Tip Growth in Filamentous Fungi: A Road Trip to the Apex

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Abstract
Fungal hyphae extend by apical growth. This process involves the polarized traffic of secretory vesicles to the Spitzenkörper (SPK) and their subsequent distribution to specific domains of the plasma membrane, where they fuse to provide all the enzymes and material needed for cell wall expansion. Endocytic recycling and localized translation of specific mRNAs play an important role in hyphal apical growth. The traffic of vesicular carriers from synthesis sites to their destinations is coordinated by the combined action of coats, tethers, Rab GTPases, motors, and SNAREs in a mechanism that is just beginning to be understood. Only recently has it been confirmed that the different-sized vesicles present at the SPK contain distinct cell wall biosynthetic activities and are distributed in a stratified manner.
APICAL GROWTH: THE RHYME AND REASON
OF FILAMENTOUS FUNGI

Fungi have a huge impact on our daily lives. They are used in biotechnology to obtain products beneficial for humankind, they play a major role in maintaining a healthy balance in ecosystems, and they pose devastating effects on plants and animals, including humans. Although unicellular yeasts are used mainly for the production of alcoholic beverages and breads, filamentous fungi have many other uses (134). The filamentous growth form is not only the more prevailing form of growth in fungi but also, according to phylogenetic analysis, more ancestral than the yeast form (72). Filamentous fungi and animals share molecular pathways involved in polarized growth that are absent in yeast. Filamentous fungi play an important role in the biosphere as decomposers of organic matter, which allows recycling of carbon, nitrogen, and phosphorus. Many filamentous fungal species are used in industrial processes for the production of organic acids, antibiotics, vitamins, and enzymes (11). Some human and animal fungal pathogens produce filamentous hyphae during the infection process (34). Currently, only a dozen fungal diseases are considered life threatening to humans, particularly immunocompromised patients (116). However, the number of emerging fungal diseases is increasing, due in part to climate fluctuations, and will pose a larger worldwide threat (53).

The basic unit of growth of a filamentous fungus is the hypha, a long cylinder with a slightly tapered apex. The term hypha was initially coined about two centuries ago to describe a “more or less filamentous, watery or fibrous stem, which is formed by repeated branching” (111, 179). Understanding the biology of the fungal cell has been a goal of mycologists for many years. Some of the salient features of fungal tip growth were identified more than half a century ago. However, the field remained semidormant for a few decades during which only a few research labs continued searching for the ultimate answer to how hyphal morphology is attained. The great technological advances in live and electron microscopy, molecular biology, and genetics, as well as next-generation sequencing tools, have contributed to dramatically changing that scenario. The number of labs studying fungal cell biology has grown, allowing for rapid and successful progress.
and identification of some of the key molecular players of tip polarized growth. The field is still lagging a great deal behind that of the budding yeast *Saccharomyces cerevisiae* or neurons, for which a wealth of information is known; however, we are now only steps away from seeing the filamentous fungi included in the same league as these well-established model systems.

Apart from all the inherent benefits of understanding fungal morphogenesis from an applied point of view, how a hypha is generated poses an interesting problem per se that has fascinated scientists eager to decipher a basic biological phenomenon unique to the fungal realm. Tip growth is an extreme example of polarized growth occurring at the apex of fungal hyphae, neurons, root hairs, and pollen tubes (75). But among these cell types, only fungal hyphae can potentially extend indefinitely under the right conditions.

Polarity is a fundamental property for cell development, which allows the asymmetrical transport of cellular components to specific sites of the cell. Polarity in filamentous fungi requires the continuous synthesis and selective targeting of proteins, lipids, and cell wall building blocks to specific domains at the apex of the hyphal plasma membrane.

Many excellent reviews cover some of the key aspects of hyphal growth, including polarity (12, 20, 71, 73, 74, 108, 149), the role of the cytoskeleton (28, 60, 99, 147, 148, 155, 181), cell wall synthesis (16, 94, 95, 145), and endocytosis (119, 164, 175). Most of the information included in this review is based on research conducted in the ascomycetes *Neurospora crassa*, *Aspergillus nidulans*, *Candida albicans*, and *Ashbya gossypii*, and the basidiomycete *Ustilago maydis*; these fungal species have been analyzed more extensively at the cell biology and molecular levels. However, in the past few years, more data have become available for other filamentous fungi, including important plant and human pathogens (47).

This review highlights earlier, key findings that inspired the research that led to our current understanding of molecular and cellular processes that participate in tip growth. It focuses on cell wall morphogenesis and its underlying secretory pathways and pinpoints some of the major challenges that impede our ability to fully understand the operation and regulation of hyphal growth.

