HYPHAL TIP GROWTH: OUTSTANDING QUESTIONS

Salomón Bartnicki-García

Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, México and Department of Plant Pathology, University of California, Riverside, California.

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I. General

The remarkable ability of fungi to make tubular cells or hyphae is an exquisite case of polarized growth and has been the subject of intense attention and experimentation for >100 years. Much has been learned about many facets of hyphal morphogenesis, but a precise understanding of the structural, biochemical, and genetic basis of apical growth remains to be attained.

In this chapter I will summarize some new developments in hyphal morphogenesis and address some of the outstanding questions in this field. Hyphal morphogenesis or tip growth of fungi has been the subject of recent reviews, each with a somewhat different perspective (1-8).

II. Key structures and processes in tip growth

Myriad genetic and biochemical processes are involved in the growth of a hypha. Its is therefore not surprising that a wide variety of genetic, biochemical, and environmental alterations have been reported to have a controlling impact on hyphal growth and morphogenesis. The challenge is to narrow the search to those events that are immediately or directly involved in the production of a tubular cell wall by tip growth. Understanding how a fungus establishes a polarized gradient of wall formation is clearly the ultimate objective in the search for the basis of tip growth.

Different researchers have employed different experimental tools and applied widely different emphasis. The end result is a number of seemingly divergent models to explain of tip growth in fungi (see III).

A. The Central Question - Polarized Secretion

Since cell wall formation in fungi is the result of a secretory process, it follows that tip growth is basically a polarization of the secretory apparatus of the cell. A most vivid example of the subtlety of the polarization mechanism can be seen during germ tube emergence in *Mucor rouxii* (Fig. 1). Without any detectable change in the overall rates of cell growth or wall synthesis, or in any other major metabolic parameter, the pattern of wall deposition in the germ sphere switched from uniformly dispersed (isotropic) to highly polarized at the site of germ tube emergence. We concluded that a corresponding spatial reorganization of the underlying secretory apparatus took place with no other perceptible change in cell physiology or metabolism (9). This morphogenetic transition seems ideal to identify genes selectively expressed during the switch from isotropic to polarized growth, an approach recently adopted (see III.D.3).

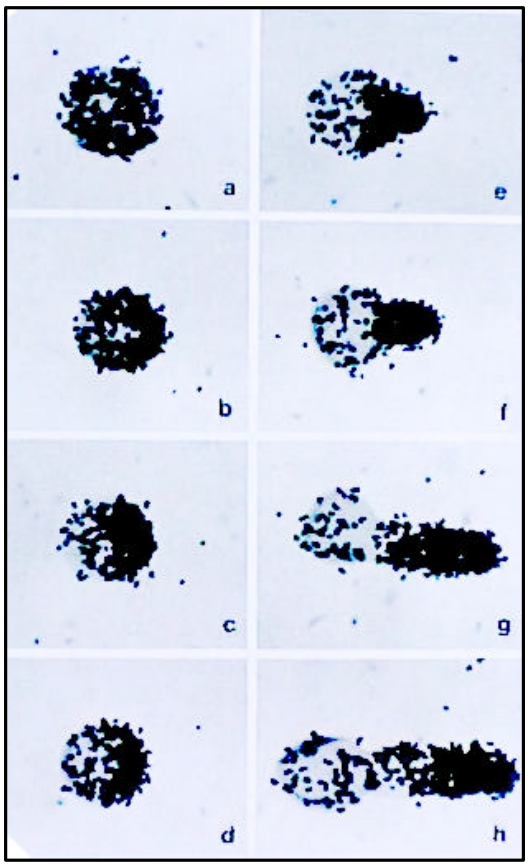

Figure 1. Autoradiographs of germinating spores of Mucor rouxii assembled to show the progressive polarization of cell wall deposition (chitin and chitosan) in the germ sphere prior, during and after germ tube emergence. Modified from Bartnicki-Garcia and Lippman (9).

B. The Spitzenkörper

Because of its location in the apical dome of growing hyphae, the Spitzenkörper has attracted much attention from those interested in understanding the mechanism of apical growth in fungi. Brunswik's (10) original discovery of an iron-hematoxylin-staining body in the hyphal tips of *Coprinus* spp. went largely unnoticed until Girbardt's studies confirmed the existence of a Spitzenkörper in living hyphae by phase contrast microscopy (11). Phase contrast microscopy provides the best optics to study the structure and behavior of the Spitzenkörper in living specimens of fungal hyphae (12-18). The video microscopic surveys made by Lopez-Franco and Bracker (13), on > 30 different fungal species, recognized eight different patterns of Spitzenkörper organization in higher fungi; the large Spitzenkörper found by Vargas et al. (19) in the lower fungus *Allomyces macrogynous* constitutes a ninth unique type. This morphological variability has yet to be reconciled with the proposed function of the Spitzenkörper.

1. Organizer of vesicle traffic

Although the exact function of the Spitzenkörper has not been well established, there is strong reason to believe, because of its position, composition and behavior, that it serves to organize or direct vesicle traffic for hyphal elongation. This characteristic accumulation of wall-building vesicles in the hyphal apex is much more than an simple case of traffic congestion. The Spitzenkörper appears to be a highly evolved distribution center for collecting vesicles and delivering them to the cell surface. The cluster of Spitzenkörper vesicles accumulates around a

core region, whose biochemical identity remains a mystery. Both microtubules (20-22) and actin microfilaments (23-24), and other unrecognized granular material have been found in the Spitzenkörper region. More recently, McDaniel and Roberson (25) discovered that γ-tubulin is a component of the central region of the Spitzenkörper of *Allomyces macrogynous* (Fig. 2), a finding that corroborated earlier suspicions that the Spitzenkörper functions as an MTOC (26). Since microtubules are considered to be cellular tracks for the long-range transport of vesicles (26-29), a system of microtubules rooted in the MTOC of the Spitzenkörper would explain the vesicle-gathering role of the Spitzenkörper.

