Spatially Segregated SNARE Protein Interactions in Living Fungal Cells*

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The machinery for trafficking proteins through the secretory pathway is well conserved in eukaryotes, from fungi to mammals. We describe the isolation of the snc1, sso1, and sso2 genes encoding exocytic SNARE proteins from the filamentous fungus Trichoderma reesei. The localization and interactions of the T. reesei SNARE proteins were studied with advanced fluorescence imaging methods. The SSOI and SNCI proteins co-localized in sterol-independent clusters on the plasma membrane in subapical but not apical hyphal regions. The vesicle SNARE SNCI also localized to the apical vesicle cluster within the Spitzenkörper of the growing hyphal tips. Using fluorescence lifetime imaging microscopy and Foerster resonance energy transfer analysis, we quantified the interactions between these proteins with high spatial resolution in living cells. Our data showed that the site of ternary SNARE complex formation between SNCI and SSOI or SSOII, respectively, is spatially segregated. SNARE complex formation could be detected between SNCI and SSOI in subapical hyphal compartments along the plasma membrane, but surprisingly, not in growing hyphal tips, previously thought to be the main site of exocytosis. In contrast, SNCI-SSOII complexes were found exclusively in growing apical hyphal compartments. These findings demonstrate spatially distinct sites of plasma membrane SNARE complex formation in fungi and the existence of multiple exocytic SNAREs, which are functionally and spatially segregated. This is the first demonstration of spatially regulated SNARE interactions within the same membrane.

Protein transport through the exocytic and endocytic pathways in eukaryotic cells occurs via vesicle trafficking between successive membrane-bound compartments. The transport of vesicles from endoplasmic reticulum to the plasma membrane requires a series of events involving budding from donor compartments and docking and fusion with acceptor compartments (1, 2). Membrane-associated proteins called SNAREs2 (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) facilitate the fusion between vesicles and target membranes. SNAREs were originally divided into two classes according to their localization to vesicle (v-SNARE) or target (t-SNARE) membranes (3). Each SNARE protein contains a motif that is able to form a parallel α-helical structure with other SNARE motifs that brings the vesicle and target membranes into close proximity, allowing membrane fusion (3). Mammalian synaptic t-SNAREs can interact as a binary complex of one syntaxin and one SNAP-25 in cells (4), in the absence of associated v-SNARE. The subsequent interaction of the appropriate v-SNARE with this binary SNARE complex allows the formation of the SNARE ternary complex and thus defines the location of vesicle fusion (4). It is unclear whether the stable ternary SNARE complex forms before the final fusion event is initiated.

In addition to the mammalian exocytic SNAREs, the best characterized SNAREs are the yeast Saccharomyces cerevisiae membrane trafficking counterparts (5, 6). SNARE complex formation in the yeast secretory pathway has been shown to begin with the formation of binary complexes between two t-SNAREs, SsoP (a syntaxin homolog) and Sec9 (the SNAP-25 homolog) (7), that are then capable of binding the v-SNARE Snpc. The Sso and Sec9 proteins have been shown to be located uniformly over the plasma membrane in these geometrically simple cells (8). In contrast to yeast cells, filamentous fungi exhibit highly polarized growth at the hyphal tips. This involves the delivery of Golgi apparatus-derived secretory vesicles to the apical plasma membrane via an apical vesicle cluster within a multicomponent complex called the Spitzenkörper (9). These secretory vesicles contain membrane proteins and lipids, enzymes involved in cell wall synthesis, and possibly cell wall precursors (10).

Although the exocytosis of extracellular enzymes (i.e. in contrast to “constitutive” secretion at growth cones or buds) into the external medium is generally believed to occur mainly from hyphal tips, few studies have actually localized the subcellular

* The work was supported by a grant from the Academy of Finland (Grant Number 106685) in the frame of the Academy of Finland research program “VTT Industrial Biotechnology” (Finnish Centre of Excellence Program 2000–2005, Project 64330) (to M. V.) and a Wellcome Trust RCFD Fellowship (to R. R. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY676605, EF121546, and EF583658.