**TIP GROWTH: FUNDAMENTAL ASPECTS**

**Getting into Hyphal Shape**

Fungal hyphae are tube-like structures with a hemispherical or hemiellipsoidal apical region. In 1892, Reinhardt (122) described that growth occurred at the hyphal apices and suggested an orthogonal pattern of tip expansion. In 1965, Robertson (133) showed that hyphae elongated by tip growth. This finding was later confirmed by studies that used external markers and fluorescent antibodies or measured distances between septa (39, 41, 102). However, Bartnicki-García & Lippman (25) provided the ultimate evidence confirming that cell wall polymers are deposited at the apex. Their autoradiographic studies in germ tubes of *Mucor rouxii* showed that the highest rate of cell wall synthesis occurred within 1 μm of the foremost apical point (25). These studies demonstrated that fungal hyphae elongate at their apices by localized deposition of new plasma membrane and cell wall presumably via exocytosis of secretory vesicles.

Mapping out how a hypha is generated is quite complex. Deciphering the mysteries of hyphal morphogenesis has motivated many labs to identify morphogenes (i.e., genes that are somehow involved in shaping an organism). Genetic analyses have identified many genes that participate in hyphal morphogenesis (90, 112, 141). There is no doubt that morphological mutants have been extremely useful for dissecting the molecular mechanisms underlying morphogenesis. For example, genetic screens of *A. nidulans* mutants affected at particular stages of conidiophore formation, a developmental process that includes switching between polarized growth and
isotropic expansion, enabled researchers to dissect the molecular pathway underlying asexual conidiogenesis and to assign specific roles to individual genes (4, 159). However, different unrelated mutations of genes involved in hyphal extension can manifest similar hyphal phenotypes (e.g., loss of polarity, increased branching, disturbed directionality), and many housekeeping metabolic genes also can result in morphology-defective phenotypes (35). These factors hinder the search for bona fide apical growth-related genes.

Although the power of molecular biology and biochemistry cannot be denied, Harold (68–70) has raised significant issues in his elegant and philosophic essays. How the linear organization of the genome is translated into the three-dimensional sophisticated architecture of the hypha is difficult to understand conceptually. Identifying genes and molecules is only one step toward elucidating how molecules organize into a higher-level order to give rise to a fungal cell. These are some of the major challenges we face in the postgenomic era. Morphogenesis requires not only the molecular building blocks but also their assembly into complex structures (68, 70). It is therefore the coordinated action of multiple enzymes, positional markers, and cytoskeletal elements that permits the synthesis and extension of the fungal filament.

### Cell Wall Assembly and Expansion

The shape of the hypha is determined by cell wall assembly (25), a polarized process that takes place at the tip. Therefore, to understand hyphal morphogenesis it is imperative that we understand the basis of the cellular machinery involved in cell wall synthesis.

Fungal cell walls are composed mainly of polysaccharides and glycoproteins (14, 16). The inner layer (alkali-insoluble fraction) contains interwoven fibrils of chitin and β(1,3)- and β(1,7)-glucans and is embedded in an amorphous gel-like matrix constituted of alkali-soluble polysaccharides [α(1,3)-glucans] and glycoproteins (mainly galactomannoproteins) (85). Evidence suggests that the matrix glycoproteins are synthesized through the secretory pathway, transported in secretory vesicles and incorporated into the cell wall by exocytosis. The enzymes responsible for the synthesis of the polysaccharides can be considered nanomachines, which are presumably inserted into the plasma membrane (Figure 1). Chitin is synthesized by a family of integral membrane proteins, the chitin synthases (CHS), which accept their substrate, UDP-N-acetylglucosamine (UDP-GlcNAc), at the cytoplasmic side of the plasma membrane and assemble linear chains of β(1,4)-GlcNAc at the external side (95, 135, 145). Filamentous fungi have more CHS gene families than yeasts do (38, 45, 125), which correlates with the higher content of chitin in their cell wall. Some of the CHS are zymogenic and are activated at the membrane site (43, 44, 162), whereas Chs3 localization and function in *C. albicans* are regulated by phosphorylation (96).

β(1,3)-glucans are synthesized by a glucan synthase complex (GSC) containing a putative catalytic subunit (Fks) and a regulatory subunit (Rho1). Unlike in yeast, where as many as four *fks* genes have been identified, in filamentous fungi there is only one *fks* gene, and it is usually essential (95, 97). FKS is a transmembrane protein that accepts the substrate UDP-glucose from the cytoplasmic side and extrudes the product outside the plasma membrane. The Rho GTPase, Rho1, is synthesized in the endoplasmic reticulum and undergoes geranylgeranylation, a process that enables its insertion into the plasma membrane (86). Both Rho1 and Fks1 are transported to the plasma membrane as an inactive complex (1). Once at the plasma membrane, Rom2 activates Rho1-GDP, and in its GTP-bound state it activates Fks1.

Transporting both CHS and GSC in an inactive form until they are inserted into the plasma membrane ensures the in situ synthesis of these two wall polysaccharides: chitin and β(1,3)-glucan. α(1,3)-Glucans are synthesized by α-glucan synthases, which are integral membrane proteins that
Figure 1
Three-dimensional view of the fungal cell wall biosynthetic nanomachinery. Chitin synthases (CHS) and glucan synthases (GS) are transported in different vesicles that fuse with the plasma membrane, into which the enzymes are inserted. Chitin and β(1,3)-glucan microfibrils are synthesized in situ to constitute the skeletal fraction of the wall. Glycoproteins are synthesized and transported through the secretory pathway and, after exocytosis, remain attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor. Figure produced by E. Sánchez-León.

appear to contain multiple functional domains (79). However, not much is known about the synthesis of this carbohydrate.