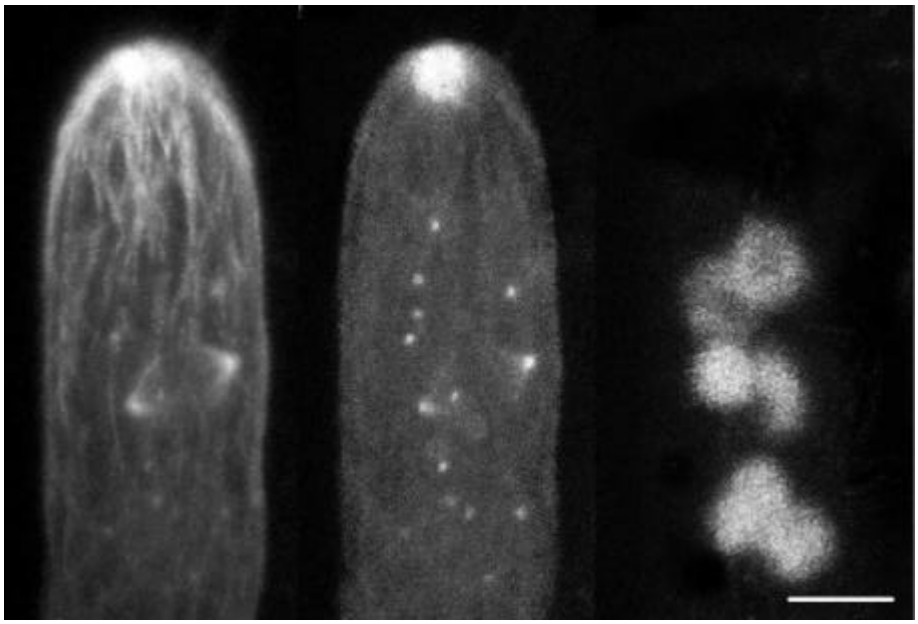

Figure 2. Confocal microscopy of the microtubular cytoskeleton in a hyphal tip of *Allomyces macrogynous*. Left. Immunofluorescnce labeling of ? -tubulin shows cytoplasmic microtubules converging at the apex. A single mitotic spindle is seen in the subapex. Center. Immunofluorescnce labeling of γ-tubulin shows a large accumulation in the Spitzenkörper region. The small discrete spots in the subapex correspond to centrosomes. Right. DAPI staining of nuclei in the same hypha. A and C reproduced courtesy of McDaniel and Roberson (25); B is a previously unpublished image supplied by McDaniel & Roberson from work first reported in (22). Scale bar = 5 um.

2. The Spitzenkörper as a Vesicle supply Center (VSC)

A vesicle-based computer simulation of fungal morphogenesis led us to the realization that different cell shapes could be generated by displacing the immediate source of wall-making vesicles, called the vesicle supply center (VSC) (30). We showed that linear displacement of the VSC generated hyphal shapes. This shape-building process was described by a simple equation

$$y = x \cot \qquad (XV/N)$$

named the hyphoid, that relates the number of wall-building vesicles released from the VSC per unit time with the rate of advancement of the VSC. The 2D shape described by the plotted hyphoid was almost identical to the shape of well-preserved hyphae in longitudinal median section (30).

In view of the remarkable coincidence between the position of the VSC in the hyphoid and the position of the Spitzenkörper in a real hypha, we proposed that the key function of the Spitzenkörper was to serve as a VSC, i.e., a movable distribution center for wall-building vesicles (30, 31). Accordingly, morphogenesis would be primarily determined by the interplay between movement of the Spitzenkörper and the amount of wall-building vesicles emanating from the Spitzenkörper.

Several examples of hyphal morphogenesis in different fungi were analyzed by computer simulation (Fungus Simulator©) (32) including normal hyphal growth, spontaneous hyphal bulging (14, 33), induced apical branching in a temperature sensitive mutant (15), hyphal meandering (17), and deformed growth of mutated hyphae (18). In each morphogenetic example, the VSC of the Simulator was programmed to follow the actual trajectories of the Spitzenkörper. In all cases, morphology could be explained by assuming that the Spitzenkörper functions as a VSC.

Figure 3. Abrupt changes in growth directionality of a hypha of *Trichoderma viride* during a Spitzenkörper chase lasting 9 min. The Spitzenkörper was forced to move in different directions by shifting the position of the laser trap (white stars). Black arrows show the Spitzenkörper at the beginning and the end of the sequence. Reconstruction made from a videotaped sequence supplied by C. E. Bracker and R. Lopez Franco (35). The montage contains eight images captured at 0, 2.0, 2.6, 3.0, 4.7, 5.8, 6.9, and 8.9 min.

3. Spitzenkörper Trajectory

Video microscopy and image analysis of living hyphae of *Neurospora crassa* showed a close correlation among Spitzenkörper position, trajectory, and the growth direction of a hypha (18). A permanent change in growth direction, i.e. the establishment of a new growth axis, was correlated with a sustained shift in Spitzenkörper trajectory away from the existing cell axis. This study confirmed Girbardt's finding (11) that an off-center displacement of the Spitzenkörper precedes a change in growth direction of the hypha.

Spitzenkörper trajectory determines not only growth directionality (Riquelme et al. 17) but also the overall appearance of a hypha (18, 14). In the straightest hyphae, the Spitzenkörper advances along a straight path with frequent but minute transverse oscillations. In hyphae with highly distorted morphology, e.g. in the *ropy* mutants of *N. crassa*, the trajectory of the Spitzenkörper became erratic; sustained departures of the Spitzenkörper from the hyphal growth axis produced corresponding distortions in hyphal morphology (18). The growing scaffolding of cytoplasmic microtubules in a hypha was proposed as the mechanism that maintained the Spitzenkörper on a rather fixed trajectory (17). An alternative explanation (34) invoking inhibitory substances secreted by advancing hyphae as the primary determinants of growth directionality was considered unlikely (17). A future challenge would be to find out how external factors such as light, chemicals, etc., affect the intrinsically fixed directionality of hyphae to bring about the well known tropic responses of fungal hyphae.

Perhaps the most compelling case for believing that the Spitzenkörper functions as a "steering wheel" responsible for growth directionality and morphogenesis of a hypha comes from the laser experiments done by Bracker and coworkers (35). By manipulating (chasing) the Spitzenkörper with laser tweezers, they discovered that it could be displaced at will to produce corresponding changes in the direction of hyphal elongation, and other morphological alterations (Figure 3). Their findings support the hypothesis that the patterns of apical exocytosis are governed by the position of the Spitzenkörper.

