Traffic of Chitin Synthase 1 (CHS-1) to the Spitzenkörper and Developing Septa in Hyphae of Neurospora crassa: Actin Dependence and Evidence of Distinct Microvesicle Populations

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We describe the subcellular location of chitin synthase 1 (CHS-1), one of seven chitin synthases in Neurospora crassa. Laser scanning confocal microscopy of growing hyphae showed CHS-1-green fluorescent protein (GFP) localized conspicuously in regions of active wall synthesis, namely, the core of the Spitzenkörper (Spk), the apical cell surface, and developing septa. It was also present in numerous fine particles throughout the cytoplasm plus some large vacuoles in distal hyphal regions. Although the same general subcellular distribution was observed previously for CHS-3 and CHS-6, they did not fully colocalize. Dual labeling showed that the three different chitin synthases were contained in different vesicular compartments, suggesting the existence of a different subpopulation of chitosomes for each CHS. CHS-1-GFP persisted in the Spk during hyphal elongation but disappeared from the septum after its development was completed. Wide-field fluorescence microscopy and total internal reflection fluorescence microscopy revealed subapical clouds of particles, suggestive of chitosomes moving continuously toward the Spk. Benomyl had no effect on CHS-1-GFP localization, indicating that microtubules are not strictly required for CHS trafficking to the hyphal apex. Conversely, actin inhibitors caused severe mislocalization of CHS-1-GFP, indicating that actin plays a major role in the orderly traffic and localization of CHS-1 at the apex.

Chitin, a nonbranched homopolymer of β-1,4-N-acetylglucosamine (GlcNAc) residues, is widely found in the cell walls of yeasts and filamentous fungi, though its content and spatial distribution in the cell vary greatly among different species. Chitin content in filamentous fungi exceeds that in the yeast Saccharomyces cerevisiae (3, 7, 8, 33, 35). Chitin synthesis is catalyzed by chitin synthase (CHS; EC 2.4.1.16), an enzyme that transfers GlcNAc from UDP-GlcNAc to the nonreducing end of growing chitin chains (15). Cell fractionation analysis in S. cerevisiae revealed that CHS is present mainly in two locations: the plasma membrane and chitosomes (19). Extensive analysis of available fungal genome sequences revealed the presence of several genes that potentially encode chitin synthases (9, 22). Predicted peptide sequences suggest that CHSs are membrane-bound proteins with multiple transmembrane domains. Fungal CHSs have been grouped in three major divisions and seven different classes (30). Classes I, II, and III belong to CHS division 1 and contain a hydrophilic domain (Pfam 01644) in the N-terminal region of the catalytic domain. Classes IV, V, and VII are included in division 2, with classes IV and V containing the same catalytic domain (Pfam 03142), preceded by a cytochrome b₅₆-like domain (Pfam 00173).

Mushrooms and fungi, as well as certain plants, produce chitin synthase (CHS) enzymes that catalyze the formation (polymerization) of chitin, a major component of fungal cell walls. CHS enzymes can be classified into seven different classes based on their structural and functional properties. Classes I, II, and III belong to CHS division 1 and contain a hydrophilic domain (Pfam 01644) in the N-terminal region of the catalytic domain. Classes IV, V, and VII are included in division 2, with classes IV and V containing the same catalytic domain (Pfam 03142), preceded by a cytochrome b₅₆-like domain (Pfam 00173). Only classes V and VII have a myosin motor head-like domain (MMD) (Pfam 00063) in the N-terminal region. Division 3, which includes only one class (class VI), has a conserved catalytic domain but none of the Pfam domains present in the other divisions. Classes III, V, VI, and VII are exclusively found in filamentous fungi (9, 22, 30). In Neurospora crassa, seven chs genes (chs-1 to chs-7) have been found, each belonging to one of the seven different classes of CHSs (6, 14, 31).

The multiplicity of CHS genes and their corresponding proteins poses important questions as to their respective functions and indispensability. Recently, we reported that two CHS proteins of N. crassa, CHS-3 and CHS-6, tagged with green fluorescent protein (GFP), had similar intracellular distributions (31). These two proteins accumulated conspicuously in the core of the Spitzenkörper (Spk) of growing hyphal tips and in nascent septa but were also found in subapical regions in the form of spherical vacuoles, and—particularly CHS-6—in a network of irregular endomembranous compartments. Fluorescence recovery after photobleaching analysis suggested that CHS-GFP-containing microvesicles—i.e., chitosomes—are transported from subapical regions to the Spk and that these proteins utilize an alternative route to the classical secretory pathway (30, 31).

The mechanisms that control the localization and movement of cell wall-synthesizing enzymes in fungal hyphae are ultimately responsible for defining cell shape. Here we report the subcellular localization and requirements for transport of the N. crassa class III CHS (CHS-1) and compared them to those previously found for class I CHS-3 and class VI CHS-6.