Cell wall thickness remains uniform in the extension zone of hyphae (59, 67, 163). The cell wall grows by incorporation of material, which occurs maximally at the tip and declines toward the base of the expansion zone (25, 63). On the one hand, the unitary model proposed by Bartnicki-García (17), in agreement with the ideas of Johnson (87) and later supported by Gooday (64), invoked the simultaneous action of cell wall–loosening enzymes and cell wall–synthesizing enzymes in apical extension. On the other hand, the steady-state model of apical wall growth proposed by Wessels (176) favored the idea of an expanding plastic wall that becomes rigid in the subapex by the action of cross-linking enzymes; this was based on the fact that *Schizopyllum commune* hyphae did not present chitin microfibrils at the very apex, only in the subapical regions. Cross-linking between β(1,3)-glucans and chitin is essential for the formation of a resistant skeletal component (56). Nonetheless, localization of cross-linking enzymes has not been attained. In *A. nidulans*, chitinase ChiA tagged with EGFP localized at the apex of germ tubes, at hyphal branching sites, and at hyphal tips (182). Preliminary unpublished results indicate the localization of a putative β(1,3)-endoglucanase at the hyphal apical plasma membrane in *N. crassa* (103). Both models are in fact...
Lytic vesicles

Cell wall

Synthetic vesicles

Lytic vesicles

Cross-linking vesicles

Figure 2

Integrated model of cell wall growth. This model includes the simultaneous incorporation of synthetic, lytic, and cross-linking enzymes at the hyphal apex. Maximal vesicle exocytosis would occur at the foremost apical region and gradually decrease at the subapex, as predicted by the vesicle supply center model. Figure produced by E. Sánchez-León.

not exclusive. The most accepted current view proposes that cell wall–loosening enzymes, such as chitinases and glucanases, participate in the breakage of polysaccharide chains, such as chitin and β(1,3)-glucans, allowing the addition of newly arrived material and generating free ends, substrate for cross-linking enzymes, that rigidify the cell wall (Figure 2).

The Spitzenkörper

The Spitzenkörper (SPK) was first identified as an iron hematoxylin body present at the apex of fixed hyphae of Coprinus sp. and was thought to be associated with hyphal tip growth (37). The SPK was later observed by phase-contrast microscopy as a phase-dark body present only in the tips of actively growing hyphae (58, 105). At the ultrastructural level the SPK corresponds to a dense accumulation of vesicles of different sizes, ribosomes, microtubules, actin, and an amorphous or granular material of undefined nature (31, 59, 67, 82, 83, 132) (Figure 3). The SPK is a highly dynamic and pleomorphic apical cluster that plays a central role in apical growth and determines hyphal morphogenesis (21, 58, 129). One function attributed to the SPK is the concentration of secretory vesicles before they are delivered to the plasma membrane, to which they fuse, to produce new cell wall surface (19). In addition, because nucleation of microtubules was observed in hyphal apices and because microtubules were found in close proximity to the SPK (77, 113, 130), the SPK was suggested to serve as a microtubule-organizing center (MTOC). This hypothesis was deemed even more plausible when γ-tubulin was found by immunolocalization at the SPK of Allomyces macrogynus (106). Nevertheless, no γ-tubulin or other components indicative of a MTOC have been found at the SPK of other filamentous fungi.

Whereas mature N. crassa hyphae present a robust SPK, germ tubes do not display a SPK at their tips (8). One explanation for this lack of noticeable SPK has been the low growth rate of germlings, in which an insufficient amount of tip-directed secretory vesicles arrive at the tip. The same argument has been used to explain the lack of SPK in hyphae of slowly growing fungal species (93).

The SPK together with the polarisome and the exocyst have been considered part of the tip growth apparatus by several authors (74, 149, 152). Although these three components share some spatial distribution at the hyphal dome (88, 93), I prefer to make a functional distinction between the actual apical secretory apparatus and the polarity apparatus.

Riquelme
Overview of a hypothetical hyphal apex displaying some of the major components participating in tip growth. The polarity machinery maintains sites of growth by activating a set of cascade pathways that lead to polymerization of the actin cytoskeleton. Vesicles move along microtubules or actin cables until they reach the Spitzenkörper. From there they are delivered to the apical plasma membrane. Figure produced by E. Sánchez-León.

