produce any severe phenotype but suppressed a *bimC* mutation (O'Connell et al. 1993).

Another kinesin with a function in mitosis is the Kip3 family member KipB, where the motor domain is localized closer to the N-terminus. Gene deletion did not cause any defect in hyphal extension or organelle movement, but in chromosome segregation (Rischitor et al. 2004). This was surprising, because a similar motor in *Sac. cerevisiae*, Kip3, is involved in nuclear migration (Miller et al. 1998). However, *Asp. nidulans* KipB results are in good agreement with results for the homologous

proteins in *Sch. pombe*, Klp5 and Klp6 (West et al. 2002).

Two motors with N-terminal motor domains and pronounced roles in polarized growth are conventional kinesin, KinA and the CENP-E family kinesin KipA. Deletion of *kinA* resulted in slower hyphal growth, which is similar to effects in other fungi (Lehmler et al. 1997; Seiler et al. 1997; Wu et al. 1998; Requena et al. 2001; Fig. 5.4). It is generally accepted that this motor transports vesicles towards the extending tip and provides cell wall components (Seiler et al. 1999). In addition, it appears to be involved in other cellular processes related to polarized growth, namely mitochondrial and nuclear distribution. Whereas nuclear distribution was affected in *N. crassa* and *Asp. nidulans*, mitochondrial distribution was changed in *N. haematococca* (Wu et al. 1998). This may be due to the fact that mitochondrial movement depends on the actin cytoskeleton in *Asp. nidulans* (Suelmann and Fischer 2000) and on the MT cytoskeleton in *N. crassa* (Fuchs et al. 2002; Fuchs and Westermann 2005). Whether mitochondrial distribution is also altered in *N. crassa* conventional kinesin mutants, has not yet been studied. The mechanism of how conventional kinesin may contribute to mitochondrial or nuclear distribution is not yet clear, but it could be that the effects are indirect. It was shown in *Asp. nidulans* that KinA is required for transportation of dynein subunits to the plus end of MTs (Zhang et al. 2003; Fig. 5.4). Dynein is a crucial motor for nuclear migration; and exclusion of dynein from the MT plus ends could cause the observed nuclear clustering (Xiang et al. 1994). In addition, it has to be considered that conventional kinesin may well be involved in delivering other components of the MT plus end complex. Lack of conventional kinesin could thus influence the dynamics of MTs as well as their cortical interaction.

KipA of *Asp. nidulans* is similar to Tea2 in *Sch. pombe* and is characterized by an N-terminal motor domain (Konzack et al. 2005). It accumulates at MT plus ends and appears to reach this place by an intrinsic motor activity. Mutant proteins, in which a crucial residue for ATP hydrolysis was replaced, lost the ability to accumulate at MT tips but decorated them evenly. These findings were in agreement with studies of Tea2 in *Sch. pombe* (Browning et al. 2003). Gene deletion caused a surprising phenotype in *Asp. nidulans*. Delta *kipA* strains grew as well as wild-type strains but the hyphal morphology was changed. In contrast to MTs in the wild type, MTs in the *kipA* deletion strain did not merge into one place at the apex. This was interpreted as a reason why hyphae would meander. If MTs do meet at one point, they would deliver the vesicles, which are transported along them at one place, the Spitzenkörper. Hence, hyphae would grow straight. If MTs do not merge into one point, vesicles would be delivered at different places and arbitrarily a majority could be deposited on the left, in the middle or on the right side of the hypha. For instance, if the majority of vesicles were deposited asymmetrically at the left, the hypha would grow to the left. The KipA protein could transport proteins, which are necessary for temporal anchorage of MT at the cortex at a specific point. Those proteins would be crucial for straight growth and, because they labelled the end of the cell, they were named "cell end markers" in *Sch. pombe*. Examples of such a protein in fission yeast are Tea1 and Tip1 (Browning et al. 2003; Busch et al. 2004). However, MT fixation at the cortex through Tea1 has not been shown. Tea1 may indeed be evolutionarily conserved among fungi, because a similar protein has been localized to the growing hyphal tip in *Asp. nidulans* (Konzack, Takeshita and Fischer, unpublished data).