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‡1 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; TCSPC, time-correlated single photon counting; FLIM, fluorescence lifetime imaging microscopy; FRET, Foerster resonance energy transfer; PDB, Protein Data Bank; TPE, two-photon excitation; FWHM, full width at half-maximum; SC, synthetic complete; MCP-PMT, multichannel plate-photomultiplier tube.

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SNARE Interactions in Filamentous Fungi

sites of secretion. Immunogold and green fluorescent protein localization of glucoamylase in Aspergillus niger supported the hypothesis that the secretion of this enzyme primarily takes place from hyphal tips (11, 12). Other studies have suggested that enzymes might also be secreted from subapical hyphal locations (13, 14), but this has never been shown directly. Understanding of the fungal secretion pathway has significantly increased recently. Based on the analysis of sequenced fungal genomes, it is clear that the secretory machinery of filamentous fungi has similar features to that of other eukaryotes (15, 16), and some genes encoding the proteins involved in vesicle trafficking have been cloned and characterized (17–22).

Here, we have used a combination of in vitro biochemistry, in vivo complementation assays, and advanced live cell imaging techniques to quantify novel SNARE protein targeting, co-localization, and interactions in filamentous fungi. As the v-SNARE interacts with t-SNAREs after formation of the fungal SSO-sec9 binary complex (7), by quantifying the interaction of a vesicular v-SNARE with two plasma membrane t-SNAREs using fluorescence lifetime imaging microscopy (FLIM)/FRET analysis, we have identified different subcellular sites for exocytic SNARE ternary complex formation in living fungal hyphae. Interestingly, the interactions between a sole v-SNARE and two alternative t-SNAREs are spatially segregated. This is the first time that SNARE protein interactions have been demonstrated directly in living fungal cells; these data reveal contrasts with mammalian SNARE cluster regulation and also suggest an alternative exocytic pathway to the constitutive secretion known to occur via the Spitzenkörper at hyphal tips. Importantly, this is the first time that spatially regulated SNARE interactions have been found on the plasma membrane.

EXPERIMENTAL PROCEDURES

S. cerevisiae Suppression and Complementation Studies—A Trichoderma reesei cDNA expression library (23) in the vector pAJ401 (24) was transformed into the SSO2 temperature-sensitive S. cerevisiae strain H1152 (Mata, sso2-1, leu2-1, trp1-1, ura3-1, sso1::HIS3 (25). Transformants were plated on synthetic complete (SC) medium (−Ura) with 2% galactose as the carbon source, and the plates were incubated at the restrictive temperature, 31 °C. Transformants that were able to grow on these plates were grown as streaks under the same conditions and then replicated for growth at 31, 33, 35, and 37 °C. Plasmids were isolated from clones that were able to grow at 37 °C and transferred into Escherichia coli for sequencing.

The suppression of the SSO2 temperature-sensitive mutation was tested by transforming the plasmid pMS84 obtained in the original screening back to the H1152 strain. After transformation, the strain was grown on SC (−Ura) plates with 2% galactose as a carbon source. For the suppression studies, the transformants were streaked on the same medium and replicated to parallel plates that were incubated at 24, 31, 33, and 37 °C.

Suppression of Sso1p and Sso2p depletion was tested in the H458 strain (Mata, ade2-1, his3-11, trp1-1, ura3-1, sso1-1::LEU2, sso2-1::LEU2:(GAL1::SSO1, HIS3) (courtesy of Hans Ronne, Uppsala University, Sweden). The strain was grown after transformation on SC (−Ura) plates with 2% galactose as a carbon source, and the transformants were replicated on either YP (1% yeast extract, 2% peptone) or SC (−Ura) plates with 2% glucose as the carbon source and grown at 30 °C.