The decisive proof that the SPK is directly involved in the cell wall–building process arrived when cell wall synthesis enzymes were localized at the SPK (126, 165). Some studies identified the presence of CHS in the tips of *A. nidulans* and *U. maydis* (156, 171); however, their precise localization within the apex could not be discerned. *N. crassa* has one of the most prominent SPKs. Using fluorescent protein tagging, we (137, 165) identified CHS at the core of the SPK, where microvesicles concentrate, and GSC in the macrovesicle-rich region surrounding the CHS core. These exciting results revealed the nature of the cargo of some vesicles constituting the SPK and established morphological-functional differences among those vesicles. The joint presence of CHS and microvesicles in the core of the SPK supports the role of chitosomes in apical growth.

Thus far, this type of SPK vesicular distribution has not been confirmed in other fungal species. Several SPK patterns among the fungal kingdom have been described (101). It would be remarkable if this variety of SPK shapes corresponded to changes in the distribution and mode of operation of the cell wall–synthesizing machinery.

**The VSC Model of Hyphal Morphogenesis**

The vesicle supply center (VSC) model of hyphal morphogenesis (24) marked a turning point in the understanding of fungal morphogenesis. Despite some of the criticisms aroused by the VSC model, it undeniably has been one of the most influential and powerful models proposed to date. Prior to this, models could only approximate the hyphal shape (121). The VSC model, derived from a computer simulation of fungal morphogenesis, yielded the hyphoid equation $y = x \cot (xV/N)$, where $N$ is the amount of cell wall–building vesicles produced per unit of time and $V$ is the rate of advancement of the VSC, which would correspond to the SPK in real hyphae. Accordingly, advancing the VSC in a linear fashion while vesicles are being released would produce an ideal hypha.
Lipid rafts: plasma membrane domains rich in sphingolipids and ergosterol, essential for F-actin polarization and for concentration of GPI-anchored proteins at specific regions

By altering the V/N ratio or displacing the VSC, several cell shapes could be generated, including germinated conidia, yeast budding, branching hyphae, and meandering hyphae (23, 123, 129).

As is true for other models in biology, the VSC model made some drastic reductionist assumptions to simplify the complex process of hyphal growth. The original two-dimensional VSC model was later modified into a three-dimensional model; the latter was used to demonstrate that the cell wall expands orthogonally, as suggested many years earlier (22, 57). In this new formulation, the role of the cytoskeleton would be to hold or displace the VSC and the turgor pressure would provide the physical force needed for cell wall expansion. Although the role of turgor in hyphal morphogenesis historically has been controversial, it plays a key role in cell wall expansion (98, 110, 145).

The lack of evidence showing that the flow of cell wall–building vesicles passes through a SPK control gate represented one of the main shortcomings to validating the VSC hypothesis (145). Although it had long been established that vesicle traffic must exist in fungal hyphae, the origin, destination, and contents of the vesicles have remained nearly unknown. As mentioned above, by using compatible fluorescent proteins to dual label enzymes with cell wall synthesis activity, namely CHS and GSC, it was possible to elucidate the fate of cell wall–building vesicles accumulating at the SPK before they reached the plasma membrane (165). Microvesicles containing CHS were identified at the SPK core, whereas macrovesicles with glucan synthase activity were identified at the SPK periphery. The VSC model did not take into account the source of the vesicles. The newly available markers provide us with new tools to unveil the origins of these vesicles.

We do not quite understand how the different vesicular layers are formed, maintained, and regulated, or whether this spatial distribution is indicative of different exocytic vesicular routes from the SPK to the plasma membrane. To take the VSC model to the next level, we should first obtain detailed information on the amount, distribution, and routes of vesicle traffic at the tip. More accurate experimental data on vesicle numbers have been obtained by electron tomography of A. nidulans hyphal apices (80).

Although some studies dispute the presence of endocytosis in fungal hyphae (160), studies in which the SPK was stained with FM4-64 suggest that the SPK might have a role in endocytosis (52). The presence of a subapical collar of actin patches in A. nidulans and N. crassa hyphae was further indication that endocytosis occurs in filamentous fungi (29, 48, 146, 152, 164, 173). Extensive experimental evidence has proven that endocytosis plays an important role in polarized tip growth (146, 173). The apical recycling model suggests that a balance between endocytosis and exocytosis must be maintained at the hyphal tip, where these processes are spatially coupled (7, 144, 152, 164).

The landmark polarity factors present at the apical crescent (e.g., cell end markers, t-SNAREs, lipid rafts), where exocytic vesicles fuse with plasma membrane, are displaced to subapical regions when new material is inserted. The model proposes that the subapical collar would allow these factors to be recycled back to the tip while impeding them from diffusing to distal regions.