4. Growth Pulses and Satellite Spitzenkörper

Two recent findings by Lopez-Franco and coworkers (12, 36) have modified our basic understanding of the physiology of hyphal tip growth. First, the well-established notion that hyphae elongate at a steady rate when grown under constant environmental conditions is, strictly speaking, incorrect. When analyzed with the high precision made practical by computer-enhanced video microscopy, i.e., measuring elongation rates at high magnification and at 1 to 5 sec intervals (rather than minutes or hours as is the usual practice), elongation was found to be always a pulsatile phenomenon (36). In the seven fungal species examined, representing major taxonomic groups, periods of slow and fast growth alternated at a somewhat regular frequency. Pulsation varied from species to species and ranged from 2.7 to 14 pulses/min on the average. The other related finding was the discovery of satellite Spitzenkörper, i.e., small packages of vesicles that arise a few micrometers behind the apical pole, migrate rapidly to the hyphal apex and merge with the main Spitzenkörper and thus appear to contribute to the growth of the hyphal apex (12). Satellites were frequently detected and their occurrence may be related to growth pulsation. In two fungi, the fast phase of the growth pulses was correlated with the merger of satellite Spitzenkörper with the main Spitzenkörper. It remains to be determined whether satellite Spitzenkörper are mainly responsible for pulsation or whether they simply exacerbate a presumed intrinsic pulsation of the secretory apparatus. Alternative interpretations have been

suggested by Johns et al. (37) who measured the force exerted by the tips of *Achlya bisexualis* on a miniature strain gauge, and found that the force fluctuated with a periodicity comparable to that of growth pulses. They speculated that such fluctuations may reflect minute changes in turgor pressure that are beyond measurement, or pulses in wall hardening and wall loosening.

5. Spitzenkörper origin

Studies by Reynaga-Peña and coworkers (15,16) on an apical-branching, temperature-sensitive mutant of *Aspergillus niger* (*ramosa-1*) addressed the question of Spitzenkörper biogenesis. Basically, at the restrictive temperature, single growing hyphal tips split into two tips. The original Spitzenkörper did not divide; instead it retracted from its polar position and disappeared. A few minutes later two new Spitzenkörper appeared, each giving rise to an apical branch. The two new Spitzenkörper arose seemingly de novo from vesicle clouds that formed in the apical region next to the future site of branch emergence. It remains to be seen whether the invisible core of the original Spitzenkörper may have divided to serve as nucleation site for the formation of the two new Spitzenkörper. The behavior of satellite Spitzenkörper (12) and observations on the origin of lateral branches (38) suggest that nucleation sites for new Spitzenkörper can appear repeatedly in the subapical region independently of the main Spitzenkörper.

Regalado (39) proposed a mathematical model to explain the accumulation of vesicles in the Spitzenkörper through changes in the rheological properties of the cytoskeleton conditioned by the Ca^{2+} gradient in the hyphal tip.

6. Questions

If a Spitzenkörper is so essential for tip growth, why don't we see one in oomycetous hyphae? This question was often a reason for skepticism about earlier claims on the significance of the Spitzenkörper. It is important to keep in mind that although no Spitzenkörper can be seen with the optical microscope, transmission electron microscopy provides ample proof that Oomycetes have a similar cluster of vesicles in their hyphal apices (40); an excellent example can be seen in the hyphal tip of *Saprolegnia ferax* (Fig. 1 in Ref. 2). Consequently, there is no reason to think that the mechanism of tip growth would be radically different between cellulosic and chitinous fungi, despite the entirely different evolutionary history of these two groups of fungi (4). As we postulated earlier (30), fungi lacking a visible Spitzenkörper must have its functional equivalent, namely, a VSC from which vesicles start on the final leg of their journey to the cell surface. Presumably, in Oomycetes the cluster of apical vesicles does not have sufficient density and/or refractivity to be visible by light microscopy. On the other hand, Harold (5) is of the opinion that the diversity of apical organization bespeaks the existence of alternative mechanisms for shaping a hypha.

Is γ-tubulin, ergo an MTOC, present in the Spitzenkörper of higher fungi? Attempts to extend the finding of γ-tubulin to the hyphal apices of higher fungi have been unsuccessful (R. Roberson, private communication). The failure to detect γ-tubulin in the apical region was not due to staining problems since the antibody did stain the γ-tubulin present in basal bodies. Given the functional significance of γ-tubulin presence in a Spitzenkörper, it is worth exhausting alternative technical reasons for the lack of γ-tubulin staining. But if γ-tubulin is truly absent in the hyphal apices of septate fungi, what replaces it? Is there a major difference in hyphal organization and function that obviates the need for an apical MTOC between the lower and the higher members of the same phylogenetic trunk line? Note that the arrangement of

microtubules in the hyphal apex also differs. In *Allomyces* (20,22), the microtubules are sharply focused on the Spitzenkörper; in higher fungi, they are not (41). Perhaps higher fungi have evolved a modified MTOC that does not depend on γ-tubulin.

Is the Spitzenkörper a maturation site for vesicles? Given that secretory vesicles undergo extensive biochemical modification in their transit from ER to plasma membrane, the Spitzenkörper may be a final station mediating or regulating a final modification that prepares the vesicles for the final leg of their exocytotic journey.

Is the Spitzenkörper a transfer station where vesicles that arrive from the subapex on microtubular tracks switch to actin tracks? The Spitzenkörper may be the place where vesicles switch motors and shift from incoming longitudinal travel on microtubules to outgoing travel on actin filaments.

Is the Spitzenkörper a vesicle-recycling center? Contrary to earlier studies, investigators from several laboratories (Hoffman and Mendgen, 42; Fischer-Parton et al. 43; R. Lopez-Franco and C. E. Bracker, personal communication) found that endocytosis does take place in fungal hyphae and that this process of membrane internalization contributes material to the Spitzenkörper. The fluorescent dye FM4-64 has been especially useful. The dye was taken up and internalized from the plasma membrane appearing progressively in structures corresponding to presumed endosomes, the Spitzenkörper, the vacuolar membrane, and mitochondria (43). The pattern of stain distribution was broadly similar in a wide range of fungal species. Accordingly, the Spitzenkörper would be a site not only for collecting exocytotic vesicles but also for recycling internalized membranes.

The preceding questions highlight the need for biochemical studies to identify the core components of the Spitzenkörper and cytological studies to label vesicles and cytoskeleton in living cells to clarify their dynamics and mutual interaction. The application of molecular genetics could be of great help in overcoming technical obstacles that heretofore have limited progress in elucidating details on the structure and function of the secretory apparatus of a fungus. Such new quest is already under way (44-46).

C. The Cytoskeleton

A good number of studies in recent years have reaffirmed the central importance of the cytoskeleton in hyphal morphogenesis. Both the F-actin and the microtubular skeletons, plus their associated proteins, have been implicated but some of the findings, and conclusions have been somewhat divergent, particularly those based on inhibitor experiments. Given the vagaries of negative results, it may be safer not to regard them as final and conclude that *both* F-actin and microtubules are of critical but different importance in hyphal morphogenesis.