Deletion of any kinesin motor (besides *bimC*) does not cause severe phenotypes. Interestingly even a strain in which KinA, KipA and KipB were deleted was still viable, although hyphal growth and development were quite severely affected (Konzack et al. 2005). This shows that kinesins can substitute for each other to some extent, which was recently shown nicely in the case of the Unc-104 homologues, Nkin-2 and Nkin-3 from *N. crassa*. Whereas Nkin-2 associates with mitochondria and connects mitochondria with MTs, Nkin-3 was found in the cytoplasm. Surprisingly, after depletion of Nkin-2, Nkin-3 was upregulated and also bound to mitochondria and MTs (Fuchs and Westermann 2005). Homologues of these two motors do also exist in *Asp. nidulans* and are currently being investigated in our laboratory. It appears that one of them, UncA, plays an important role in hyphal tip extension whereas the other one, UncB, is likely to play a role in the nucleus and during septation (N. Zekert, unpublished data).

As mentioned above, fungi usually contain only a single dynein protein, although in some basidiomycetes the heavy chain is encoded by two genes (Eshel et al. 1993; Xiang et al. 1994; Straube et al. 2001; Yamamoto and Hiraoka 2003; Martin et al. 2004). Dynein has a crucial role in nuclear migration but is also implicated in vesicle transport

(Seiler et al. 1999). Because dynein moves towards the MT minus end, it is difficult to imagine that it is directly involved in polarized growth, given that MTs are mainly oriented with their plus ends to the membrane. Indeed, deletion of dynein does not cause an immediate block of hyphal extension and the impact on colony growth could partly be due to the lack of nuclei and other organelles, which are translocated with the help of dynein (Xiang et al. 1994).

Besides the concerted action of the cytoskeleton and associated motor proteins to translocate organelles, cytoplasmic streaming has to be considered as another mechanism to push forward the cytoplasm and organelles. Mouriño-Pérez et al. (2006) showed recently in *N. crassa* that the MT array was able to advance as a unit as the hypha elongates. The basis for this bulk flow has not yet been resolved.

If MTs play a role in vesicle delivery to the growing hyphal tip, the question remains how the places for cell extension are marked. First insights into this process came from studies in *Sch. pombe*.

F. Cell End Makers at the Cortex

One of the first proteins which labelled a growing yeast end was discovered in *Sch. pombe* in a screening for polarity mutants. One of the corresponding genes, which was cloned by complementation, encodes the Tea1 protein (Mata and Nurse 1997). The protein is hitchhiking with growing MT ends and is delivered at the pole, where it associates with the cortex. The second one, Tea2, encodes a kinesin-like motor protein (Browning et al. 2000; Fig. 5.5). It was shown recently that the main membrane anchor, which recruits proteins such as Tea1, is Mod5 (Browning et al. 2003; Snaith and Sawin 2003). This protein is posttranslationally modified with a prenyl residue, which confers membrane association. Among the proteins recruited to the Mod5 anchor is also the formin, For3 (Bretscher 2005; Martin et al. 2005; Martin and Chang 2006). This protein initiates the growth of actin filaments away from the growing tip. These cables can be used as tracks for the vesicles necessary for cell extension.

Given that the machinery is largely conserved in filamentous fungi and that the crucial component, Mod5, could not yet be identified, the question remains what targets Mod5 to the membrane at the pole of the cell rather than along the cell. This points to a key function of the membrane itself. Indeed, it was proposed some time ago that sterol-rich lipid rafts exist which may cause asymmetric distribution of proteins in a membrane (Grossmann et al. 2006; Hancock 2006). There is recent evidence that these membrane domains play a role in the polarized growth of filamentous fungi (Martin and Konopka 2004); and the laboratory of S. Harris has shown that a ceramide synthase is important for hyphal morphogenesis (Li et al. 2006).