POLARITY ESTABLISHMENT

Establishment of polarity implies generating cellular asymmetry. Although the budding yeast has been a very useful model system to investigate polarized growth and its determinants, it is certainly not the ideal model fungus for understanding polarized tip growth in fungi. Yeasts are unicellular and divide asexually by budding or fission. In budding yeast, once polarity has been established, there is a short initial phase of apical growth at the bud tip, but shortly after, the daughter cell expands isotropically to give rise to an ellipsoidal shape, with an even distribution of new material all around the cell surface. In fission yeast, polarized growth is restricted to the two tips of the rod-shaped cells. In both budding and fission yeast, polarity is controlled during the cell cycle and sites of polarized cell growth and cell division are established by spatial cues determined at sites...
POLARITY REGULATION: POSITIVE AND NEGATIVE TROPISMS

Polarity factors are responsive to extracellular signals (chemostatic signals, pheromones, cell-to-cell contacts); therefore, the hyphal tips are the regions susceptible to experience changes in growth direction in response to multiple factors (33, 114, 180). Some plant pathogens present thigmotropism and penetrate the host when they detect certain topographical characteristics. For instance, the bean rust Uromyces appendiculatus detects ridges and grooves on plant surfaces by mimicking the topology of the host by using inert surfaces (78). In some arbuscular mycorrhizae, hyphal branching occurs in response to chemical factors produced by the host (5). Chemotropism also occurs during cell-cell fusion and hyphal avoidance (54, 55, 61). The morphological switch from yeast to hyphal growth in C. albicans, one of the best-studied dimorphic fungi, is regulated by an environmental change (reviewed in 34).

of previous cell division (42). In contrast, in filamentous fungi, upon establishment of polarized growth, the hyphal tube needs to be continuously extended by apical growth. Forward and reverse genetic screens have allowed researchers to identify and characterize genes and their products important for polarity establishment and maintenance (109, 141).

Breaking the Symmetry: Switch from Isotropic to Polarized Growth

Spore germination involves a switch from isotropic to polarized cell expansion. The VSC model of fungal morphogenesis can duplicate spore germination by advancing the VSC from its central position in the spore to the periphery (23). This simulation predicts the existence of a SPK in such a process. However, during the early stages of germ tube emergence and elongation in N. crassa, no SPK was observed (8), perhaps because of the small amount of tip-directed vesicles.

As for other morphogenetic processes, the emergence of a new tube from a preexisting rigid cell wall presumably requires softening of the existing cell wall (95). The details on how this may be accomplished are not really known. Some GPI-anchored fungal and plant chitinases may have a role during morphogenesis. A. nidulans mutants lacking the corresponding genes showed lower spore germination and hyphal growth rates (154). In addition, upregulation of chitinase activity has been observed during yeast-to-hypha morphogenetic transition in C. albicans (142).

Although in principle branching and germination could be considered similar processes that require the establishment of a new polarity axis, their biogenesis is quite different. Contrary to what has been observed during germination, lateral branches display polarity factors and a small SPK at their incipient tip practically from their onset (9, 124). Simulation of branch emergence with the VSC model would imply the formation of a new VSC, whereas germination could be achieved simply by displacing the preexisting VSC (see sidebar, Polarity Regulation: Positive and Negative Tropisms).

Cues for Polarity: Positional Markers, Polarisome, and Beyond

How does a spore know where to grow a germ tube? All the plasma membrane surrounding a cell could potentially be a new growth site. However, most fungal species produce only one or two germinating tubes, which emerge at specific domains in the plasma membrane (155).

In haploid budding yeast, new buds grow adjacent to previous sites of growth division marked by the landmark proteins Bud3, Bud4, and Bud10 (42). These cortical markers are poorly conserved
or absent in filamentous fungi (73). In *N. crassa* Bud3 and Bud4 are essential proteins for the establishment of septation sites (89). In fission yeast, growth occurs first at previous sites of cell growth and then at previous cell division sites. This process involves the delivery of the landmark protein Tea1 via microtubules (50). Among the filamentous fungi, a cell end-marking system of Tea proteins has been described so far only for *A. nidulans*, where it plays a critical role in hyphal growth (76).

Once a site is marked at the cell surface by polarity landmark proteins, a cascade of small GTPases (Ras-like factors and Cdc42) is activated to transduce the positional information to several effectors that promote the localization of the polarisome, a well-studied polarity-establishing multiprotein complex that mediates the nucleation of actin cables (150). In *S. cerevisiae*, Cdc42 is essential for cell viability. Filamentous fungi, in addition to Cdc42, have a Rac1 homologue. Although both GTPases have partial functional overlap and localize at the apical plasma membrane, Cdc42 is more important for polarity establishment and Rac is more important for polarity maintenance (9, 168). The yeast polarisome is composed of Spa2, Pea2, Arp3/Bud6, and the formin Bni1. Except for *A. gossypii*, no Pea2 homologues have been found in other filamentous fungi. The localization and role of the different polarisome components in *C. albicans* (46, 88, 149), *A. nidulans* (167), *A. gossypii* (91), and *N. crassa* (10, 100) have been analyzed. Bud6 localizes mainly at the apical cell surface as a crescent, whereas Spa2 partially colocalizes with the SPK at the apex. Formins nucleate actin microfilaments while they cap their barbed end (143). They have two domains, FH1 and FH2. The FH1 domain binds profilin, allowing G-actin to become incorporated into the growing barbed ends, a process mediated by the FH2 domain. In *A. gossypii*, *C. albicans*, and *A. nidulans*, formin homologues localize at hyphal tips in one of two forms, as a crescent at the cell surface or as a spot at the SPK (46, 138, 143).

