The *Neurospora crassa* exocyst complex tethers Spitzenkörper vesicles to the apical plasma membrane during polarized growth

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**ABSTRACT** Fungal hyphae are among the most highly polarized cells. Hyphal polarized growth is supported by tip-directed transport of secretory vesicles, which accumulate temporarily in a stratified manner in an apical vesicle cluster, the Spitzenkörper. The exocyst complex is required for tethering of secretory vesicles to the apical plasma membrane. We determined that the presence of an octameric exocyst complex is required for the formation of a functional Spitzenkörper and maintenance of regular hyphal growth in *Neurospora crassa*. Two distinct localization patterns of exocyst subunits at the hyphal tip suggest the dynamic formation of two assemblies. The EXO-70/EXO-84 subunits are found at the peripheral part of the Spitzenkörper, which partially coincides with the outer macrovesicular layer, whereas exocyst components SEC-5, -6, -8, and -15 form a delimited crescent at the apical plasma membrane. Localization of SEC-6 and EXO-70 to the plasma membrane and the Spitzenkörper, respectively, depends on actin and microtubule cytoskeletons. The apical region of exocyst-mediated vesicle fusion, elucidated by the plasma membrane–associated exocyst subunits, indicates the presence of an exocytotic gradient with a tip-high maximum that dissipates gradually toward the subapex, confirming the earlier predictions of the vesicle supply center model for hyphal morphogenesis.

**INTRODUCTION**

Exocytosis constitutes the last stage of the secretory pathway. It is a key process for cell growth and morphogenesis and entails the fusion of secretory vesicles to the plasma membrane (PM) to maintain the normal complement of proteins and lipids at specific PM subdomains (Shandala et al., 2012). During exocytosis, the vesicle membrane fuses with the PM via a fusion pore that dilates until the secretory vesicle collapses into the PM and the contents of the vesicles are deposited into the extracellular space (Rizzoli and Jahn, 2007).

The exocyst, a conserved multiprotein complex required for the final steps of exocytosis (Novick et al., 1980, 1981; Hsu et al., 2004), tethers vesicles to the PM before their soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–dependent fusion at specific PM sites (TerBush and Novick, 1995) and consists of eight components: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (TerBush et al., 1996; Guo et al., 1999a,b). All exocyst subunits are encoded by single genes in fungi and animals, whereas plants often have several genes for individual components (Elias et al., 2003; Zhang et al., 2010). It was suggested...
that this expansion of exocyst-encoding genes in plants might reflect developmental expression during differentiation in various tissues or multiple types of exocytosis (Zhang et al., 2010).

All exocyst components are hydrophilic, cytosolic proteins, which can also associate with membranes (TerBush and Novick, 1995). In Saccharomyces cerevisiae, exocyst components are localized at the site of bud emergence and the tip of small daughter cells and relocate to the mother–daughter connection during progression of the cell cycle (TerBush and Novick, 1995; TerBush et al., 1996; Finger et al., 1998). The exocyst complex regulates the docking of vesicles to specific sites of the PM during polarized secretion. The exocyst complexes of plants and mammals have more-diversified roles than that of budding yeast (Wang and Hsu, 2006; Zhang et al., 2010). In undifferentiated neuronal cells, for example, the exocyst shows perinuclear localization, but on differentiation it is redistributed to the extending neurite and concentrates at the growth cone (Vega and Hsu, 2001). In epithelial cells, the exocyst accumulates at the level of the adherens junction (Grindstaff et al., 1998). As in yeast, the mammalian exocyst is also essential for cellular processes that require polarized membrane traffic (Hertzog and Chavrier, 2011). The exocyst complex exists as two subcomplexes in yeast and mammalian cells, and the interaction between the two assemblies allows vesicle docking to the PM (Wang and Hsu, 2006). In yeast, Exo70p and Sec3p components are tethered to the PM (Boyd et al., 2004), whereas the remaining exocyst subunits associate with secretory vesicles through Sec4p (Guo et al., 1999b), a Rab GTPase that controls assembly of the exocyst, and the vesicle-SNARE Snclp/Snc2p (Shen et al., 2013). In mammals the PM-associated subcomplex contains Exo70, Sec3, Sec5, Sec6, and Sec8, whereas Sec10, Sec15 and Exo84 are complexed at the vesicle membrane (Moskalenko et al., 2003). Coassembly of the two animal subcomplexes is governed by the Rab-GTPases RabA and RabB via their interaction with Sec5 and Exo84 (Moskalenko et al., 2003). In plants there is less information on where exocyst subunits localize (Zhang et al., 2010). Immunolocalization studies in tobacco pollen tubes showed SEC6, SEC8, EXO70, and EXO70A1 at tip regions (Hala et al., 2008). In Arabidopsis, the exocyst facilitates initiation and maintenance of polarized growth in pollen tubes (Cole et al., 2005) and participates in secretory processes during cytokinesis (Fendrych et al., 2010).