1. Actin Cytoskeleton

There seems to be general agreement that actin is abundantly present in growing hyphal tips though its organization varies. In Oomycetes, actin forms a cap next to the apical plasma membrane apices (47) while in the rest of the mycelial fungi actin is more likely to appear in small plaques (48-50). Intriguingly, in some fungi actin is sometimes more abundant in the subapical than in the apical region (51-53); since the functional significance of this arrangement is not obvious, the possibility of it being a fixation artifact has been advanced (7). Inhibitor experiments with cytochalasin A by Torralba et al. (53) confirmed that a polymerized actin

cytoskeleton is required for normal apical growth, hyphal tip shape and polarized enzyme secretion in *Aspergillus nidulans*. Likewise, actin inhibition by Latrunculin B disrupted tip growth in *Saprolegnia ferax* hyphae (54-55). Bachewich and Heath (55) reported that radial arrays of F-actin precede new hypha formation in *Saprolegnia ferax* and suggested that F-actin participates in establishing polar growth. Heath et al. (41) concluded that there was an obligatory role for F-actin in hyphal polarization and tip morphogenesis. By a different, molecular route, Harris et al. (56) determined that the normal pattern of germtube emergence in *Aspergillus nidulans* is dependent on the integrity of the actin cytoskeleton.

2. Microtubular cytoskeleton

Although past studies questioned the importance of microtubules in hyphal growth and morphogenesis (2, 57), new studies with mutants deficient in motor proteins have shown that a fully functional microtubular cytoskeleton is necessary to maintain normal growth rates, normal nuclear distribution, and regular hyphal morphology (18, 58-63). Inoue et al. (62) reported that the heavy chain of dynein was required for normal secretory vesicle transport to the hyphal apex and normal hyphal tip cell morphogenesis in *Nectria haematococca*. Riquelme et al. (18) concluded that dynein and dynactin deficiencies of two *ropy* mutants *Neurospora crassa* distorted hyphal morphogenesis by destabilizing the Spitzenkörper and causing it to deviate widely from an axial trajectory. Kinesin deficiency in *Nectria haematococca* also caused various effects including severe reduction in colony growth rate, helical or wavy hyphae with reduced diameter, and reduction in Spitzenkörper size. Wu et al. (63) noted that these effects were not due to altered microtubule distribution, as microtubules were abundant throughout the length of hyphal tip cells of the mutant. These studies suggest that both the anterograde and retrograde movement of vesicles on microtubules are important in maintaining the large Spitzenkörper size and high growth rate of the wild-type strains. Findings implicating dynein in apical transport (18, 62) differ from the negative conclusion reached by Seiler et al. (61) but are in accord with the video microscopy observations of McDaniel and Roberson (25) who showed that microtubules were required for vesicle movement in hyphae of *Allomyces macrogynous* and noted that movement can occur in both directions along a common path.

The involvement of opposite motors in apical transport suggests that cytoplasmic microtubules in a hypha may not all have the same orientation. Thus the finding of a bona fide MTOC in hyphal apices (22) and the accumulation of dynein at the tip (61) indicate that the minus ends of microtubules are at the apex. On the other hand, a reverse orientation is indicated by the observation that kinesin, a plus-end motor, accumulates at growing tips (60). A similar discrepancy in microtubule orientation, found in nuclear migration, prompted Xiang & Morris (8) to conclude that fungi may have a "need for different microtubule polarities" and may even result in "different mechanisms to move nuclei in the hyphae." The same could be said for vesicle traffic in the growing tips.

3. Questions

Are actin and microtubules required for tip growth? Based on inhibitor experiments with Nocodazole, MBC and Latrunculin B on *N. crassa* and *Saprolegnia ferax*, Heath et al. (41) concluded that there was an obligatory role for F-actin in hyphal polarization and tip morphogenesis but only an indirect role for microtubules. A reevaluation of their evidence leads to a modified conclusion: inhibition of either actin or microtubular functions has a direct impact

on tip growth albeit with different consequences on hyphal morphogenesis. Actin inhibition by cytochalasin often (54, 64, 65) but not always (17) stops elongation and leads to formation of bulbous tips; microtubule inhibition by nocodazole (41) or MBC (17) also inhibits growth but causes morphological distortions that may extend over a long stretch of the hyphal tube. It is essential to keep in mind that most of the distortions in the shape of a hypha originate when the distorted or deviated portion was part of the growing tip, i.e., the observed alterations in the cylindrical portion of the hypha were direct effects on tip growth. Actin inhibition causes a more drastic loss of polarized growth, but the effect does not usually persist, either because the hypha stops growing completely or because it ceases to respond to the inhibitor. Microtubule inhibition tends to produce a less severe but more persistent disruption or disorientation of tip growth. The different effects are probably a reflection of the different localization and different roles that actin and microtubules play in vesicle traffic.

Is tip growth initiated by a localized site of actin polymerizarion? The strong correlation between actin presence and tip growth poses the question of whether a localized site of actin polymerization at the plasma membrane may be the initiator of tip growth. Such view, however, needs to be reconciled with other findings showing no changes in levels of actin or gene expression during extensive induction of growing points. Thus, in *Achlya ambisexualis*, Brunt et al. (66) reported that antheridiol increased transcription of the heat shock protein chaperones (Hsp90 and Hsp70 family), but there was no similar increase in the level of transcripts encoding actin even though 90% of hyphae in the hormone-treated thalli were undergoing antheridial branching. Likewise, Tinsley et al. (50) used the the temperature-sensitive mutants *cot-1* and *mcb* of *N. crassa* to show that there was no increase in actin following a > 20-fold increase in the number of hyphal tips. They suggested that the level of actin monomers within *N. crassa* hyphae is sufficient to accommodate the need for additional actin in the new tips. These two different studies provide evidence that actin reorganization, but not necessarily new synthesis, is needed to establish new growth zones. In turn, this is evidence that actin per se is not the initiator of hyphal growth but that something else in the incipient or future apex provides the signal for actin polymerization at the growing tip. Rho GTPases have been implicated in establishing cell polarity by controlling the localization of F-actin in budding of *Saccharomyces cerevisiae* (67), and in the apical growth of pollen tubes (68), and fungal hyphae (69).

Do nuclear events compete with apical growth for microtubule resources? Since microtubules are involved in both mitosis and tip growth, the question arose as to whether the two processes compete for the same pool of tubulin. This was found no to be the case (70). Presumably, the population of cytoplasmic microtubules involved in apical growth operates independently of those involved in mitosis.

D. Turgor

1. Evolutionary and Ecological Significance

Thanks to the work and writings of Money and coworkers, the role of turgor in fungal biology has received a great deal of attention in recent years (71-77, 37). The importance of turgor in growth, morphogenesis, pathogenesis, and ecology of fungi has been extensively discussed. As I noted earlier (78-79), the development of high turgor concomitant with the formation of microfibrillar cell walls of high tensile strength has been a central factor in the evolution of the Fungal Kingdom. In the same vein, Johns et al. (37) remarked that the evolution of the fungal

cell wall and the generation of turgor pressure afforded by this structure are at the root of the ecological diversity of the filamentous fungi (37). The enormous turgor that fungal cells can generate explains the penetrating physical power of fungal hyphae (80-81).