One critical question is how are the polarity factors delivered and maintained continuously at the hyphal tip. One possibility is that a septin barrier located subapically could prevent the diffusion of cortical proteins to distal hyphal areas (see below). Also, an actin-dependent endocytosis mechanism at the subapex could recycle polarity factors back to the apex as mentioned above. Finally, some polarity factors can be locally translated at the tip (see below).

**POLARIZED CARGO TRANSPORT AND DELIVERY**

**Vesicles and the Secretory Pathway**

Different types of vesicles are involved in fungal cell wall growth (18). The two main populations of vesicles differ in size and function (19). Secretory or apical macrovesicles generally have diameters larger than 100 nm. Microvesicles have diameters smaller than 100 nm (30–40 nm on average). Chitosomes are a population of microvesicles proven to carry CHS for the synthesis of chitin at the plasma membrane (126, 136, 137). However, macrovesicles have not been isolated in pure form and therefore have not been fully characterized biochemically. Early data show that macrovesicles carry preformed polymers that will be incorporated into the amorphous part of the cell wall. Our own data have shown that glucan synthase is also transported in macrovesicles (165). Because the secretion of large amounts of diverse proteins occurs primarily at growing tips, it would not be unreasonable to predict that these are transported also in different subpopulations of macrovesicles. Further research needs to be conducted to decipher (a) whether functionally different enzymes are transported within the same or different vesicles and (b) whether all cell wall–building vesicles are generated via conventional (endoplasmic reticulum–to–Golgi equivalent) or unconventional secretory pathways.
Coating, Sorting, and Coupling: Coats, Rabs, Tethers, and SNAREs

Membrane trafficking is essential for generating and maintaining polarity. The classical secretory pathway sorts synthesized proteins from the endoplasmic reticulum, through the Golgi equivalent, to their final destination (e.g., plasma membrane, vacuole). Vesicle biogenesis and delivery include vesicle budding (scission from the donor membrane–vesicle formation), vesicle transport, vesicle docking (vesicle and target membrane brought into close proximity), and vesicle fusion (30, 66). Vesicular transport and active sorting between these organelles occur through a series of steps that require the participation of (a) coat complexes, which promote vesicle formation and recognize cargo-sorting signals; (b) tether complexes, which interact with coat proteins and mediate docking; and (c) SNAREs [soluble-NSF (N-ethylmaleimide-sensitive-factor) attachment protein receptors], which facilitate the fusion between the donor and target membranes (30). These processes are regulated by Rab GTPases, which act as molecular switches that cycle between a cytosolic, inactive GDP-bound form and a membrane-bound, active GTP-bound form, and by motor complexes that coordinate the vesicular transport along cytoskeletal elements. The type of interaction between the Rab GTPases and the actin- or microtubule-dependent motors is still unclear (65). In yeast and mammals, the secretory pathway has been well dissected and the mechanisms that maintain compartment identity and ensure fidelity of transport are rather well characterized, particularly for the endoplasmic reticulum–to–Golgi apparatus traffic events (15). Post–Golgi apparatus traffic is less well understood. Given the notorious differences in organelle distribution in hyphae (34), it would be sensible to also anticipate conspicuous differences in the mechanisms regulating vesicle traffic in filamentous fungi.

The Golgi apparatus traditionally has been considered a major hub for sorting, packaging, and transporting cargo. More recently, the endosomes have been identified as an additional hub, with a role in sorting and trafficking cargos internalized by endocytosis and participating in processes such as cell signaling, cytokinesis, polarity, cell adhesion, and cell migration (65, 84). Internalized cargoes, such as receptors or polarity factors, first reach the early endosome. They can then be degraded after entering the late endosome/vacuolar pathway or recycled through recycling endosomes to the plasma membrane in regions of constant reorganization (104). There are different types of recycling endosomes with heterogeneous tubular-vesicular morphology, which suggests dynamic and intense trafficking activity and connectivity between the endocytic and the exocytic pathways (6) (Figure 4).

Therefore, it is increasingly apparent that the exocytic and endocytic pathways converge at specific stages, sharing some of the lipid and protein loads. It is the precise combination of cargo and associated factors that ensures the correct directionality and fidelity of a vesicle (40, 66, 178). Indeed, phosphoinositides are important signaling molecules in organelle traffic. They serve as binding platforms for proteins. Specific phosphoinositides and small Rab GTPases cooperate at hubs that sort membrane traffic. Localized production of specific lipids (such as PI(3)P) causes the recruitment of molecules to subdomains of membranes.