Although the basic exocytotic machinery is conserved among eukaryotes, organisms have evolved different strategies to regulate endomembrane traffic. In contrast to unicellular yeasts, in which growth is directed to the bud tip and site of cell division in a cell cycle–dependent manner, in tip-growing cells, such as root hairs, pollen tubes, and developing neurons, expansion occurs primarily at the tip. Thus polarized exocytosis at sites of cell growth must be maintained permanently. Sustained exocytosis is focused to the hyphal apex of filamentous fungi, where a Spitzenkörper collects secretory vesicles temporarily, from which they are delivered to the apical PM (Bartnicki-Garcia et al., 1989). The localization of exocyst components was correlated with the hyphal growth rate in a filamentous member of the Saccharomycycota, Ashbya gossypii (Köhli et al., 2008). AgSec3, AgSec5, and AgExo70 localized as a cortical cap at the hyphal tip of slow-growing hyphae, whereas in fast-growing hyphae they accumulated at the Spitzenkörper. In the dimorphic yeast Candida albicans, exocyst components Sec3, 6, 8, 15, Exo70, and Exo84 localized to an apical crescent and remained stably located (Jones and Sudbery, 2010; Sudbery, 2011). In Aspergillus nidulans, SECC, the homologue of Sec3p, was localized in a small region of the PM, immediately anterior to the Spitzenkörper, at the apices of fast-growing hyphae (Taheri-Talebi et al., 2008). In Aspergillus oryzae, AoSec3 was localized to cortical caps at the hyphal tip as in A. nidulans but was also found in septa (Hayakawa et al., 2011).

Neurospora crassa, one of the fastest tip-growing organisms, has single genes for all eight exocyst components (Borkovich et al., 2004; Riquelme et al., 2011). Hyphae of N. crassa present a multilayered Spitzenkörper with a core of microvesicles that contain chitin synthase and an outer layer of macrovesicles that contain glucan synthase activity (Verdin et al., 2009; Sánchez-León et al., 2011). To study the final steps of the secretory pathway for these vesicles and their cargoes, we constructed translational fusions of the exocyst-encoding genes with gfp at their endogenous loci and analyzed their expression in relation to Spitzenkörper components.

**RESULTS**

**Identification of N. crassa exocyst complex**

Genes encoding putative N. crassa exocyst components were identified by in silico analysis of the N. crassa genome (www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html; Galagan et al., 2003) with fungal plant and human exocyst proteins as “bait” (Table 1). Eight clear homologues of exocyst proteins were identified and named SEC-3, -5, -6, -8, -10, and -15 and EXO-70 and -84. In addition, we found one gene (NCU03658) with a truncated Sec10-like motif, encoding the homologue of secretory pathway protein Sls2/Rcy1 (Figure 1). Overall, amino acid conservation of exocyst subunits between S. cerevisiae and N. crassa subunits is low (14–24% identity; Table 1). As one would expect, conservation is higher when comparing Ashbya gossypii to S. cerevisiae and N. crassa to A. nidulans, respectively.

All exocyst components but sec-10 were tagged with the split marker technique (Supplemental Figure S1 and Materials and Methods). The modified green fluorescent protein (GFP) fusion proteins expressed from the endogenous loci were functional, and all generated strains displayed wild-type (WT) morphology. To determine the composition of the N. crassa exocyst, we performed GFP-trap affinity purification experiments coupled to mass spectrometry (AP-MS). These AP-MS raw data (Supplemental Table S1) were filtered against a control data set obtained from purifications with GFP-expressing cells and precipitates of GFP-tagged proteins that have functions unrelated to the exocyst (Dettmann et al., 2013; Heilig et al., 2013). Precipitates of the six tagged exocyst subunits SEC-3, -5, -6, and -8 and EXO-70 and -84 recovered the entire octameric complex (Table 2). SEC-15 could not be precipitated. No additional proteins were identified that copurified with all exocyst components in a stable manner, and most proteins identified in these preparations were obvious contaminants. On the basis of these data, we conclude that an octameric exocyst complex exists in N. crassa.

**Exocyst subunits display two distinct localization patterns at the hyphal apex**

The tagged exocyst components were detected primarily at two cellular locations at the hyphal apex: the Spitzenkörper outer layer and apical PM (Figure 2). In addition, we observed some cytoplasmic fluorescence, probably representing a fraction of cytosolic components. Exocyst components SEC-5, -6, -8, and -15 accumulated primarily as a PM crescent at the hyphal dome and were excluded from the FM4-64–stained Spitzenkörper (Figure 2 and Supplemental Figure S2). In contrast, EXO-70 and EXO-84 accumulated in the proximal region of the external layer of the Spitzenkörper (Figure 2 and Supplemental Figure S2). SEC-3 accumulated at both the crescent and the Spitzenkörper outer layer, suggesting that it
may be the component bringing the remaining subunits together (Figure 2).

Coexpression of SEC-6 and EXO-70 labeled with either GFP or mCherry showed lack of colocalization of these two subunits (Supplemental Figure S3). Therefore SEC-6 and EXO-70 were chosen as representative markers for the two putative exocyst localizations—plasma membrane and Spitzenkörper outer layer (Supplemental Figure S3)—for further experiments. Both proteins showed positional changes within the N. crassa apex that correlated with changes in the position of the Spitzenkörper and thus determined growth.

### Table 1: Exocyst components and their localization from four members of the Ascomycota that serve as model organisms: two Pezizomycotina (N. crassa and A. nidulans) and two Saccharomycotina (S. cerevisiae and A. gossypii).