2. Role of Turgor in Hyphal Growth and Morphogenesis

Money and coworkers (37, 72) are probably correct in challenging claims for turgor having a *controlling* role in hyphal growth and morphogenesis. The conclusions reached by Eamus and Jennings (82) and Gervais et al. (83) linking growth rate with internal pressure have been disputed (37). Previously, Kaminskyj et al. (84) had also questioned that turgor was a determinant factor in growth rate. Probably, growth rate has far more to do with metabolic activity, wall loosening and exocytosis than the degree of internal osmotic pressure (37).

Putting aside the controversy of turgor being a controlling agent of growth rate, a more basic question emerges. Is turgor essential for hyphal growth, i.e. does turgor provide the physical force needed to expand the cell wall? Here, Money is probably incorrect in doubting whether turgor fulfills this role and limiting the role of turgor to invasive growth (37,72). His argument is based on some unique observations made by Harold et al. (71) on the ability of hyphae of *Saprolegnia ferax* to grow at high concentration of osmolytes without adjusting their turgor. The turgor of such hyphae fell below the limit of detection of the micropipet pressure probe used (0.02 MPa), i.e., 5% or less of the turgor in control hyphae. Despite the fact that the actual pressure of osmotically stressed hyphae could not be determined, these hyphae were prominently regarded as "turgorless" and held as reason to contradict the common belief that turgor supplies the driving force for hyphal extension. But the same article contains more measured conclusions that I believe paint the true picture. The role of turgor was not completely ruled out but rather conditioned: "If hydrostatic pressure plays a role it is one that can be filled by 3% of the normal turgor." Since hyphae grown at such high concentration of osmolytes are known to have weak cell walls (85), such low value of turgor may have been sufficient to expand the debilitated wall. In a parting answer to the question Is turgor required for extension of *Saprolegnia* hyphae?, they concluded: "In the extreme case when turgor is essentially 'zero' and the wall most plastic, the answer is no. But when turgor is high and the wall rigid, hydrostatic pressure may well be required to stress the wall allowing it to expand and admit new wall material." This last statement addresses the normal condition of fungal hyphae. But it is no longer necessary to dispute the validity of the claims on logic alone; experimental evidence showing that the wall expands orthogonally over the entire growth zone of a hyphal tip (86) (Fig. 4) leaves turgor as the only viable candidate to provide the physical force for wall expansion. But beyond a minimum threshold value to permit expansion, the magnitude of turgor has no role in the 3-D model of hyphal morphogenesis (87). Presumably, as Harold et al. (71) hinted, most of the turgor pressure in a growing fungus is in excess of that needed for normal hyphal growth.

As to hyphal morphology, until recently there was no experimental basis to suspect that turgor would have a morphogenetic role (72). By forcing cell wall expansion to follow an orthogonal pattern, turgor modulates the 3-D shape of a hypha (86). However, turgor does not dictate the tubular shape of a hypha, and its overall effect on hyphal shape is relatively minor (87) compared to the major factor in hyphal morphogenesis, namely, the highly polarized distribution of cell wall building vesicles created by the Spitzenkörper (see II.A).

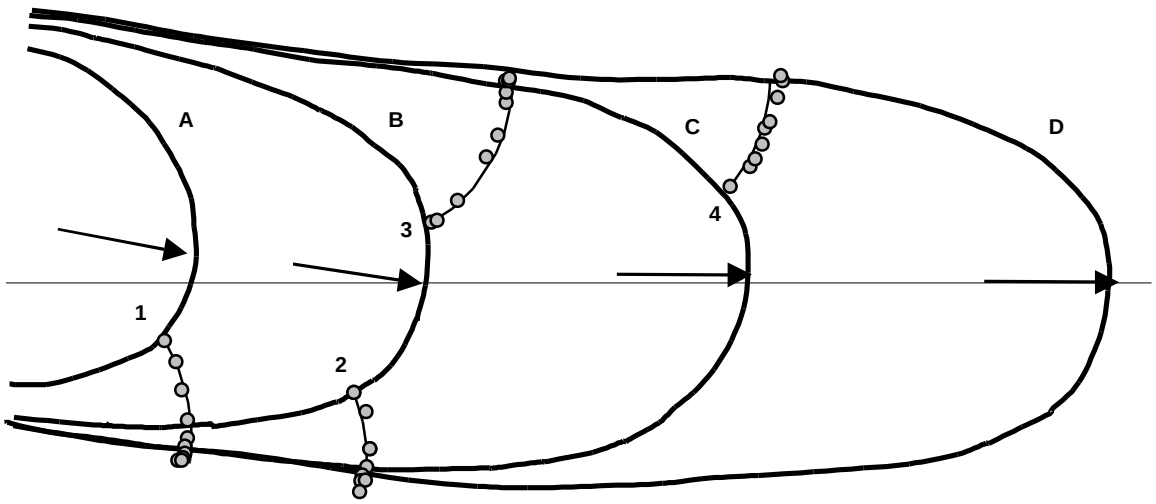

Figure 4. Orthogonal pattern of surface expansion in hyphal tip growth. Circles depict the trajectories of four carbon particles that became attached to the surface of an elongating hypha *Rhizoctonia solani*. Growth was followed for 219 sec (D). Particle 1 was attached at 0 time (A), particles 2 and 3 after 58 sec (B) and particle 4 at 136 sec (C). The thin solid lines are the theoretical curves calculated for orthogonal displacement of each particle. Arrows show the growth axis for each tip profile. Modified from Bartnicki-Garcia et al. (86).

3. Question

If not turgor, what, then, controls cell wall extensibility? This is one of the most fundamental questions in cell wall growth, particularly tip growth, for which there is no decisive answer. The extension of an inelastic cell wall of high tensile strength requires a plasticizing action that would yield to turgor to increase the surface area of the cell. Accordingly, the crucial issue is the need for a mechanism to coordinate synthetic and lytic processes so that the wall attains an exact measure of controlled plasticity necessary for cell wall extension (79). As I suggested earlier (88), the proper balance of wall synthesis and lysis in the growing regions of the wall may be established through a coordinated discharge of different types of vesicles carrying different ingredients for wall extension. In other words, the well-proven gradient of wall synthesis (89-91) needs to be accompanied by a parallel gradient of wall softening. So far, all evidence for the involvement of a softening, lysing, or plasticizing action is circumstantial and qualitative (92-94, 75). Here, molecular genetics could have a crucial impact by helping to elucidate and quantitate the fleeting but crucial biochemical process that softens the fungal cell wall to permit extensibility.