Because the installation of the growth machinery at a specific place determines growth directionality, one would expect that external signals influence the architecture of proteins. Indeed recently a kinase with such a potential was described in *Asp. nidulans* (Li et al. 2006). This kinase has a well characterized role in DNA damage response, but Li et al. (2006) found that deletion also affects the establishment of polarized growth. The reason appears to lie in a disorganization of MTs in the apex, similar to the defect in the kinesin mutant Δ*kipA* (Konzack et al. 2005). Whereas MTs merge in one point in the wild type, they are dispersed in the *atmA* and the *kipA* mutants. In both cases the authors argue that MT–cortex interaction might be affected. Two further candidates for regulation of protein activity are Pod6 and Cot1, which were described in *N. crassa*, although they are distributed evenly along the hypha and to not show an accumulation at the growing tip (Seiler et al. 2006).

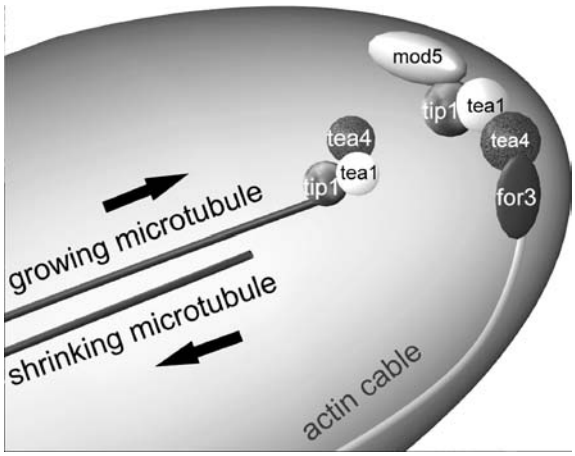

Fig. 5.5. Model of polarized growth in *Sch. pombe*. Reprinted from Martin and Chang (2003), with permission from Elsevier

IV. The Actin Cytoskeleton

A. Organization of the Actin Cytoskeleton

Immunostaining of actin or visualization with phallloidin derivatives revealed a spot-like distribution of the protein along the cortex in many fungi with a high concentration at the tip. In comparison, in *Ash. gossypii* actin cables are frequently seen (Schmitz et al. 2006). Meanwhile, actin fuses to GFP, which allows in vivo studies of the dynamics of actin (B. Oakley, personal communication; Fig. 5.1). Furthermore, Peñalva et al. fused an actin-binding protein with GFP, which is a nice tool to study actin localization and behaviour in living *Asp. nidulans* cells (M. Peñalva, personal communication). The important role actin plays in polarized growth becomes obvious when depolymerizing agents, such as latrunculin B or cytochalasin, are added to growing hyphae. Sampson et al. showed that addition of latrunculin B causes a fast block in hyphal extension (Sampson and Heath 2005; Fig. 5.6). Likewise, deletion of the myosin gene, *myoA*, is lethal (McGoldrick et al. 1995). There are two likely contributions of the actin cytoskeleton to polarized growth. On the one hand, the actin–myosin cytoskeleton is used for vesicle transportation and secretion and thus the delivery of cell wall components. On the other hand, cortical proteins are brought into place by this system in *Sac. cerevisiae* and guarantee the proper attachment of MTs to the cortex (Schuyler and Pellman 2001b). Because MT attachment sites required for polarized growth seem to be very defined in the apical dome (see below), it is conceivable that the actin cytoskeleton plays a role at this point as well. However, further experiments are required to unravel the exact mechanisms.

As another aspect of polarized growth, we should consider the existence of a Ca^{2+} gradient along the hypha with a high concentration at the tip of *Phyllosticta ampelicida* and *N. crassa* (Shaw et al. 2001; Silverman-Gavrila and Lew 2003; see also Chap. 9 in this volume). In the absence of this gradient, hyphal polarity is affected (Schmid and Harold 1988). Although the effect has been known for a long time, a direct link to the machinery described above has not emerged yet. One explanation for the role of Ca^{2+} ions is the stimulation of vesicle fusion with the membrane. The Ca^{2+} concentration appears to be regulated through a stretch-activated phospholipase C at the tip, which catalyses the formation of inositol (1,4,5)-triphosphate (IP_3) and in turn causes the release of Ca^{2+} from special vesicles (Silverman-Gavrila and Lew 2002).