<table>
<thead>
<tr>
<th>Component</th>
<th>Locus ID</th>
<th>Subcellular localization</th>
<th>Identity with Sc (%)a</th>
<th>Identity with Nc (%)</th>
<th>Reference</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sec-3</td>
<td>NCU09869</td>
<td>Between Spk outer layer and PM</td>
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<td>Surface crescent</td>
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<tr>
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<td>Surface crescent</td>
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<td>This study</td>
</tr>
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<td>NCU08012</td>
<td>Spk outer layer</td>
<td>23</td>
<td>N/A</td>
<td>This study</td>
</tr>
<tr>
<td>exo-84</td>
<td>NCU06631</td>
<td>Spk outer layer</td>
<td>20</td>
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<td>This study</td>
</tr>
<tr>
<td><strong>A. nidulans</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>secC (sec3)</td>
<td>AN0462.2</td>
<td>Surface crescent</td>
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<td>36</td>
<td>Taheri-Talesh et al. (2008)</td>
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<td>secE (sec5)</td>
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<tr>
<td>secF (sec6)</td>
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<td>22</td>
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<td>secJ (sec10)</td>
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<td>secO (sec15)</td>
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<td>45</td>
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<td>Surface crescent</td>
<td>22</td>
<td>46</td>
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<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
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<td>SEC3</td>
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<td>14</td>
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<tr>
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<td>N/A</td>
<td>20</td>
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<td>Bud, bud neck</td>
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<td>N/A</td>
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<tr>
<td><strong>A. gossypii</strong></td>
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<td></td>
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<td>Agexo70</td>
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<td>51</td>
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<td>Agexo84</td>
<td>ADL321W</td>
<td>S, Surface crescent F, Spk</td>
<td>46</td>
<td>21</td>
<td>–</td>
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</table>

Percentage identity of exocyst subunit protein sequence compared with S. cerevisiae (Sc) and N. crassa (Nc). Based on identity and coverage of the aligned regions (usually 80–100%), Sec3 is the least-conserved exocyst component (coverage in various comparisons, 46–83%). Spk, Spitzenkörper; S, slow; F, fast.

ablast2seq (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to align sequence pairs. Exact amino acid identities of all aligned hits were summed and divided by the total length of the protein in the corresponding organism to get the final percentage.
germlings, there was a weaker and smaller fluorescent crescent at the apices of SEC-3-GFP, SEC-5-GFP, SEC-6-GFP, SEC-8-GFP, EXO-70-GFP, and EXO-84-GFP (Supplemental Figure S4). No fluorescence was observed at the apex of germ tubes for SEC-15-GFP (unpublished data).

Because components of the exocyst complex are concentrated in subdomains of the PM that represent sites of active vesicle fusion (TerBush and Novick, 1995; Hertzog and Chavrier, 2011), we analyzed the behavior of SEC-6-GFP by total internal reflection fluorescence microscopy (TIRFM). TIRFM allowed detection of directionality (Figure 3). No correlation between localization pattern of SEC-6-GFP and EXO-70-GFP and the tip extension rates was observed (Figure 3). In slow-growing hyphae of strains expressing SEC-6-GFP the fluorescence had a tendency to be more spread out, but it was always distributed to form an apical crescent (Figure 3). In

FIGURE 2: Localization of exocyst components at apices of mature hyphae of \textit{N. crassa} imaged by laser scanning confocal microscopy. For details see text. First column, GFP fluorescence; second column, FM4-64–stained cells; third column, merged; fourth column, phase contrast. Note that exocyst components EXO-70 and EXO-84 partially colocalize with the frontal outer part of the FM4-64–stained Spitzenkörper. Scale bar, 10 μm.
fluorescence-tagged proteins that are very close to the cell surface in regions of the hyphae in close contact with the coverslip. High-intensity fluorescent clusters, which represent individual exocytic events, were detected at delimited locations within the apical PM (Figure 4 and Supplemental Movie S1), suggesting an orderly discontinuous vesicle discharge mechanism for exocytosis during tip growth. These exocytic events were further evidenced when the times series obtained by TIRFM were converted into surface plots (Supplemental Movie S2).

The vesicle-associated exocyst components colocalize with the macrovesicular outer layer of the Spitzenkörper in an actin- and microtubule-dependent manner

We previously showed that the *N. crassa* Spitzenkörper consists of functionally and structurally distinct layers of vesicles (Verdin et al., 2009). To analyze the role of the exocyst complex in tethering of the different populations of vesicles at the Spitzenkörper, we coexpressed the GFP-tagged exocyst components SEC-6 or EXO-70 in strains with CHS-1–mCherry fluorescent protein (mChFP; microvesicular Spitzenkörper core) and strains with GS-1-mChFP (macrovesicular Spitzenkörper outer layer). The vesicle-associated component EXO-70 did not colocalize with the Spitzenkörper core labeled with CHS-1-mChFP but partially colocalized with the Spitzenkörper outer layer, occupied by the glucan synthase regulator GS-1-mChFP (Figure 5 and Supplemental Figure S5). The PM-associated component SEC-6, in contrast, did not colocalize with any layer of the Spitzenkörper (Figures 2 and 5, Supplemental Figure S5, and Supplemental Movie S3).