Very few labs have studied the components of the secretory machinery in filamentous fungi (2, 3, 32, 118, 174), as there is practically null information on vesicle biogenesis and traffic. Of particular interest are the processes involved in the exocytosis of vesicles constituting the SPK, which upon arrival to the apical plasma membrane are presumably tethered via the exocyst (Figure 5). This conserved octameric complex (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) was first identified in budding yeast (157). Filamentous fungi have one homologue for each component. In A. nidulans, C. albicans, and slowly growing cells of A. gossypii, the fluorescently tagged exocyst components localized at the apical plasma membrane as a crescent (88, 93, 149, 152).
The hyphal apical secretory apparatus. Vesicles generated in the subapex associate with GTP-bound SEC4 Rab GTPase and motor proteins and concentrate at the Spitzenkörper. From there, the exocyst mediates their docking to the plasma membrane, where v-SNAREs and t-SNAREs interact to promote fusion of both membranes. Depicted is an example of a vesicle moving through an actin microfilament as described for *Saccharomyces cerevisiae* and *Candida albicans*. Figure produced by E. Sánchez-León.

Only in fast-growing cells of *A. gossypii* were the exocyst components localized at the SPK (88). In *N. crassa*, EXO-70 and EXO-84 are localized in close proximity to the SPK macrovesicular outer layer, whereas the rest of the components are distributed as a crescent at the apical plasma membrane (M. Riquelme, E.L. Bredeweg, O. Callejas-Negrete, S. Ludwig, R.W. Roberson, A. Beltrán-Aguilar, S. Seiler & M. Freitag, manuscript in preparation).

Exocytosis, the last stage of the secretory pathway, therefore provides membrane and delivery of specific cargo to sites of growth. In mammalian systems, it can occur at least by two described mechanisms: the classical full collapse fusion, in which the vesicle collapses into the plasma membrane, or by kiss-and-run, an alternative process in which the vesicle fuses transiently through a pore with the target membrane (131). In the latter, nonclassical, less-studied mode of fusion mechanism, the internal content of the vesicle can pass to the external medium through the pore, or transmembrane proteins can pass from the vesicle membrane to the target membrane by diffusion or a flip-flop mechanism.
**Moving Messages**

In recent years mounting evidence has shown that the transport of specific mRNAs to localized regions is important for fungal polar growth (26, 62, 169, 183). mRNAs and associated factors, such as RNA-binding proteins, form messenger ribonucleoprotein complexes (mRNP), which are actively transported along the actin or microtubule cytoskeletons. mRNA localization participates in targeting proteins involved in polarized secretion or spatial organization of the cytoskeleton (183).

In yeast, transcripts encoding polarity factors and some exocyst components are transported by this machinery along with cortical endoplasmic reticulum and Myo4, a class V myosin involved in moving mRNPs in one direction along F-actin cables (13). As mentioned above, the SPK contains polysome clusters in several filamentous fungi (82), suggesting local translation of mRNAs at the hyphal apex. Ample evidence confirming localized translation is now available (158). However, in *U. maydis* the shuttling of mRNP occurs in both directions along microtubules (26, 27). As the nature of some of the transported mRNAs is starting to be unraveled (92), new clues about unconventional routes for tip-enriched proteins with nonapparent secretory canonical signals emerge.

Why would it be advantageous for the hyphal cell to move and localize mRNAs? It is known that many copies of a protein can be made from one mRNA molecule, which is more cost-effective than protein transport. In addition, localized translation can allow a fast protein synthesis response to signals. More importantly, mRNA localization can serve to establish protein gradients, which may participate at the hyphal tip to maintain the precise and regulated exocytic gradient.

**The Role of the Cytoskeleton in Tip Growth**

The cytoskeleton maintains cytoplasmic organization, controls organelle positioning and movement, and plays an important role in tip growth and hyphal morphogenesis (20). The main components of the cytoskeleton are microtubules, actin, motors, associated proteins, and septins.

Whereas it is clear that actin, associated proteins (e.g., fimbrin, coronin), and myosins (actin-dependent motors) are essential for tip growth (49, 75, 107), the role of cytoplasmic microtubules has been more controversial. The main argument was that conidia germination and polarized growth were not inhibited when microtubules were depolymerized (115). However, it was proved that cytoplasmic microtubules and associated motor proteins have an important role in maintaining SPK stability, hyphal shape, and direction of growth (128, 129).
It had long been widely accepted that *A. nidulans* cytoplasmic microtubules are disassembled during mitosis (115). Yet, it was later shown in *A. nidulans* hyphae that during mitosis (lasting 5–10 min), when presumably all cytoplasmic microtubules were disassembled, growth rate was not reduced (127). This finding suggested that some cytoplasmic microtubules might remain during mitosis, supporting tip growth. Analysis of *A. nidulans* expressing GFP-tagged tubulin showed that cytoplasmic microtubules disassemble completely during mitosis in germ tubes but not in rapidly growing mature hyphae, where some cytoplasmic microtubules remained intact near the tip (81). It was observed that the microtubules remaining intact during mitosis were detyrosinated (184). These modified microtubules were the preferred tracks for UncA, a kinesin-3 motor with no homologues in yeast, to transport endosomes and vesicles (184).