B. The Polarisome

A protein complex related to the actin cytoskeleton is localized at the incipient bud of *Sac. cerevisiae* and is named the polarisome (see Chap. 6 in this volume). This structure is involved in the organi-

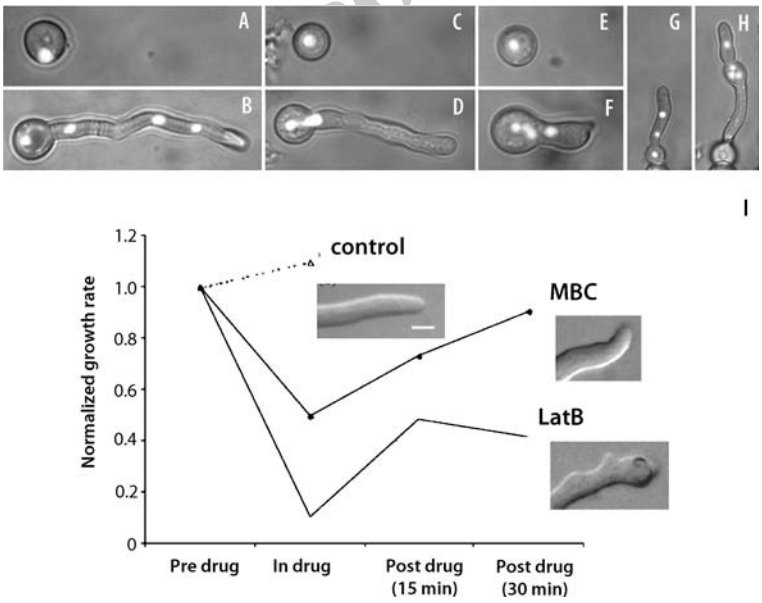

Fig. 5.6. Effect of anti-microtubule and anti-actin drugs on polarized growth of *Asp. nidulans*. **a**, **c** Conidia of a wild-type strain with a GFP-tagged nucleus. **b**, **d** Germlings of the strain in **a**, **c** grown for 10 h at 30 °C in minimal medium (**b**) or minimal medium supplemented with 1.5 µg|ml benomyl. Hyphae elongated and the nucleus divided but nuclei did not migrate into the germtube. **e–h** Germination of a conidium in the presence of 50 µg/ml cytochalasin A. Some spores just swelled but did not form germtubes (**e**) whereas others formed short, more or less deformed hyphae (**f–h**). Nuclear distribution was not specifically affected and sometimes normal (**f**, **h**). **i** Comparison of the growth rate of hyphal tips in the presence of the MT drug MBC or the actin drug latrunculin B (LatB). Taken from Sampson and Heath (2005), with permission

zation of the actin cytoskeleton and its appearance resembles the Spitzenkörper in filamentous fungi (Sagot et al. 2002). There is evidence that this protein complex also exists in filamentous fungi as structure separate from the Spitzenkörper (Harris and Momany 2004). The existence of polarisome components in filamentous fungi was shown first in *Asp. nidulans*. Sharpless and Harris demonstrated that SepA – an orthologue of a key component of the yeast polarisome, Bni1 – colocalize with the Spitzenkörper (Sharpless and Harris 2002). Similarly in *Ash. gossypii*, a filamentous fungus very closely related to *Sac. cerevisiae* (Wendland and Walther 2005), a homologue of the *Sac. cerevisiae* polarisome protein Spa2 was analysed (Knechtle et al. 2003) and recently also the Bni1 orthologue, AgBni1 (Schmitz et al. 2006). Whereas Spa2 is not essential in *Ash. gossypii*, but is necessary for fast polarized growth, deletion of *Agbni1* causes loss of polarization and swelling of the cells to a potato-like appearance. A Spa2 orthologue has been characterized in *C. albicans* as well and its role studied during filamentous growth (Zheng et al. 2003). The protein persistently localize at hyphal tips and deletion causes defects in polarity establishment. Recently, Crampin et al. suggested that the polarisome and the Spitzenkörper are distinct structures which coexist in hyphae (Sagot et al. 2002; Crampin et al. 2005; Fig. 5.2, see page 120). Similar results for Spa2 (SpaA) have been obtained in *Asp. nidulans*, suggesting that a polarisome or the existence of polarisome components at the growing hyphal tip could be a general theme for filamentous fungi (Virag and Harris 2006b). According to this model, filamentous fungal cells employ both the MT and the actin cytoskeleton and, related to these structural elements, the Spitzenkörper as vesicle supply centre and the polarisome for actin organization.