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### MATERIALS AND METHODS

#### Strains and culture conditions

Bacterial and Neurospora strains used or generated in this study are listed in Table 1. Escherichia coli DH5α was grown on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (100 μg/ml) and incubated at 37°C. Neurospora cells were routinely grown on Vogel’s minimal medium (VMM) with 1.5% sucrose and, when required, 0.5% histidine (0.5 mg/ml) and 0.5% fructose (0.5 mg/ml) and incubated at 37°C.

Plasmids and oligonucleotides used in this study are listed in Table 1. Strains used or generated in this study were identified in Neurospora genetics. Conidia of *N. crassa* host strains FGSC9717 and FGSC9718 were obtained from the Fungal Genetics Stock Center (UMKC, Kansas City, MO).

Recombinant DNA techniques and plasmid constructions. Plasmids and oligonucleotides used are described in Table 1. Standard PCR and cloning procedures were used to fuse the *sgfp* gene to the carboxy terminus of *chs-1* in plasmids pMF272 (12) and pMF357. The 2.8-kb open reading frame (ORF) of the *chs-1* gene (class III CHS; NCU03611.3) and a 0.97-kb fragment downstream of *chs-1* were identified in *N. crassa* (www.broad.mit.edu/annotation/genome/neurospora) and amplified by PCR from *N. crassa* N1 (FGSC988) genomic DNA with custom-designed primers that included XbaI/PacI and PstI/KpnI restriction endonuclease sites at their 5' termini, respectively. PCR was performed in a Bio-Rad Thermal Cycler using Platinum Taq high-fidelity DNA polymerase (Invitrogen). For the *chs-1* gene, denaturation at 95°C (2 min) was followed by 25 to 30 cycles of 95°C (30 s), 58°C (30 s), and 72°C (6 min) and by a final extension at 72°C (5 min). For the *chs-1* 3'-flanking region, the conditions were similar, except that the annealing temperature was 61°C and the extension time was 70 s. The amplified and gel-purified *chs-1* PCR product was digested with XbaI and PacI and inserted into XbaI/PacI-digested pMF272 (12) (GenBank accession no. AY598428), yielding plasmid pESL01-1 (see Fig. S1A in the supplemental material). All plasmids were propagated in *E. coli* DH5α and purified with QIAprep Spin Miniprep kits (Qiagen). Plasmid inserts were sequenced at the Core Instrumentation Facility of the Institute for Integrative Genome Biology at the University of California, Riverside, CA, with primers pMF272F and pMF272R.

### Table 1. Plasmids, strains, and oligonucleotides used

| Plasmid, strain, or oligonucleotide | Description, genotype, or sequence | Reference and/or source |
|------------------------------------|------------------------------------|-------------------------|
| pMF272                             | Pccg1::sgfp<sup>a</sup>            | 12                      |
| pMF357                             | Pccg1::H1<sup>a</sup>             | This study              |
| pESL01-1                           | Pccg1::chs-1::sgfp<sup>a</sup>    | This study              |
| pESL02-3                           | Pccg1::chs-1::sgfp<sup>a</sup>    | This study              |

### Oligonucleotides<sup>b</sup>

| Oligonucleotide | Description | Source |
|----------------|-------------|--------|
| Chs1-Xba1-F-N  | 5'-GTCCCTCTAGAATGGCGGTACCGGTC-3' | This study |
| Chs1-PacI-R-N  | 5'-GGGCTTAATAGGCGGCGGAACG-3'     | This study |
| Chs1-3' flank-F | 5'-ACTGGGTACCCTACACACCGC-3'      | This study |
| Chs1-3' flank-R | 5'-TACGCTGCAGAACCCTACACCGTAC-3' | This study |
| Mrp10          | 5'-AGAGACACAGAATAACCGTTTCTT-3'   | This study |
| Mrp11          | 5'-AATCTACACAGCCCAAGCTTATATC-3'  | This study |
| Mrp12          | 5'-ATTAAAGACGGAGTGTTGGAAAG-3'    | This study |
| Mrp13          | 5'-ATGGATATAATGTGGCACGGTGAAG-3'  | This study |
| pMF272F        | 5'-CAAAATCACAACAAACTCTCACAACCCT-3' | 12 |
| pMF272R        | 5'-AGATGAACCTTGAGGTACGTTG-3'     | 31 |

<sup>a</sup> Heterokaryon.

<sup>b</sup> Bold type indicates restriction endonuclease sites.
FIG. 1. LSMC analysis of the distribution of CHS-1–GFP in the growing apices of Neurospora crassa hyphae. All hyphae show one intense fluorescent spot coinciding with the Spk core and punctate fluorescence in the subapical regions (A to K). Comparison of N. crassa strains N150 (Pccg-1::chsx-1::sgfp) and NES2-11 (chs-1::sgfp) showed no apparent differences between CHS-1–GFP expressed under the control of the native chs-1 gene promoter (A, C, G, and J) and that expressed under the control of the ccg-1 promoter (D, F, and H). The Spk core area occupied by CHS-1–GFP in all growing hyphae coincided with the phase-dark Spk observed in leading hyphae (C and F). Panels H and J show evidence of accumulation of fluorescence at the cell surface of the apical and subapical regions (white arrows). Panels I to K show comparison of the localization of CHS-1–GFP with the fluorescent dye FM4-64. Note mostly the lack of coincidence with only partial overlap at the core of the Spk and at some subapical vesicles (arrowheads). Bars, 5 μm.