The localization of EXO-70-GFP was impaired upon exposure to the secretory pathway inhibitor brefeldin A (BFA; Figure 6), consistent with the association of this exocyst component with secretory vesicles. In contrast, SEC-6-GFP remained at the hyphal apical plasma membrane after drug treatment (Figure 6), providing additional support for incorporation of this exocyst component at plasma membrane independently of the secretory pathway.

TABLE 2: Proteomics analysis of the *N. crassa* exocyst complex.
An intact exocyst complex is necessary for organization of the Spitzenkörper and regular hyphal growth

The *N. crassa* functional genome project (Colot et al., 2006) generated deletion mutants for sec-5, sec-8, sec-10, sec-15, exo-70, and exo-84 (available from the Fungal Genetics Stock Center [FGSC], Kansas City, MO; see Table 3). Homokaryotic deletion mutants were available for sec-5 but could not be obtained for any of the remaining exocyst components, suggesting that these genes are essential. Although viable, the growth of *N. crassa* Δsec-5 was highly disturbed, with a phenotype similar to that previously described for sec-5+/−, a mutant strain obtained by ultraviolet mutagenesis (Seiler and Plamann, 2003). We sequenced the sec-5 locus in this strain and found several nucleotide insertions that introduced a nonsense codon early in the sequence, suggesting that sec-5+/− represents a loss-of-function mutant. Both Δsec-5 and sec-5+/− grew as highly compact, button-like colonies, in contrast to the spreading colony appearance typical of the WT strain (Figure 8A and C). At low magnification, Δsec-5 and sec-5+/− produced hyperbranched and distorted hyphae (Figure 8D and F).

WT hyphae stained with FM4-64 showed fluorescence at the plasma membrane (PM) and in apical vesicles that were concentrated at the Spitzenkörper (Figure 8J). Δsec-5 and sec-5+/− mutants showed lower accumulation of FM4-64 fluorescence at the tip (Figure 8, K and L), indicative of a lower concentration of vesicles. In fact, *N. crassa* Δsec-5 and sec-5+/− did not form a Spitzenkörper under phase-contrast microscopy (Figure 8, H and I). This observation was further investigated by transmission electron microscopy. In wild-type *N. crassa* hyphae, the Spitzenkörper was composed of an aggregation of macrovesicles (vesicles ranging from 70 to 100 nm in diameter) surrounding a differentiated core region composed of microvesicles (vesicles ranging from 25 to 40 nm in diameter) embedded in a dense granular to fibrous matrix, most likely actin microfilaments (Figure 9, A and B). Macrovesicles in wild-type hyphae contained a fine, granular, electron-opaque matrix (Figure 9, B and D) and had on average a diameter of 83 nm (n = 109; SD = 8.5). Directly subtending the Spitzenkörper was a cluster of ribosomes (Figure 9B). Ribosomes were also present within the core but were less commonly observed there. Microvesicles were on average 32 nm in diameter (n = 217; SD = 4.2) and were spherical when viewed in thin section (Figure 9, B and D). Occasionally, microvesicles were polyhedral. Macrovesicles and microvesicles extended into the apical cortex (Figure 9, A, C, and D), and, on occasion, macrovesicles were noted in juxtaposition to the cytosolic surface of the apical PM (i.e., docked; Figure 9C). Invaginations of the apical PM seen at the hyphal apex (Figure 9D) represent exocytosis events. These exocytotic profiles were only rarely observed.

To address the importance of the cytoskeleton for the localization of the two exocyst subunits, we determined the behavior of SEC-6-GFP and EXO-70-GFP after depolymerization of the actin and microtubule (MT) cytoskeletons. The effects of each inhibitor on growth and morphology of *N. crassa* hyphae were previously reported (Riquelme et al., 2000, 2002). Both SEC-6 and EXO-70 required the actin and the MT cytoskeletons for proper localization (Figure 7). On benomyl treatment, the apical localization of SEC-6-GFP was retained when hyphae started curling and lost growth directionality, although only very weak and dispersed fluorescence could be observed. After treatment with latrunculin A, hyphae ceased to grow in a polar manner and started to swell. During that period no SEC-6-GFP could be seen at the apical PM (unpublished data). When the hyphae started recovering and growth resumed apically, SEC-6-GFP was again observed at the apical PM (Figure 7, arrowhead). EXO-70-GFP also required both the microtubule and the actin cytoskeleton for proper localization. Both benomyl and latrunculin A treatments hindered localization of EXO-70 at the Spitzenkörper.

**FIGURE 4:** Individual frames extracted from a TIRFM time series of a *N. crassa* hypha expressing SEC-6-GFP. Bursts of exocytosis occurring at different positions within the apical plasma membrane can be observed. Bottom, magnified apices of the corresponding panels. Scale bar, 10 μm. Time in minutes:seconds.