The machinery discussed so far describes how fungi could extend their hyphae, but this picture does not yet allow any adaptation of the process to external (e.g. nutrient gradients) or internal signals (e.g. the stage of the cell cycle). Little is known so far about the transduction of such signals into e.g. changes of growth direction, although several regulatory proteins have been described which influence polarized growth, probably through an interaction with the actin cytoskeleton. The principle of this possible regulation is best studied in *Sac. cerevisiae* (Tcheperegine et al. 2005) and some of the components appear to be conserved in filamentous fungi. Among those are members of the Rho and Rac families, small GTPases which act as molecular switches (Boyce et al. 2001, 2003, 2005; Guest et al. 2004; Momany 2005; Virag and Harris 2006a). However, a detailed analysis for the exact role in polarized growth in filamentous fungi remains to be done.

C. Actin-Dependent Motor Proteins

The function of the actin cytoskeleton depends on the activity of actin-dependent motor proteins, the myosins. Myosins serve a broad range of cellular functions and are grouped into 18 different classes. In *Asp. nidulans* a class I myosin was identified and shown to be required for protein secretion and polarized growth and with an essential role for viability (McGoldrick et al. 1995). It localizes to the growing hyphal tip (Yamashita et al. 2000).

Given that myosin motors are involved in vesicle transportation towards the cell cortex and vesicle fusion with the cell membrane, it is very interesting that *Asp. nidulans* employs a myosin-derived motor domain for the transportation of class V and class VI chitin synthases, where the motor domain is directly fused to the enzyme (Horiuchi et al. 1999; Takeshita et al. 2005, 2006).

Myosin motor proteins of other classes have been described, e.g. in *Sac. cerevisiae*, where a class V myosin motor is involved in the inheritance of peroxisome and other organelles (Bretscher 2003; Fagarasanu et al. 2006). A second class V myosin is required for RNA transportation (Bretscher 2003).

D. The *swo* Mutants and the Establishment of Polarity

So far we have discussed polar growth in the sense of maintaining polarized extension by recruiting the cellular machinery for cell wall assembly to the tip of an existing hypha. An interesting remaining question is how polarity is established starting from round spores, such as conidiospores in the case of *Asp. nidulans*. This crucial question has been addressed in *A. nidulans* by the isolation of three temperature-sensitive mutants, *swoC*, *swoD* and *swoF*, in which spores swell at restrictive temperature but do not produce a germ tube (Momany et al. 1999). The SwoC protein displays homology to rRNA pseudouridine synthases of yeast and the role in polarized growth still remains obscure. In contrast SwoF has a high identity with N-myristoyl transferases and it is speculated that a polarity determinant could be the substrate for myristolya-

tion (Shaw et al. 2002). This posttranslational protein modification is found in proteins which switch between membrane-bound and cytoplasmic states (e.g. G protein α-subunits) and could be important for the localization of cell end markers or other landmark proteins, as discussed above (Bathnagar and Gordon 1997). Therefore the identification of prenylated or myristoylated proteins appears to be of prime importance for understanding polarity establishment in filamentous fungi.

V. Conclusions

The past few years have provided many new insights into the role and interplay of actin and the MT cytoskeleton in the polarized growth of fungi. It appears that the main function of MTs is to deliver vesicles and cell end markers. Especially the latter function needs much more attention, since only one putative cell end marker protein has been identified in *Asp. nidulans* so far. If homologues of *Sch. pombe* cell end markers exist in filamentous fungi, questions remain: what exactly are their biochemical functions, which downstream events do they trigger to allow straight hyphal growth and which upstream regulatory circuits are integrated? The publication of several fungal genome sequences along with the continuous improvement of molecular and microscopy techniques promise a fruitful future for cytoskeletal research in fungi.

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