A chs-1::sgfp+ B Phase C Merged D Pccg-1::chs-1::sgfp+ E Phase F Merged G Pccg-1::chsx-1::sgfp+ H Phase I Merged J Pccg-1::chs-1::sgfp+ K Phase L Merged

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CHS-1–GFP in all growing hyphae coincided with the phase-dark Spk observed in leading hyphae (C and F). Panels H and J show evidence of accumulation of fluorescence at the cell surface of the apical and subapical regions (white arrows). Panels I to K show comparison of the localization of CHS-1–GFP with the fluorescent dye FM4-64. Note mostly the lack of coincidence with only partial overlap at the core of the Spk and at some subapical vesicles (arrowheads). Bars, 5 μm.
Cytochalasin A (CA; Sigma) stock solution (10 mg/ml) was prepared in dimethyl sulfoxide and used at a final concentration of 10 μg/ml in VMM. Latrunculin A (LatA; Sigma) stock solution (1 mM) was prepared in dimethyl sulfoxide and used at a final concentration of 20 μg/ml in VMM. A stock solution (20 mg/ml) of the fungal metabolite brefeldin A (BFA; Sigma) was prepared in dimethyl sulfoxide and used at a final concentration of 5 μg/ml in VMM. For each dye or inhibitor, the agar blocks with hyphae were inverted onto coverslips with 5- to 10-μl drops of the diluted working stock. Stained or treated cells were imaged 3 to 5 min after recovery.

**RESULTS**

In mature hyphae, CHS-1–GFP localizes predominantly in the Spk core region. In all growing hyphae examined, CHS-1–GFP was conspicuously found in the core of the Spk; this was true for both leading hyphae and lateral branches (Fig. 1; see Fig. S3 and Movie S1 in the supplemental material). The fluorescence of CHS-1–GFP coincided with the position of the phase-dark Spk. The membranous marker FM4-64 stained a larger Spk area than that outlined by CHS-1–GFP; the latter was mainly restricted to the central or “core” region of the Spk.
(n = 19) (Fig. 1I to K). As the hypha elongated, the Spk with its CHS-1–GFP-containing core moved forward, accompanied by characteristic minor lateral movements and pleomorphic changes (see Movie S1 in the supplemental material). There was also accumulation of CHS-1–GFP at the cell surface in the apical and subapical hyphal regions (arrows in Fig. 1H and J). Small fluorescent particles were found scattered over the entire length of the hyphae examined (Fig. 1A, D, H, and J). Since in several other studies in Neurospora the expression of GFP constructs has been driven by the ccg-1 promoter, we determined if expression of CHS-1–GFP was affected by different promoters, specifically whether his-3-targeted CHS-1–GFP from the ccg-1 promoter significantly changed localization patterns compared to expression of CHS-1–GFP at the endogenous locus. LSCM analysis of NES1-15 (Pccg-1::chs-1::sgfp) and NES2-11 (chs-1::sgfp) in both cases (n ≥ 30) revealed no significant differences in CHS-1–GFP localization (Fig. 1). The only noticeable difference was that under the native promoter, less background fluorescence was observed and therefore that strain was the one selected for further analyses.

Wide-field epifluorescence microscopy showed a high concentration of CHS-1–GFP directly behind the Spk (see Movies S1 and S2 in the supplemental material). This mass of small, rapidly moving fluorescent particles appeared to merge with the Spk core. With the finer detail and better temporal resolution of TIRFM, we could observe the movement of this mass of particles reaching the Spk (Fig. 2E and F; also see Movie S4 in the supplemental material). Some of the fluorescent particles evidenced by TIRFM seemed to move along the hyphal periphery (Fig. 2H to N; see also Movie S3). However, by changing the angle of incidence of the laser, we were able to observe beneath the cell periphery and could image the particles (vesicles) moving in the interior of the hypha (Fig. 2A to G). Most vesicles moved in an anterograde fashion, although some moved in a retrograde manner (Fig. 2H to N). Analysis of the dynamics of three fluorescent vesicles in a lateral branch showed one vesicle moving fast toward the tip and the other two moving in a retrograde manner but at different speeds (Fig. 2O). Kymograph analysis in different hyphae revealed different motion patterns of vesicles (Fig. 2P and Q). The speeds of anterograde and retrograde moving vesicles were on average 3.8 ± 0.9 and 3.6 ± 0.9 μm/s, respectively (n = 20 for each).