**FIGURE 5:** Coexpression of exocyst components and Spitzenkörper components. (A) Exocyst components SEC-6 and EXO-70 do not colocalize with chitin synthase CHS-1, although EXO-70-GFP can be found surrounding CHS-1-mChFP at the core of the Spitzenkörper. (B) SEC-6 and the glucan synthase regulator GS-1 do not colocalize, whereas EXO-70 and GS-1 are partially colocalized. Scale bars, 10 μm.
An organized Spitzenkörper was not recognized in hyphae of the sec-5^{−9} mutant strain (Figure 9E), although at the apex of some hyphae a differentiated spherical zone was noted (Figure 9E). This zone contained a granular-to-fibrous matrix within which only a few microvesicles were embedded (Figure 9F). The differentiated spherical zone was similar in size, location, and content to the Spitzenkörper core noted in wild-type hyphae (Figure 9A). In the mutant a massive number of macrovesicles occupied the cytoplasm (Figure 9, A and E). Macropores on average were slightly larger in diameter (88 nm, n = 252; SD = 9.2) and contained a more diverse content, ranging from electron dense to translucent with varying degrees of granularity. In addition, larger bodies ranging from 100 to 350 nm in diameter were also observed in the cytoplasm of the mutant. Few microvesicles could be observed in the differentiated apical spherical zone in the sec-5^{−9} mutant, and these were similar to those observed in wild type. Mitochondria, endoplasmic reticulum, and other inclusions were observed among vesicles in subapical regions of hyphae (Figure 9, E and G).

To further investigate the nature of the spherical zone found by transmission electron microscopy at apices of the sec-5^{−9} mutant, we expressed Lifeact-GFP in the mutant. By the same approach, actin was previously found at N. crassa Spitzenkörper, forming patches in the subapical collar and as cables (Berepiki et al., 2010; Delgado-Alvarez et al., 2010). In sec-5^{−9}, however, Lifeact-GFP did not accumulate at the Spitzenkörper or as patches in the subapical collar (Supplemental Figure S6). Instead, it was observed as thick actin bundles that depolymerized after exposure to latrunculin A (Supplemental Figure S6). To confirm the importance of an intact exocyst for Spitzenkörper organization, we expressed SEC-6-GFP and EXO-70-GFP in sec-5^{−9} background. Both SEC-6-GFP and EXO-70-GFP were completely mislocalized in sec-5^{−9} and thus no longer observed at the apical hyphal surface or Spitzenkörper outer layer, respectively (Figure 10).

**DISCUSSION**

One of our long-term goals is the mechanistic understanding of how secretory vesicles are concentrated at sites of growth and how they are distributed from there to their final destination at the PM. We previously showed that the Spitzenkörper of N. crassa is a multilayered structure, with centered chitin synthase–containing microvesicles (chitosomes) and peripheral glucan synthase activity–containing macrovesicles (Verdin et al., 2009). In this study we identified association of the exocyst complex with a population of secretory vesicles, suggesting that the exocyst is involved in the PM tethering of the vesicles concentrated at the Spitzenkörper. We did not detect any exocyst component in the Spitzenkörper core region. Therefore it remains elusive how microvesicles occupying the Spitzenkörper core reach the PM. The genome sequence of N. crassa, like that of other filamentous fungi that have a high secretory capacity, revealed the existence of additional secretion-related Rab and Arf genes (e.g., ARF6/Rab2/5/18) with no homologues in yeast (Borkovich et al., 2004). Moreover, deletion of the sec4 homologue, srgA, is not lethal in Aspergillus niger (Punt et al., 2001). Taken together, the evidence suggests the
existence of additional secretory routes in filamentous fungi that are missing in yeast.

Exocyst complexes are present as two major subcomplexes of different composition in mammalian cells and budding yeast. Our localization experiments also suggest the existence of two dynamic assemblies, although it has been impossible to isolate them separately by biochemical means, not just in N. crassa, but also in other organisms. SEC-5, SEC-6, SEC-8, and SEC-15 accumulate as a crescent at the foremost apical region of the hyphal PM, in front of the Spitzenkörper, whereas EXO-70 and EXO-84 accumulate at the frontal region of the outer macrovesicular layer of the Spitzenkörper (Figure 11, A and B). SEC-3 adopts an intermediate position between both putative assemblies. This is quite different from the situation in yeast, in which Sec3p and Exo70p are associated with the PM, and in mammals, in which additional exocyst components are associated with the PM (Boyd et al., 2004; Wang and Hsu, 2006). Our GFP-trap experiments followed by MS analyses did not reveal the existence of large quantities of stable subcomplexes in N. crassa, as each bait component recovered the whole octameric complex. Thus more specific approaches are necessary to characterize these subcomplexes by biochemical means. Moreover, our MS analyses also showed that presumably functionally interacting proteins, such as the predicted Rho and Rab-type GTPases (Borkovich et al., 2004), were not recovered by this approach, suggesting that they only interact in a weak or dynamic manner with specific exocyst subunits.

EXO-70 and EXO-84 associate with the Spitzenkörper in N. crassa hyphae regardless of their growth rate. This contrasts with observations in A. gossypii (Köhli et al., 2008) but may be explained by the fact that the growth rates of this fungus (0.2–3.5 μm/min) are one to two magnitudes slower than that of N. crassa (Seiler and Plamann, 2003). This hypothesis is supported by the fact that all exocyst components form a crescent at the apical PM in N. crassa germlings, as in A. gossypii’s slow-growing hyphae. This could be due to the fact that no Spitzenkörper (apical vesicle cluster) can be seen in N. crassa until the germling reaches maturity and a certain growth rate (Araujo-Palomares et al., 2007, 2009). Therefore we propose that the differential localization of the exocyst components in different fungal species is due to the fact that in slow-growing N. crassa germlings, A. gossypii, S. cerevisiae, and C. albicans yeast cells and pseudohyphae no Spitzenkörper exists (Köhli et al., 2008).