An alternative explanation for the control of wall extensibility assigns this role to the F-actin membrane skeleton underlying the apical wall (see III. B). This explanation, however, fails to take into account that wall extensibility requires autolysis (92).

III. Models of hyphal tip growth

Different models have been proposed to explain how the tubular cell of a hypha is generated by apical growth. Each model focuses on a different aspect of hyphal biology and invokes or emphasizes different morphogenetic criteria. Although each of the models or approaches listed below is usually presented to the exclusion of the others, in reality no single model provides a satisfactory answer. Each model explains but a portion of the mystery of the deceivingly simple process of hyphal tip growth.

A. The Hyphal Apex Is Shaped by Gradual Rigidification of the Cell Wall

The steady-state (SS) model of tip growth (3, 95) is a refinement of previous ideas invoking changes in the physical properties of the wall to explain tip growth (96-98). Accordingly, the hyphal tube is manufactured by a steady-state process that transforms and expands the newly deposited plastic wall of the apical dome into a rigid cylindrical wall at the base of the dome.

The newly added wall material is plastic owing to the presence of individual polymer chains and the lack of crosslinkages. Vermeulen and Wessels (99-100) showed that the newly synthesized chitin at the hyphal tips was not microfibrillar and was highly susceptible to chitinase; this susceptibility diminished as the wall progressed from apex to subapex, and the chitin chains crystallized into microfibrils. As the nascent wall becomes progressively crosslinked by covalent bonds between β-1,3-glucan and chitin (101-102), it develops a greater resistance to turgor pressure.

1. Comments

Even though there is much merit in the discovery that the overall physical properties of the apical wall may change significantly because of crosslinks between β-1,3-glucan and chitin polymers, it is questionable whether any changes in rigidification of the apical wall would regulate its shape. The wall of the apical dome, even at its most plastic point, must be strong enough to resist deformation by the high turgor pressure of the cytoplasm; otherwise, the cell would explode under normal growth conditions. The steady-state model assumes that the apical dome adopts a hemiellipsoidal shape (95), but it is not evident why a plastic dome adopts this particular shape, and there is no quantitative formulation to correlate the gradual change in wall rigidity with the shape of the hyphal apex.

B. The Expanding Cytoplasm Molds the Hyphal Apex

In this model, favored by Heath and coworkers (2, 7, 57), the fungus is viewed primarily as a protozoon living inside a rigid tubular casing. Morphogenesis is thought to originate from the same elements that mold the shape of a wall-less protozoon, namely the underlying cytoskeleton. Specifically, in its more recent version, a scaffolding of F-actin (104) in conjunction with spectrin- and integrinlike components (105-106) forms a membrane skeleton associated with the inside of the apical plasma membrane. This membrane skeleton is believed to regulate tip extensibility and thus hyphal morphology (106). There is some circumstantial

evidence to support these ideas. The high concentration of actin found in hyphal tips of many fungi (47-48, 50, 52, 107-108) indicates that actin must play a major role in apical growth. The evidence gathered by Degousee et al. (106) showed that F-actin is attached to a membrane skeleton that is rich in spectrinlike protein and also contains an integrinlike protein. This complex is concentrated in growing hyphal tips of *Neurospora crassa*.

1. Comments

The idea that actin has a direct physical role in shaping a hypha (41, 57, 104) runs contrary to new experimental findings showing that the apical wall of a hypha expands orthogonally, i.e. expansion is always perpendicular to the cell surface (86) (Fig. 4). There is no evidence that actin is deployed perpendicular to the cell surface over the entire growing region, which includes the apex and the neighboring subapex. The ever-present high force generated by the turgor of the cytoplasm does have the orientation, and the strength, to explain expansion of a walled cell. More likely, the high concentration of actin in the apex is an indicator of intense exocytosis and plays a crucial role in the transport of vesicles to the cell wall. Inhibition experiments with Latrunculin B are consistent with F-actin regulating polar vesicle delivery and controlling vesicle fusion at the plasma membrane (55).

Studies by Heath and coworkers (see reviews 2, 7, 57) have contributed greatly to our understanding of the fungal cytoskeleton and its role in fungal growth. Although knowledge is still too fragmented, there is little doubt that the cytoskeleton is intimately involved in the elongation of a hyphal cell. But the intense focus on the cytoskeleton relegates the cell wall to a secondary role, and downplays the importance of the ultimate defining process of fungal morphogenesis: the making of the cell wall. The importance of cell wall biogenesis can not be underestimated. The evolution of fungi and their extraordinary ecological role were attained largely because eons ago fungi learned to construct microfibrillar cell walls to satisfy innumerable ecological challenges (73, 78, 79). The conceptual approach to tip growth adopted in the membrane skeleton model, and its earlier versions (2, 57), was not strengthened by reviving 19th-century ideas equating a fungus with a tube-dwelling amoeba (7). Such analogy may have been appropriate for the days when the cell wall was regarded merely as a hardened exudate of the cell, and when nothing was known about the chemical and structural complexity of the cell wall, and the existence of a highly sophisticated secretory apparatus to construct the cell wall. By equating tip growth with pseudopodium formation (7), the membrane skeleton model disregards the crucial difference between an amoeba and a hypha, namely, the existence a polarized secretion process for building and shaping a tubular cell wall.

C. The Hyphal Apex Is Shaped by a Moving VSC

The VSC model described in II. B.2. was based on an earlier qualitative model that postulated that hyphal shape is determined by a polarized pattern of distribution of vesicles involved in cell wall construction (88). The model assumed that extension growth was the result of three concomitant actions: synthesis and deposition of new cell wall polymers, enzymic plasticizing action to loosen a basically rigid wall structure, and turgor to force cell wall expansion (88). The basic premise of the qualitative model stipulates that the biochemical gradients needed for hyphal wall construction are created by a gradient of exocytosis. From these qualitative assumptions, we developed a mathematical model (30) that postulated that the spatial discharge of wall-building vesicles was centrally controlled by a VSC. The VSC model provides a

plausible mechanism to explain how a continuous sharp gradient of vesicle-discharge can be generated by a growing hypha. By the simple action of moving forward while continuously releasing wall-destined vesicles, the Spitzenkörper could generate such gradient (30).