Spatial relationship between vesicular populations involved in glucan and chitin synthesis. Previously, we found that GS-1, a glucan synthase-related protein, was localized in a subpopu-
lation of vesicles that accumulated in the outer stratum of the Spk (39). To further analyze the dynamic relationship between the different Spk components, strains NJV4.2.1 (GS-1–GFP) and TJV12-1 (CHS-1–mChFP) were vegetatively fused and examined by confocal laser scanning microscopy (Fig. 3). CHS-1–mChFP appeared as a solid round apical body surrounded by a layer of GS-1–GFP. Overall, the intensity, position, and behavior of CHS-1–mChFP (Spk core) remained relatively constant as the hypha advanced, although with the typical minor oscillations of a Spk. In contrast, the structure highlighted by GS-1–GFP (Spitzenring) was more dynamic, adopting horseshoe-like forms with the opening facing different directions as the hypha grew (Fig. 3; see also Movie S5 in the supplemental material), thus suggesting that it rotated around the microvesicular core. A sudden retraction of the Spk showed both the core and the Spitzenring behaving as a unit (Fig. 3B). Once the Spk regained its position at the center of the apical dome, the hypha continued to grow, although it initially had a more pointed appearance and a reduced diameter.

In distal regions of mature hyphae, CHS-1 is localized in the tubular vacuolar network. The vacuolar dye CCDFDA was used to analyze the nature of the compartments harboring CHS-1–mChFP or CHS-1–GFP fluorescence. CCDFDA staining in the Neurospora strain TJV12-1 revealed a very dynamic and extensive tubular and spherical vacuolar network along the hyphae (Fig. 4B, E, and H), in both distal and subapical regions. CHS-1–GFP or CHS-1–mChFP was observed within globular vacuoles as well but only in distal regions of hyphae (Fig. 4A and J). The FM4-64 lyophilic probe stained the membrane of globular vacuoles, confirming that fluorescently labeled CHS-1 occupied the lumen of these presumed vacuolar structures (Fig. 4J to L).

Coimaging of CHS reveals differential localization of CHS-1, CHS-3, and CHS-6. To analyze whether CHS-1–GFP was an integral part of the same subcellular compartments where CHS-3–GFP and CHS-6–GFP were found to be present (31), we generated heterokaryons by hyphal fusion between each one of these strains and strain TJV12-1 (expressing CHS-1–mChFP). Although we found that CHS-1–mChFP and CHS-6–GFP were located in the Spk, the fluorescence of CHS-1–mChFP occupied a larger area than that of CHS-6–GFP fluorescence (Fig. 5A to C). Also, CHS-1–mChFP did not label the numerous globular and tubular structures labeled by CHS-6–GFP in the subapical regions of the hyphae (Fig. 5B and C). Although we found no distinction between CHS-1–mChFP and CHS-3–GFP did not label the numerous globular and tubular structures labeled by CHS-6–GFP in the subapical regions of the hyphae (Fig. 5B and C). Although we found no distinction between CHS-1–mChFP and CHS-3–GFP in the Spk, where both occupy the same area of the Spk core (Fig. 5E to G), in the apical and subapical cytoplasm, two distinctly separate subpopulations of vesicles were clearly evident (Fig. 5G).

**CHS-1–GFP localization in developing septa.** A strong fluorescent band of CHS-1–GFP was observed in all developing septa (Fig. 6; see Fig. S3 in the supplemental material).
two-dimensional optical sections, the first indication of impending septation was a growing accumulation of CHS-1–GFP that appeared as symmetrical thickening of the hyphal wall (Fig. 6A). In three dimensions (3-D), this corresponds to a cylindrical “sleeve” of CHS-1–GFP. As this sleeve shortened, a fluorescent ring began to grow inwardly from the middle of the sleeve, with the typical morphology of a fungal septum (Fig. 6B; see also Movie S6 in the supplemental material). The fluorescent ring can be seen growing centripetally until a full septum is formed (B to E). FM4-64 staining revealed the colocalization with CHS-1–GFP in a developing septum (F to H). Bars, 5 μm.

**FIG. 6.** CHS-1–GFP accumulation in developing septa. A sleeve of CHS-1–GFP accumulated in cortical areas of the hypha prior to septum formation (A) (arrowheads). A fluorescent ring can be seen growing centripetally until a full septum is formed (B to E). FM4-64 staining revealed the colocalization with CHS-1–GFP in a developing septum (F to H). Bars, 5 μm.

CHS-GFP localization in germinating conidia. During germ tube emergence, there was no conspicuous accumulation of CHS-1–GFP, CHS-3–GFP, or CHS-6–GFP at the sites of polarized growth (Fig. 7). Young germlings (<100 μm in length or grown for less than 9 h) of *N. crassa* expressing CHS-1–GFP and CHS-3–GFP showed discrete fluorescent patches of different sizes, scattered through the germ tube (Fig. 7A and D) and not visible in the corresponding strain used for the transformations (Fig. 7J). In some of the germlings, a diffuse phase-dark cloud was observed by phase-contrast microscopy at the apical region (Fig. 7E and H). Yet, at this stage no distinct Spk could be observed.