The positional changes of the exocyst components mirrored the positional changes of the Spitzenkörper, confirming a link between the Spitzenkörper vesicles containing cell wall–synthesizing enzymes and the target PM region, where vesicles are delivered and fuse to PM as previously suggested (Bartnicki-Garcia et al., 1995; Riquelme et al., 1998). Recently a role for the exocyst in determining where the cell wall–synthesizing enzymes are delivered in the PM has been proposed in C. albicans (Caballero-Lima et al., 2013). In addition, our results indicate that the region of exocyst-mediated vesicle fusion at the hyphal apical PM follows a tip-high gradient as seen for the target-SNARE SSO-2 (Gupta and Heath, 2000) and as predicted earlier by the vesicle supply center model (Figure 11C) to be sufficient to generate the hyphal shape (Bartnicki-Garcia et al., 1989).

In S. cerevisiae, the exocyst components associated with secretory vesicles are transported to the cell surface in an actin-dependent manner (Finger et al., 1998; Pryne et al., 1998). In N. crassa, transport of secretory vesicle–associated component EXO-70 and PM-associated component SEC-6 is dependent on both actin and MTs, in line with the dependence of long-distance transport on both cytoskeletal elements in filamentous fungi (Seiler et al., 1997; Harris et al., 2005; Steinberg, 2007). Analyses in animal cells also suggest a functional interaction between the exocyst and MTs (Vega and Hsu, 2001; Wang et al., 2004). One of the possible roles suggested for the exocyst in animal cells is the regulation of vesicle release from MTs to cortical actin for vesicle docking and fusion (Wang and Hsu, 2006), which would fit with previously proposed models of vesicle traffic in fungal hyphae (Bartnicki-Garcia, 2002; Harris et al., 2005), in which the Spitzenkörper was suggested to be a switching station from MTs to actin cables. It remains to be proven whether the exocyst has a role in executing the switch.

FIGURE 8: Comparison of N. crassa WT and sec-5 mutant phenotypes. (A–C) After 7 d of incubation (28°C) on a Petri dish, both sec-5Δ and sec-5(7–9) mutants formed small, button-like colonies of a much smaller diameter than WT, which covered the Petri dish in 3 d (D–I). At low magnification, sec-5 mutants showed hyperbranched and distorted hyphae. The sec-5 mutants did not show a Spitzenkörper under phase-contrast microscopy (H, I) or when stained with FM4-64 (J–L). Scale bars, 200 μm (F), 5 μm (I),10 μm (L).
Components are essential (Novick et al., 1980). Conditional loss-of-function mutants showed decreased exocytosis and accumulation of secretory vesicles in the cytoplasm (Novick et al., 1980; Munson and Novick, 2006). In C. albicans, deletion of Sec3 is also not lethal and leads to accumulation of vesicles in yeast buds; here, Sec3 is required for polarized growth in hyphae (Li et al., 2007). We determined that most subunits are also essential for viability in N. crassa. This conclusion is based on the lack of homokaryotic knockout strains for most components and our inability to construct such strains (unpublished data). In contrast, sec-5 mutants are viable but severely affected. Lack of SEC-5 impairs exocyst function and leads to accumulation of exocytic macromolecules in the cytoplasm, decreased growth rate, induction of multiple new tips, and hyperbranching, illustrating the importance of the exocyst for Spitzenkörper formation and organization, polarized growth, and hyphal morphology.

In summary, this work shows that N. crassa is an excellent model system to analyze exocytosis and elucidate the role of the exocyst in polarized growth. Unlike other model systems, in which the analysis of loss-of-function mutants was required to identify the function of the exocyst, N. crassa allows direct manifestation of exocyst cycling between vesicles and target PM. More important, the present study suggests the existence of different mechanisms regulating the exocytosis of different cargo-transporting vesicles.

**MATERIALS AND METHODS**

**Strains and culture conditions**

Host strains for tagged exocyst components were N. crassa strains FGSC9718 (NMF263; Δmus-51::bar) and FGSC9717 (N2928, Δmus-51::bar-his-3; Ninomiya et al., 2004; Colot et al., 2006). Culture conditions and Neurospora methods are described elsewhere (Davis, 2000). Strains were grown on Vogel’s minimal medium (VMM; Vogel, 1956) supplemented with 1.5% sucrose. To select putative transformants, VMM supplemented with 0.05% fructose, 0.05% glucose, and 2% sorbose (FGS) and hygromycin B (Hyg, 200 μg/ml; Invitrogen, Carlsbad, CA) was used. An N. crassa sec-5 mutant (strain 7-9) was previously obtained by UV mutagenesis (Seiler and Plamann, 2003). An N. crassa sec-5 his-3 mutant strain was generated by crossing sec-57-9 to N625. Deletion mutants of sec-5 (FGSC11526), sec-8 (FGSC11510), sec-10 (FGSC11723), sec-15 (FGSC11509), exo-70 (FGSC11414), and exo-84 (FGSC11506) were generated by the N. crassa functional genome project (Colot et al., 2006) and obtained from the Fungal Genetics Stock Center (FGSC; Kansas City, MO). Heterokaryotic mutant strains were backcrossed to Δsad-2 strains (SMRP277 and SMRP278) on synthetic crossing medium (Westergaard and Mitchell, 1947) to isolate homokaryotic deletion strains; sad-2 mutants were used to circumvent the possibility of meiotic silencing (Shiu et al., 2001). Progeny were screened by PCR to identify homokaryons lacking the various exocyst genes. All strains are listed in Table 3. Plasmids VMRP27-3 and VMRP28-6 containing sec-6::8xGly::mchfp and exo-70::8Gly::mchfp, respectively, cloned into XbaI- and PacI-digested pJV15-2 (Verdin et al., 2009), were transformed into N. crassa strain FGSC9717. Plasmid pRM49-OC30 (Delgado-Alvarez et al., 2010) containing Lifeact-GFP was transformed into N. crassa sec-5 mutant strain (SMRP33). N. crassa his-3 sec-5 mutant strain (SMRP32) was crossed with strains expressing either SEC-6-GFP or EXO-70-GFP.