1. Comments

Aside from a VSC solution, Harold (5) considered two other alternative explanations for shaping the hyphal tip through exocytosis. One was the calcium hypothesis in which secretory vesicles carrying calcium channels merge with the plasma membrane creating an influx of calcium ions. The calcium gradient, known to be present in hyphal tips (109), would promote actin polymerization, vesicle fusion, and localized cell wall deposition. The other one was targeted exocytosis where vesicles are carried to marked fusion sites, an idea explored by Gupta and Heath (110). Both alternatives represent viable mechanisms to support exocytosis, but neither one can explain the origin of the exocytosis gradient since they both depend on molecules that have to be carried to the surface by the vesicles themselves. One virtue of the VSC model is that it does not need pre-existing signals or targets on the cell surface to initiate or maintain its operation. The control of morphology obtained by laser manipulation of the Spitzenkörper (Fig. 3; II.B.3) supports an internal origin for the gradient of wall-building vesicles.

The VSC model was a deliberate exercise in physiological reductionism that attempted to extract the essence of cell wall biogenesis. Cell wall construction was reduced to its minimum expression: one vesicle discharge equals one unit of cell surface. The model does not address the complex interaction between wall synthesis and wall softening and, in its original 2D version, it did not need to address the role of turgor pressure (see below). Despite supporting circumstantial evidence (II.B.2), the ultimate validity the VSC hypothesis depends on demonstrating that the flow of wall-building vesicles passes through a Spitzenkörper control gate. Such traffic of vesicles in/out of the Spitzenkörper is yet to be demonstrated and measured. If the Spitzenkörper is proven unequivocally to be a VSC, it follows that the mechanism that advances the Spitzenkörper would be a key regulator of hyphal morphogenesis. Circumstantial evidence suggests that the microtubular cytoskeleton is involved (17) but one cannot rule out a complex interplay between various components of the cytoskeleton providing the propelling force (30).

A common criticism of the VSC model, variously voiced by Koch (111), Green (112), and Harold (5), was that it "dealt with the two-dimensional analogue of the hypha" and as such it accounted for cell substance and not for surface area. We had initially assumed that mere rotation of the 2D hyphoid model, along its longitudinal axis, would automatically supply a 3D model of hyphal morphogenesis (30), but Koch predicted correctly that the 2D VSC model would be indetermined in three dimensions and concluded that "the velocity of the VSC is not a sufficient condition to define the shape". Indeed, when an attempt was made to derive a 3D model based on the VSC concept, an indetermination was encountered whose solution required defining a priori the pattern of expansion of the wall, i.e. defining the overall spatial movement of the wall as the newly inserted wall elements displace the existing wall fabric (87). The actual mode of wall expansion was determined experimentally, and found to follow orthogonal trajectories (86) (Figure 4), as depicted by Reinhardt (113) in 1892! Fortunately, the close similarity in profile between the 2D hyphoid and the 3D orthogonal hyphoid (87) validates the use of the simpler 2D model in morphogenetic studies described in II.B.2. Although not claimed by the authors, the data presented by Shaw et al. (114) on the mode of surface growth of root hairs show clearly that orthogonal expansion applies to tip-growing cells of the plant world.

Harold (5) pointed out that the VSC model lacked a mechanism for the performance of physical work. Although we did not set out to deal with this issue, the mathematical impasse mentioned above compelled us to consider wall expansion patterns and, in turn, the physical forces behind them. We now have experimental reason to invoke turgor as the physical force that expands the wall of a hypha (see II.D.2)

D. The Molecular Genetics Approach

A number of laboratories have taken advantage of the methodology of molecular genetics to unravel the genetic components involved in fungal tip growth. No major breakthrough has emerged but steady progress has been made in detecting genes mainly on three several fronts: cytoskeleton, signal transduction, and polarized cell wall formation.

1. Cytoskeleton genes

That many of the mutated genes responsible for distortions in hyphal morphogenesis belong to the cytoskeleton and associated molecules underscores the importance of the cytoskeleton. As already described in II.C., mutants deficient in microtubule motor proteins (dynein and kinesin) show overall growth rate reduction, a smaller and often unstable Spitzenkörper, and distorted morphogenesis. Harris et al. (56) identified the genes *podB* and *sepA* as necessary for the organization of the actin cytoskeleton at sites of polarized growth in *Aspergillus nidulans.* With a conditionally null *myoA* strain of *Aspergillus nidulans*, McGoldrick et al. (115) showed that MYOA, a gene encoding an essential myosin I, is required for secretion and polarized growth.

Much work has focused on mutations disrupting nuclear distribution, mainly *nud* in *Aspergillus nidulans* (116) and *ro* in *Neurospora crassa* (49) which led to the identification of dynein as the major motor for nuclear migration in hyphae. The subject has been reviewed by Xiang and Morris (8), who concluded that the models proposed to explain nuclear migration are still controversial.

2. Signal transduction

A variety of mutations point to the involvement of signal transduction pathways in hyphal morphogenesis. For example, studies by Feng et al. (117) with the *ras1-2/ras1-3* mutant of *Candida albicans* indicated that low-molecular-weight molecules in serum induce hyphal differentiation in *C. albicans* through a Ras-mediated pathway. Similarly, Truesdell et al. (118) found that a mutationally activated Ras homolog (*CT-Ras*) induced abnormal hyphal proliferation and defects in polarized growth, an indication that proper regulation of Ras is required for normal growth in *Colletotrichum trifolii*. Disruption of another RAS-related gene (CaRSR1) in *C. albicans* indicated its involvement in polarized growth: initiation of budding in yeast, germ tube emergence, and pseudohyphal elongation (119). Alex et al. (120) found that COS1, a gene encoding a two-component histidine kinase, is involved in hyphal but not yeast morphogenesis. Mutants of *C. albicans* lacking both copies of COS1, produced normal yeast cells but showed defective hyphal formation in response to nutrient deprivation or serum. Yarden and coworkers (121) reported that *cot-1*, a kinase-encoding gene required for hyphal cell elongation in *Neurospora crassa* hyphae, was photoregulated by blue light, an effect blocked by L-sorbose. These interactions indicate the involvement of alternative and potentially interdependent signaling pathways for the regulation of hyphal elongation/branching (122).

Examples of induction of polarized (hyphal and pseudohyphal) growth abound in the dimorphic and mating responses of fungi. The signalling pathways play a fundamental role connecting external signal with some internal metabolic or regulatory response (see review by Banuett, 123). But in dissecting the ultimate basis of tip growth, it is important to keep in mind that the polarization of wall growth that leads to hyphal development is usually initiated internally with no obvious external input (Figure 1).