The actin cytoskeleton is required for CHS-1–GFP localization at the Spk and the septum. Inhibitor studies were undertaken to examine whether MTs or actin was involved in CHS-1 transport. To study the effect of benomyl, we first generated dually labeled heterokaryons of strains TJV12-1 (CHS-1–mChFP) and N2526 (tubulin-GFP) to image GFP-labeled tubulin and mChFP-labeled CHS-1 simultaneously. In heterokaryotic hyphae, GFP clearly labeled the expected large number of cytoplasmic MTs (12, 25), while mChFP labeled the Spk core with intense red fluorescence (Fig. 8A and F). Within 4 min after benomyl was applied to the heterokaryon, all MTs had become depolymerized, leaving only small fluorescent particles corresponding to tubulin subunits (arrows in Fig. 8B). During the first 20 to 25 min after benomyl treatment, hyphae showed highly distorted morphology and decreased growth (Fig. 8C), as previously reported (25). During this period of strong growth impairment, no Spk could be observed in the distorted hyphae and therefore there was no accumulation of CHS-1–mChFP at the apex (Fig. 8C). However, 5 to 10 min later, when still no repolymerized GFP-labeled MTs were visible, hyphae started to recover and grow, and CHS-1–mChFP continued to accumulate at the Spk of distortedly and slow-growing hyphae (Fig. 8D and E). Once the cells recovered from the treatment (grew out of the inhibitory zone) and reached high growth rates, MTs gradually became visible again, concomitantly with the reestablishment of normal hyphoid morphology (Fig. 8F). CHS-1–mChFP accumulation at sepa was also unaffected by the benomyl treatment (Fig. 8G).

In contrast to the results with benomyl, CA and LatA, which bind to F-actin and G-actin, respectively, therefore preventing actin microfilament assembly, affected CHS-1 localization drastically. LSCM observations of hyphae treated with 10 μg/ml CA revealed gross mislocalization of CHS-1–GFP (Fig. 8H to N). In apical regions, fluorescence was observed distributed in large patches that grew inwardly, developing one or multiple concave structures (Fig. 8H to L; see also Movie S8 in the supplemental material). At the septa, fluorescence accumulated unevenly, probably representing abortive nascent septa (Fig. 8M). One of the overall effects of CA was the previously described dichotomous apical branching giving the appearance of a candelabrum (Fig. 8N) (32). LatA-treated hyphae showed also cortical accumulations of CHS-1–GFP at subapical areas (Fig. 8P to S). Immediately after addition of LatA, the hyphal tips swelled and a Spk was no longer observable (Fig. 8O). As observed for CA, in LatA-treated cells, fluorescence accumulated unevenly at septa (Fig. 8T and U). Another intriguing effect of LatA was the accumulation of fluorescence at the membrane of globular vacuoles (Fig. 8T), instead of the typical accumulation at the vacuolar lumen.

**CHS-1 reaches the Spk unaffected by brefeldin A.** To evaluate if CHS-1–GFP follows the conventional secretory path-
way, we used brefeldin A (BFA), an inhibitor of endoplasmic reticulum (ER)-to-Golgi complex traffic, on strain NES2-11. BFA reduced the hyphal elongation rate drastically in a dose-dependent manner (Fig. 9A). LCSM revealed that the distribution of CHS-1–GFP was not altered in treated hyphae (Fig. 9). More interestingly, we found a slight increase in CHS-1–GFP accumulation at the Spk and apical plasma membrane in some hyphae (Fig. 9B and C). In addition, colonies exposed to BFA showed an increase in branching (Fig. 9D and E).

Effect of chs deletions on hyphal phenotype. Southern blotting and PCR analyses of selected strains confirmed the expected genotypes (see Fig. S2 in the supplemental material). The absence of chs-1 in the Δchs-1 strain was confirmed by PCR (see Fig. S2F in the supplemental material). Growth, colony, and hyphal morphology were examined in three deletion mutants (Δchs-1, Δchs-3, and Δchs-6 strains) and compared to the CHS-1–GFP and the corresponding host strains used for transformation (Fig. 10). Elongation rates of both Pccg-1::chs-1::sgfp+ and chs-1::sgfp+ strains and their corresponding host strains were comparable to those obtained with Neurospora Pccg-1::chs-3::sgfp+, Pccg-1::chs-6::sgfp+, and wild-type strains, reported earlier (29, 31). Elongation rates of the chs-1::sgfp+ (39.2 ± 3.9 μm/min, n = 3) and Pccg-1::chs-1::sgfp+ (36.9 ± 4.4 μm/min, n = 8) transformants were not significantly different from those of the Δmus-51::bar+ (46.2 ± 3.0 μm/min, n = 6) and his-3; Δmus-51::bar+ (39.2 ± 5.9 μm/min, n = 6) parental strains, respectively. Growth of Δchs-1 and Δchs-3 strains (37.0 ± 1.1 μm/min, n = 3, and 35.2 ± 2.2 μm/min, n = 3, respectively) showed a slight growth rate reduction compared to that of the Δmus-51::bar+ strain. Growth of the Δchs-6 strain (23.2 ± 4.4 μm/min, n = 3) was significantly reduced (Fig. 10N). Observations of the colony margins revealed no gross differences between host strains used for transformations, chs-1::sgfp+–expressing strains, and the Δchs-1 strain (Fig. 10). Hyphal diameters and overall branching patterns were similar in all strains.