**Generation of C-terminal GFP fusions**

Genomic DNA from N. crassa WT (FGSC988) was extracted either with a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) or according to a previously published method (Pomraning et al., 2009). Plasmids pMF272 (Freitag et al., 2004) and pGFP::hph::loxP (Honda and Selker, 2009; GenBank accession number FJ457011) were purified with plasmid isolation kits (Qiagen).
Some strains were obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, MO.

TABLE 3: N. crassa strains used.

We searched the N. crassa genome sequence for orthologues of all known exocyst components previously identified in yeast (Table 1) and designed primers to tag the endogenous genes with superfolder GFP by a split marker gene replacement procedure (Smith et al., 2011). Six primers were designed for each replacement cassette (Table 4). We followed a standard naming convention for each gene to be tagged, where forward and reverse primers “gene”GlyF and “gene”GlyR, respectively, amplify ~1 kb upstream of each open reading frame. Primers designated “gene”GlyR are reverse primers to the ~20 nucleotides (nt) at the 3′ end of each coding region immediately upstream of the stop codon and preceded by 30 nt of the 5′-most sequence of the tags, usually a linker sequence of 10 glycine residues (Honda and Selker, 2009). Primers “gene”LoxF and “gene”LoxR amplify ~1 kb downstream of the coding region of interest, sometimes interrupting the 3′ untranslated region. Primers designated “gene”LoxF are forward primers to ~30 nt of the differential interference contrast images. Scale bar, 10 μm.
To generate replacement cassettes, primers “gene”GlyF and “gene”GlyR, or “gene”LoxF and “gene”LoxR, respectively, were used to amplify the 5′ and 3′ flanks for gene replacements with wild-type Neurospora genomic DNA as template. Amplicon 1 (5′PCRp1) and amplicon 2 (3′PCRp2) were gel purified and used as templates for a second round of PCR, where 5′PCRp1 and 3′PCRp2 were individually mixed with different tag and marker cassette amplicons (TagPCRp3). Primers used for this second PCR were “gene”GlyF and hphSMR, or hphSMF and “gene”LoxR(Supplemental Figure S1). The resulting fragments were gel purified, mixed in equal proportions (~0.5 μg DNA/transformation), and used to transform Neurospora conidia by electroporation (~2.5 x 10⁶ cells/ml, 1.5 kV, 600 Ω, 25 μF; Margolin et al., 1997). Successful transformation is believed to require three homologous recombination events (between the 5′ flank and the 3′ flank of the genomic sequence and the two PCR fragments, as well as the overlapping region of the marker sequence—that last event reconstitutes the selectable marker).

Host strains for transformations were NMF263 (FGSC9718) and N2928 (FGSC9717). Electroporated conidia were plated on VMM with FGS, histidine (His), and Htg (200 or 300 μg/ml). Primary transformants were picked into slants with His and Htg or grown on VMM plates with His and Htg (100 μg/ml). Transformants with fluorescence detected under an epifluorescence or confocal microscope were chosen for further analysis.

**Mass spectrometry and database analysis**

Liquid N. crassa cultures were grown at room temperature, harvested gently by filtration using a Büchner funnel, and ground in liquid nitrogen. The pulverized mycelium was mixed 1:1 with standard IP buffer (50 mM Tris/HCL pH 7.5, 100 mM KCl, 10 mM MgCl₂, 0.15% NP-40, 5 mM NaF, 1 mM PEFA, 1 mM Na₃VO₄, 25 mM β-glycerophosphate, 2 mM benzamidine, 2 ng/μl pepstatin A, 10 ng/μl aprotinin, 10 ng/μl leupeptin) and centrifuged (1 h, 10,000 rpm, Sorvall SS34 rotor) to obtain crude cell extracts as previously described (Dettmann et al., 2012; Maerz et al., 2012). The cell extracts were incubated with 2 μl GFP trap beads (ChromoTek GmbH, Planegg-Martinsried, Germany)/15 ml of cell extract on a rotator for 2 h at 4°C. The beads were washed three times with IP buffer, and immunoprecipitated proteins were recovered by boiling the beads for 10 min at 98°C in Laemmli buffer and separated by SDS–PAGE.