3. Polarized Cell Wall Construction

Although the basic structure and major wall components of fungi are known, much remains to be learned about minor components of the wall, about the linkages among different components, and about the entire process of cell wall assembly. Molecular studies may help characterize proteinaceous components of the wall that play a key role in hyphal morphogenesis. For instance, Staab and Sundstrom (124) have cloned the complete hyphal wall protein 1 gene (HWP1) of *C. albicans*. The Hwp1 is a glucan-linked protein with serine/threonine-rich regions that are predicted to function in extending a ligand-binding domain into the extracellular space.

In search of genes involved directly in conversion from isotropic to tropic cell wall growth, i.e., the establishment of polarity, Momany's group (125) screened for temperature-sensitive swollen-cell mutants (*swo*) of *Aspergillus nidulans*. The screen yielded eight genes involved in polarity establishment, polarity maintenance, and hyphal morphogenesis. They concluded that *swo C, D,* and *F* are required to establish polarity, and that *swoA* is required to maintain polarity. *swo B, E, G,* and *H* are involved in later hyphal morphogenesis. Their results suggest that polarity establishment and polarity maintenance are genetically separate events and that a persistent signal is required for apical extension in *A. nidulans*. Wendland and Philippsen (69) searched for molecular similarities between the onset of polarized growth in germinating spheres of *Ashbya gossypii* and bud emergence in unicellular yeastlike fungi. They found a common requirement of rho-GTPase modules for the establishment and maintenance of polarized hyphal growth. Most recently, Knechtle et al. (126) investigated the dynamics of polarized growth of *A. gossypii* with a polarity marker (AgSpa2p) homologous to the one in *Saccharomyces cerevisiae* (Spa2p). GFP-labeled AgSpa2p was sharply localized in hyphal tips of *A. gossypii* The video microscopy images shown at the conference gave the distinct impression that the fluorescence of this polarity marker coincided with the usual position of the Spitzenkörper in a hyphal tip.

Much of our knowledge on the cytology (127-128), biochemistry (129-130), and genetics of cell wall formation in fungi pertains to chitin synthesis. Chitin synthases are coded by a multigene family divided into at least 5 classes. This genetic multiplicity may be related to morphogenesis and pathogenesis as there is ample evidence for differential gene expression (131-135); however, the redundancy of function complicates interpretation. Thus, two different *CHS* genes *Umchs1* and *Umchs2* were identified in *Ustilago maydis*. Transcripts of both genes appeared more abundant in the mycelial form, but both genes were found not to be essential (136). Single-gene disruption and replacements of class I, II, or IV enzymes of various fungi including *Neurospora crassa* (137-138), *A. nidulans* (139-141) and *Ustilago maydis* (136, 142) did not affect development. On the other hand, mutations affecting chitin synthases of class III of *Neurospora crassa* (143), *A. nidulans* (140, 144) and *Aspergillus fumigatus* (145) did affect hyphal growth. Also the chitin synthases with a myosin domain (class V) found in *A.*

nidulans (146) and *Pyricularia oryzae* (147) appear to be important for the maintenance of hyphal wall integrity and the polarized synthesis of the cell wall.

To resolve the issue of whether the polarized growth of a hypha requires a specific kind of chitin synthase, it would be helpful to find out if the spatial redirection of chitin synthesis that occurs during polarization of wall growth (Fig. 1) requires expression of a specific chitin synthase gene or, if the same population of chitosomes (148, 149) involved in isotropic growth are simply rerouted to a polar destination.

4. Comment

The molecular approach is largely an attempt to identify individual genes and gene products that play a role in hyphal morphogenesis. A good number have already been implicated, but their relative relevance, hierarchy, and chronology are far from clear. Will molecular reductionism eventually succeed in explaining the basis of hyphal morphogenesis? Beyond identifying specific molecules affecting apical growth, can reductionism provide a coherent scheme that explains tip growth? Is polarized growth triggered by the action of a single gene setting up a cascade of metabolic events that establishes a polarized gradient of exocytosis? Or do we need a holistic approach (150,151) to understand how a complex interplay of simultaneous events coalesce to trigger the onset of polarized growth?

E. Model Reconciliation

The aforementioned models of hyphal morphogenesis deal with structures and processes that occur in living hyphae, and all, or substantial parts of them, need to be considered in any final picture of hyphal development. Thus, a comprehensive model would include changes in physical properties of the wall (III.A), the cytoskeleton to provide the scaffolding for vesicle dynamics (III.B), and a VSC to orchestrate the exocytosis gradient (III.C). Ultimately, none of the current models define the precise biochemical reaction(s) that confer(s) polarity to a hypha. It remains to be seen whether any of the genes so far identified (III.D), would provide a biochemical answer to the ultimate question--the origin of polarity.

Despite firmly entrenched past postures, model reconciliation is possible and desirable. For instance, our VSC model and Wessels' steady-state model are not necessarily incompatible (152)–they account for different features of the wall-building process. A key conceptual difference between these models was in the role of lysins (i.e. wall-softening or wall-plasticizing enzymes). In the VSC model, lysin action is implicitly needed to permit wall extension. In the SS model, lysins were deliberately excluded; although Wessels (103) agreed that lysins were needed for wall growth, he limited the need for lytic action to the initiation of growth from a rigidified wall, such as in branching or spore germination. I contend that there is no basic difference between these processes: both initiation and continuation of apical growth require a steady supply of plasticizing agents. This contention is perhaps most vividly affirmed by the findings of Bracker et al. (35) during manipulation of the Spitzenkörper with a laser beam (Fig. 3). When a Spitzenkörper that was actively engaged in the elongation of a growing tip was forced away from its usual position in the apex, it would start deforming any wall adjacent to it. Clearly, the vesicles emanating from the Spitzenkörper have the power to render any wall region plastic. The inclusion of lysins in the steady-state model strengthens its key premise, namely the progressive change in physical properties of the expanding wall. Lysin action would allow the

growing wall, which is always a mixture of pre-existing plus new wall, to be plasticized adequately. The newly deposited wall would be plastic by virtue of the nascent nature of the polymer chains and the lack of crosslinks, while the existing rigid wall would be rendered plastic by lytic action. Once plasticized, the wall would expand under the force of turgor. But such expansion needs to be discrete, or the wall would bulge out and eventually break. The expansion-limiting factors would be the rigidifying processes invoked in the steady-state model plus rapid inactivation of both lytic and synthesizing enzymes. The latter is supported by autoradiographic evidence showing that the apical accumulation of chitin synthetase disappears rapidly in the subapical region (153). Similarly, lysin action must have an intrinsic short half-life to limit its plasticizing effect. A tandem VSC-steady-state model would embody the spatial and temporal controls needed for wall biogenesis. The VSC model would explain the *spatial* control of wall synthesis, while the steady-state model would account for the *temporal* control of wall extensibility.

IV. References

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