DISCUSSION

Different populations of chitosomes. In addition to showing that CHS-1 is distributed in the hyphae of N. crassa mainly in the same locations previously reported for CHS-3–GFP and CHS-6–GFP (31), namely, the sites of new cell wall synthesis (growing apices and developing septa) plus numerous particles of various sizes distributed along the cyto-

FIG. 7. Localizations of CHS-1, CHS-3, and CHS-6 tagged with GFP during early germ tube development. CHS-1–GFP and CHS-3–GFP fluorescence was randomly distributed along the germlings (A and D, respectively), whereas CHS-6–GFP fluorescence accumulated at small globular and tubular vacuoles (G). No fluorescence could be observed accumulating at the apical region of germ tubes expressing any of the fusion proteins (A, D, G, and J). No fluorescence was observed in germlings of N. crassa strain FGSC9718, the parental strain used for transformations. Bars, 5 μm.
plasm, we discovered a crucial difference in that each of these three chitin synthases is contained in a separate compartment, clearly resolved by simultaneous labeling with green and red fusion proteins. Most striking are the double-labeling images showing CHS-3 and CHS-1 as entirely separate populations of small particles. In the Spk, CHS-6 occupies a smaller area than CHS-1 and CHS-3. Earlier biochemical and cell fractionation studies on S. cerevisiae (19), N. crassa (20, 23), and other fungi had established the presence of CHS in chitosomes, a homogenous population

FIG. 8. Effect of cytoskeletal inhibitors on CHS-1–GFP localization. Microtubule inhibition (A to G). LSCM analysis of N. crassa hyphae coexpressing CHS-1–mChFP and tubulin-GFP treated with 10 μg/ml of benomyl. Untreated hyphae showing CHS-1–mChFP in the Spk (A) (arrowhead) and a great abundance of long GFP-labeled microtubules along the hypha (arrow) that seem to reach the apical dome. Microtubules are quickly and completely depolymerized, and small fluorescent patches—tubulin subunits—appear (B) (white arrows); nuclei change from pear shaped to round (B) (white arrowheads); the hypha loses its hyphal shape and grows more isotropically. During that time, no Spk could be observed (B and C), but when polarized growth was resumed (D and E), CHS-1–mChFP fluorescence reappeared at the growing tips, even though microtubules remained depolymerized. When the inhibitory effect had disappeared, repolymerization of MTs could be observed (F). CHS-1–mChFP localization at the septal ring was unaffected by benomyl (G) (white arrowheads). Actin inhibition (H to N). Treatment of the CHS-1–GFP-expressing strain NES2-11 with 10 μg/ml cytochalasin A (CA) disrupted hyphal growth and modified the distribution of fluorescence in apical and subapical regions. CHS-1–GFP no longer accumulated at the Spk; instead, several fluorescent crescent-shaped structures were observed in the apical and subapical regions immediately under the cell surface (H to L). Deposition of CHS-1–GFP at nascent septa was disrupted under the effect of CA (M) (white arrowhead). A typical dichotomous branching (candelabrum) pattern at the edge of colonies exposed to CA was observed by phase-contrast microscopy (N). Effects of 20 μg/ml latrunculin A (LatA) on NES2-11 (O to U). Initial swelling of hyphal tips as result of the drug exposure observed by phase-contrast microscopy (O). CHS-1–GFP no longer accumulated at the Spk (P to S); as for CA treatment, several fluorescent crescent-shaped structures were observed in the apical and subapical regions immediately under the cell surface. Localization of CHS-1–GFP at septa was also disturbed (T and U) (white arrowhead). In addition, fluorescence was observed at the membrane of globular vacuoles (black arrowheads). Bars, 5 μm (500 μm in panel N).
of microvesicles of low buoyant density, plus a less homoge-
neous population of membranes of higher density. Although
the actual size of the CHS-1–GFP fluorescent particles
could not be ascertained from the confocal images, several
studies of transmission electron microscopy of hyphal tips
showed the presence of a population of microvesicles in the
Spk core—the region where fluorescence is most intense.
Hence, we conclude that some, if not most, of the fine
fluorescent particles are microvesicles whose enzymatic
cargo identified them as the previously characterized chito-
somes. Collectively, our present data suggest the existence
of different populations of chitosomes, each of them con-
taining a class of CHSs, which eventually congregate at the
apex within the Spk core. Further biochemical studies in-
volving fractionation to separate specific subpopulations of
chitosomes will be required to characterize these vesicles.
In addition, studies on the remaining four other CHSs present
in N. crassa are needed to show if they also are contained in
discrete novel chitosome classes or are part of already-
imagined chitosomes.