For protein identification by mass spectrometry, peptides of the in-gel trypsinized proteins were extracted from gel slices of Coomassie-stained protein bands. Peptides of a 5 μl-sample solution were trapped and washed with 0.05% trifluoroacetic acid on an Acclaim PepMap 100 column (75 μm × 2 cm, C18, 3 μm, 100 Å, P/N164535; Thermo Scientific, Waltham, MA) at a flow rate of 4 μl/min for 12 min. Analytical peptide separation by reverse-phase chromatography was performed on an Acclaim PepMap RSLC column (75 μm × 15 cm, C18, 3 μm, 100 Å, P/N164534; Thermo Scientific) running a gradient from 96% solvent A (0.1% formic acid) and 4% solvent B (acetonitrile, 0.1% formic acid) to 50% solvent B within 25 min at a flow rate of 250 nl/min (solvents and chemicals were from Fisher Chemicals, Fair Lawn, NJ; HPLC grade). Peptides eluting from the chromatographic column were on-line ionized by nanoelectrospray using the Nanospray Flex Ion Source (Thermo Scientific) and transferred into the mass spectrometer. Full scans within m/z = 300–1850 were recorded by the Orbitrap-FT analyzer at a resolution of 60,000 at m/z = 400. Each sample was analyzed using two different fragmentation techniques applying a data-dependent...
Light microscopy

Observations of growing cells (germlings or mature hyphae) were performed as previously described (Sánchez-León et al., 2011; Richthammer et al., 2012) using a Zeiss LSM-510 META inverted laser scanning confocal microscope (Carl Zeiss, Jena, Germany) and an Olympus FluoView FV1000 Confocal Microscope (Olympus, Japan), both equipped with Ar/2 ion and He/Ne1 lasers to detect GFP (λex = 488, λem = 505–530 nm) and mChFP (λex = 543, λem = 600–630 nm). We used a Plan Neofluor 100× oil immersion objective (numerical aperture 1.3) and a Plan Apochromat 60× oil
immersion objective (numerical aperture 1.42). The images were obtained in all channels simultaneously and handled digitally with LSM-510, version 3.2, software (Carl Zeiss) and with Olympus Fluoview software (Version 4.0a).

For total internal reflection fluorescence microscopy (TIRFM), an IX-70 inverted microscope equipped with a 60x/1.45 Apochromat objective lens (Olympus America, Center Valley, PA) and a krypton/argon laser (488 nm; Melles Griot, Carlsbad, CA) was used. Images were recorded with a Cascade 512B electron-multiplying, charge-coupled device camera (Photometrics, Tucson, AZ) for 2–3 min at 512 × 512 resolution and 10 frames/s. MetaMorph 6.1 software (Universal Imaging, Downingtown, PA) was used to control the camera and capture images. MetaMorph 7.5.0 was used to analyze and process TIRFM time series.

Because germ tubs have a tendency to grow into the agar, making microscopy difficult for the analyses of germlings, we inoculated conidia of each strain into VMM plates containing 3% agar to force conidia to germinate on top of the medium surface and observed them by laser scanning confocal microscopy after 6, 7, 8, and 9 h of incubation.

Transmission electron microscopy
Actively growing hyphae were prepared for transmission electron microscopy using cryopreparation methods. Cells were grown on thin, sterile, deionized dialysis membrane segments overlaying VCM at 23°C. After ~24 h of growth, cells and supporting membranes were trimmed with a sharp razor blade to ~5 × 5 mm and after 30-40 min (time to recover from trimming) were removed from the agar surface and immediately cryofixed by rapid plunging into liquid propane cooled to ~186°C with liquid nitrogen (Roberson and Fuller, 1988; McDaniel and Roberson, 2000). Freeze substitution took place in 1% glutaraldehyde and 1% tannic acid (wt/vol) in anhydrous acetone at ~85°C for 72 h. After washing in cold acetone (~85°C), the samples were warmed slowly to room temperature in 1% OsO4 (wt/vol) in acetone, washed in acetone, and infiltrated and flat embedded in Spurr’s resin (Spurr, 1969). Selected hyphae were sectioned and poststained in 2% uranyl acetate in 50% ethanol and bedded in Spurr’s resin (Spurr, 1969). Sections were examined using the styril dye N-(3- triethylammoniumpropyl)-4-[(4-(diethylamino) phenyl) hexatrienyl] pyridinium dibromide (FM4-64; 5 μM, λ = 514/670 nm absorption/emission; Molecular Probes, Invitrogen, Carlsbad, CA). A stock solution (500 μg/ml) of the anti-microtubule agent methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl; Sigma-Aldrich, St. Louis, MO) was prepared in ethanol. A stock solution (1 mg/ml) of the actin inhibitor latrunculin A (Sigma-Aldrich) was prepared in dimethyl sulfoxide. Different concentrations of benomyl (10, 20, and 30 μg/ml) and latrunculin A (20 and 40 μg/ml) were initially tested to assess the optimal concentration at which morphological defects were visible in cells growing at a reduced growth rate. A concentration of 20 μg/ml in VMM was used thereafter for both inhibitors. To inhibit the endoplasmic reticulum–Golgi secretory pathway, brefeldin A (Sigma-Aldrich) was added to growing hyphae at a final working concentration of 20 μM in VMM from a stock solution in dimethyl sulfoxide. Agar blocks were cut from the edge of growing colonies and inverted onto coverslips containing 10 μl of the corresponding inhibitor or fluorochrome. After 10 min, they were observed by laser scanning confocal microscopy.

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REFERENCES