Cytoplasmic microtubules have been considered the main tracks for the long-distance traffic of secretory vesicles to the SPK, and actin microfilaments would be involved in short-distance transport from the SPK to the plasma membrane (82, 106). Accordingly, several authors have viewed the SPK as a switching station from microtubule to actin microfilament transport (20, 74). Although an appealing hypothesis, no direct evidence exists showing actual transfer of vesicles from one cytoskeleton to the other. The only supporting data are the presence of cytoplasmic microtubules reaching the apex and traversing the SPK (77, 106, 113, 130) and the localization of actin and myosin V at the apex (153, 166, 172). Transmission electron microscopy analysis has shown very few vesicles near microtubules and the fluorescent microscopy methods currently used still do not have the resolution needed to show vesicle traffic along microtubular tracks. Dyneins (minus-end-directed microtubule molecular motors) have a clear role in maintaining nuclear dynamics and distributing hyphae of *A. nidulans*, *N. crassa*, *U. maydis*, and *C. albicans* (36, 51, 120). The distorted hyphal morphology and erratic diminished SPK in dynein mutants and wild-type cells treated with depolymerizing microtubule inhibitors is suggestive of an additional role of dynein in vesicle traffic (128). Kinesins (plus-end-directed molecular motors) are required for exocytosis and fast hyphal extension (140). In *U. maydis*, kinesin-3 is required in endosomal movement and exocytosis (139).

In yeast, Myo2, a second class V myosin, interacts with Sec4 and participates in the transport of post Golgi vesicles along F-actin cables (170). In *U. maydis* and *A. nidulans*, class V myosins are important in hyphal morphogenesis and exocytosis (153, 172). Class V CHS are a unique class of CHS in filamentous fungi consisting of a myosin motor–like domain (MMD) fused to a membrane-spanning CHS region. The apical localization of these CHS depends on the MMD, which facilitates their transport along actin microfilaments, as shown for Mcs1 in *U. maydis* and CsmA in *A. nidulans* (156, 161).

Septins are crucial scaffolding proteins for cell morphology that have a GTP-binding domain and a phosphoinositide-binding domain (60, 117). In filamentous fungi, septins display different appearances and functions, but how they contribute to cell shape is not fully understood. They form a diffuse cap at the tips of *C. albicans* (151), as well as rings composed of discrete bars in subapical regions and emerging branches in both *A. gossypii* and *A. nidulans* (177). They have been suggested to participate in vesicle exocytosis, maintain hyphal diameter and geometry, recruit polarity factors, and retain polarized domains within the plasma membrane (60).

**SUMMARY POINTS**

1. Hyphal tip growth is a complex and highly regulated process that involves the continuous traffic of secretory vesicles to the apex and their fusion with the apical plasma membrane.
2. Polarity factors need to be maintained continuously at the apex to regulate the assembly of the actin cytoskeleton and targeted exocytosis.

3. The different-sized vesicles in the SPK contain distinctive cell wall–synthesizing activities and adopt a layered distribution. Chitosomes at the core are surrounded by GS-containing macrovesicles.

4. An exocyst-mediated mechanism is necessary for the last steps of exocytosis to dock SPK vesicles to the apical plasma membrane.

5. The actin cytoskeleton is essential for polarity establishment and maintenance, and the microtubular cytoskeleton plays a key role in maintaining hyphal morphology, direction of growth, and fast growth rates.

6. Localized translation of mRNAs and endosome-derived storage compartments play an important role for polar apical growth. These mechanisms presumably enable fast delivery of proteins to specific regions of the hyphae.

FUTURE ISSUES

1. Further studies using microscopy techniques with better temporal and spatial resolution are required to elucidate the routes of vesicles in and out of the SPK and the associated cytoskeletal tracks and motors.

2. Comparative analyses of the composition and organization of SPK vesicles in different filamentous fungi could provide clues to advance our understanding of hyphal morphogenesis.

3. It remains to be determined whether all vesicles undergo a full fusion process, during which the vesicle collapses and the entirety of its membrane merges with the target membrane. Whether a kiss-and–run fusion mechanism occurs in fungal hyphae requires further exploration.

4. Certain organelles, such as the Golgi equivalent, are more abundant in the apical compartment. Future work will help discern whether they serve to generate apical vesicles or whether they have a specialized role to sort specific cargo depending on their hyphal position.

5. Evidence of the first steps of endocytosis (markers becoming internalized at the subapical actin collar) is lacking. Transmission electron microscopy has shown in very few instances that secretory vesicles fuse with the plasma membrane at the apex. However, no records of vesicle invagination exist.

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LITERATURE CITED


7. Describes an actin subapical collar and proposes coupling of apical secretion and subapical compensatory endocytosis (see also 164).


114. Oakey BR, Oakley CO, Yoon Y, Jung MK. 1990. γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*, *Cell* 61:1289–301

148. Contains links to 3D animations of major cytoskeletal elements and organelles in U. maydis.

149. Provides a current overview of the apical secretory and polarity apparatus.
165. First article showing evidence of a stratified SPK.

**RELATED RESOURCES**