Another difference between the chitin synthases was the
conspicuous accumulation of CHS-6 in subapical tubular vac-
uoles, a feature not observed for either CHS-1 or CHS-3. The
finding of the three different CHSs in distal globular vacuoles
and the observed retrograde movement of some vesicles (pu-
tative endosomes), plus the fact that endocytosis has been
reported to occur mainly in the subapical regions of the hyphae
(1, 38), lead us to suggest that the CHS-GFP released at the
apex becomes, as the hypha grows, displaced to a subapical
position, where it is endocytosed and transported to the vacu-
ole for degradation.

Accumulation of CHS-1 to the Spk depends on an intact
actin cytoskeleton. Benomyl had little effect on the delivery
and distribution of CHS-1 at the Spk and septa, which strongly
suggests that transport of CHS-1 is independent of the micro-
tubular cytoskeleton. Conversely, under actin depolymeriza-
tion conditions, CHS-1–GFP did not accumulate in the Spk but
was found instead in subapical crescent-like structures close to
the cell surface. This suggests that actin is necessary for Spk
core assembly. A role of actin as scaffold regulating the distri-
bution and stability of the Spk has been suggested. Accord-
ingly, actin may be regulating the rate of vesicle flow, as de-
duced by the observation that the Spk in Neurospora act-1
mutants was smaller than that in wild-type strains (40). Tro-
pomysin, an actin binding protein, and LifeAct labeled with
fluorescent proteins were localized at the core of the Spk in
hyphal tips of N. crassa (4, 11). In Ustilago maydis, mutations or
deletion of the myosin motor-like domain (MMD) of Mcs1
(class V CHS) resulted in mislocalization, although not in loss
of motility of Mcs1-GFP vesicles, suggesting that Mcs1 de-

de
depend s on a myosin-actin-based cytoskeleton for localization
but requires MTs for motility (37). Unlike CHS classes V and
VII, class III CHSs do not have any apparent MMDs and their
mechanism of motility remains unknown. More studies are
needed to investigate whether CHS-1 is transported along in
chitosomes containing other CHSs with MMDs or is trans-
ported in another population of vesicles propelled by other
motor proteins.

CHS-1 traffic is independent of the conventional ER-to-
Golgi complex secretory pathway. The drug BFA has been
traditionally used as an inhibitor of ER-to-Golgi complex traf-


FIG. 9. Effect of brefeldin A (BFA) on the growth rate of N. crassa and in localization of CHS-1–GFP. Radial growth rate of N. crassa (n = 3) under different concentrations of BFA (A). LSCM analysis of hyphae of N. crassa strain NES2-11 exposed to BFA (5 μg/ml) (B to E). CHS-1–GFP was observed at the Spk of leading hyphae and branches of BFA-treated cells. Note how in some cells fluorescence occupied a larger Spk area than in untreated hyphae (B, inset). Accumulation of CHS-1–GFP fluorescence was also seen at the apical and subapical cell surface (C) (white arrows). Hyperbranching and dichotomous growth due to BFA effect were observed by LSCM (D) and bright-field microscopy (E). Bars, 10 μm (B and D), 2 μm (C), and 100 μm (E).
inhibits the membrane recruitment of the small GTPase ADP-ribosylation factor 1 (ARF1), one of the first steps in the formation of the coat protein complex I (COPI) that covers Golgi complex-derived vesicles (16). In filamentous fungi, as in mammalian cells, BFA leads to the disassembly of the Golgi complex equivalents and eventually to the arrest of apical extension (28). As the accumulation of CHS-1–GFP at the apex was not impeded by BFA, we suggest that the transport of CHS-1 may occur by an alternative nonconventional secretion pathway, as has been reported for some eukaryotic proteins (26). Similar results had been obtained with CHS-3 and CHS-6 (31). In addition, more intense and stable fluorescence was noticed in the Spk after BFA treatment, suggesting that a protein inhibited by BFA may be participating in the transport of CHS-containing vesicles from the Spk core to the plasma membrane, thus increasing the half-life of CHS-1–GFP in the Spk.

**CHS-1 during germination.** In contrast to the confocal images of well-developed hyphae, we failed to observe polarized distribution of CHS-1–GFP, CHS-3–GFP, or CHS-6–GFP during conidium germination and germ tube elongation. Earlier studies suggested either that young germ tubes (~12 h) did not contain chitin (glucosamine polymers) or that chitin was deposited at low densities in their cell walls (34). The latter explanation seems more plausible given that the growth rate of a germ tube is but a minuscule fraction of the growth rate of a mature hypha (2). In addition, transcriptional profiling data have shown that dormant conidia present minimal expression for 10 genes predicted to be involved in glucan and chitin synthesis and a peak expression at 0.5 h and afterwards (18). We did not see polarized expression of any of the GFP-tagged CHSs during conidial germination, but they were scattered through the cell, indicating that even though they are being synthesized, they are not being transported to the tip or not accumulating in sufficient quantities to be observed.

**A role for class III chitin synthases?** Class III CHSs are the only subfamily in division I of chitin synthases exclusively found in filamentous fungi (30). Previously, class III chitin synthase genes have been implicated in a variety of functions, including growth and pathogenicity. In *Coccidioides posadasii*, *CpCHS3* appears more highly expressed during the saprobic mycelial phase than at stages of parasitic spherule development (22). Disruptants of *chsB* of *Aspergillus nidulans* grow as minute colonies, without conidia and with highly branched hyphae (5). In *A. nidulans* ChsB was localized at the tips of germ tubes, hyphal tips, and forming septa and during conidio genesis (13). These authors showed that enhanced GFP (EGFP)-ChsB was distributed as a crescent in tips of germlings 60 to 70 μm in length and also during germination but coincided with the Spk in mature hyphae. In *Fusarium oxysporum*, no *chs3* mutants could be obtained, suggestive of the essential role of *chs3* for cell viability (24), and in *Botrytis cinerea Bcchs3a* has been reported to be involved in virulence (36). However, deletion of *CgChsIII* in *Colletotrichum graminicola* did not have obvious effects (42). Collectively, these data suggest that class III CHSs have undergone evolutionary divergence. The original *Neurospora chs-1* (class III) mutant, isolated after repeat-induced point mutation (RIP), exhibited aberrant morphology, hyphal swelling, thin aerial hyphae, deteriorated tips, scarce conidiation, and significant growth re-

![FIG. 10. Colony and hyphal morphology of the *N. crassa* strains analyzed in this study. Images of hyphae from the growing edges of the colonies of the different strains (A to G). Colony appearance after 24 h of growth under white light (H to N). Images correspond to the following strain genotypes: Δmus-51 (FGSC9718) (A and H), Δmus-51 (FGSC9717) (B and I), *chs-1::sgfp*’ (NES2-11) (C and J), *Pccg-l::chs-1::sgfp*’ (NES1-15) (D and K), Δchs-1 (FGSC14318) (E and L), Δchs-3 (FGSC14320) (F and M), and Δchs-6 (FGSC13408) (G and N). Bar, 200 μm. Petri plates, 9 cm.](image-url)
duction, which suggested an important role of this enzyme in cell wall biosynthesis (44). In contrast, our examination of the Δchs-I deletion mutant showed only a slight decrease in growth but, based on our unpublished data, a significant decrease in chitin synthase activity. One plausible explanation for these discrepancies is that chs-I mRNA synthesis was greatly reduced in the RIP strain but that likely some read-through occurred, generating CHS-1 protein with RIP-induced missense mutations, which are sufficiently severe to cause dominant synthetic effects. These effects are not expected to be obvious in a deletion strain because other CHS proteins can partially complement the complete lack of CHS-1.

CHS-1–GFP turnover. The generally accepted view is that chitin synthases operate at the cell surface presumably integrated into the plasma membrane. Yet, at the growing apex, we found only scant evidence of CHS-1 accumulation on the cell surface (apical plasma membrane). Conceivably, the density of CHS-1, as well as those of CHS-3 and CHS-6, positioned in action, may be too low to be easily detected in the membrane profiles, or alternatively, there may be a high rate of turnover after the CHSs reach their destination. On the other hand, accumulation of CHS-1–GFP, as well as of CHS-3 and CHS-6, can be clearly seen in the invaginating membrane during septum formation. Whereas CHS-3–GFP and CHS-6–GFP fluorescence disappeared about 20 min after septum completion (31), CHS-1–GFP fluorescence remained detectable for ~1 h after septum formation was completed. This suggests different half-lives for the three different enzymes, depending on their localization. Whether recycling or endocytosis explains the observed turnover remains an unexplored area in fungal biology.

Clues toward understanding cell wall synthesis. It is not clear if the horseshoe-shaped Spitzenkörper, composed of large secretory vesicles (macrovacuoles), rotates around the Spk core or if a fraction of the peripheral macrovacuoles are transiently lost. The last possibility is particularly exciting since the horse-shaped-like structure could be the consequence of the delivery of secretory vesicles to the apical dome. Alternatively, both chitin synthases and glucan synthases could be delivered at different times, the horseshoe opening allowing the exit of CHS from the core to the cell surface. These observations suggest a spasmotic release of vesicles that could be related to the previously reported pulsed growth of N. crassa tips (21).

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