For the analysis of the complementation of Snc1p and Snc2p depletion, the T. reesei snc1 cDNA was cloned as an EcoRI-Xhol fragment into a S. cerevisiae single copy plasmid pKK1 with the PGK1 promoter and terminator and LEU2 selectable marker. The resulting plasmid was called pMV20, and it was transformed into the JG8 T15:85 (Mata his3 leu2 trp1 snc1::URA3 snc2::ADE8 pGAL-TSNCI) (26) strain. Transformants were grown on SC (−Leu−Trp) plates with 2% galactose as the carbon source. For complementation testing, the transformants were grown on SC (−Leu−Trp) plates with 2% galactose as the carbon source for 3 days and then replicated on YPD (1% yeast extract, 2% peptone, 2% glucose) or SC (−Leu−Trp) plates with 2% galactose as the carbon source and grown at 30 °C for 4 days. To test the complementation of the temperature sensitivity defect, transformants were replicated on SC (−Leu−Trp) plates with 2% glucose as the carbon source and grown at 24, 30, and 37 °C for 4 days.

For the analysis of the complementation of Sso1p and Sso2p depletion, the T. reesei sso1 cDNA was cloned into the S. cerevisiae expression vector pAJ401, either as a fusion with the mCerulean fluorescent protein (27) or as a non-fusion, creating vectors pMV61 and pMV62, respectively. The T. reesei sso2 cDNA was cloned into the pYES-DEST53 Gateway vector (Invitrogen), creating the pMV72 plasmid. All constructs were transformed into the SSO2 temperature-sensitive S. cerevisiae strain H1152. Transformants were plated on SC (−Ura) with 2% galactose as the carbon source, and the plates were incubated at the permissive temperature, 24 °C. Transformants that were able to grow on these plates were incubated as streaks under the same conditions and then replicated for growth at 24, 31, 33, 35, and 37 °C.

Invertase Assay—To study the ability of T. reesei SNC1 to suppress the defect in invertase secretion in a strain depleted of both Snc proteins, an experiment was done with modifications as described by Ref. 26. The invertase assay was done using the JG8 T15:85 strain transformed with the pMV20-construct or the expression vector pKK1 alone and with the wild type strain NY15 (Mata, ura3-52, his4-619, courtesy of Peter Novick, Yale University, New Haven, CT). Two parallel cultures of the strains were grown in SC (−Leu−Trp) medium with 4% galactose as the carbon source at 30 °C for 2 days. The cultures were then diluted to an A600 of 0.2 in the same medium and grown at 30 °C until the A600 was 2.5. After this, the cultures were transferred to SC (−Leu−Trp) medium with 2% glucose as the carbon source and grown at 24 or 30 °C. This was done to deplete the cells of the endogenous Snc1 protein. The cultures were kept in the early logarithmic growth phase throughout the experiment by repeated dilutions in the same medium. After growth in glucose-containing medium for 12 h, samples of 4 × 10⁶ cells were removed from each culture, centrifuged, and resuspended in 20 ml of SC-Leu-Trp medium with 0.05% glucose as the carbon source to derepress the invertase gene. The samples were incubated at 24 or 30 °C for 2 h. A sample of A600...
2.5 was taken from each culture for the invertase assay. The invertase assay was performed as described in Ref. 28.

Confocal Live Cell Imaging—For imaging, the yeast transformants with the pMV62 expression construct and the controls (transformants with pAJ401 vector alone) were grown in SC (−Ura) with 2% galactose for 2 days. 200-μl drops of diluted cell suspension were placed in an eight-well slide culture chamber (Nalgene Nunc International, Rochester, NY).

For imaging T. reesei, the snc1 cDNA was cloned into the pContV vector (courtesy of Dr. N. Curach, Macquarie University, Sydney, Australia), where it was expressed as an N-terminal fusion to the fluorescent protein Venus (29) under the endogenous cbhl promoter. The resulting expression vector was called pMV63. For the construction of the mCer-SSO1 and mCer-SSOII fusion proteins under the cbhl promoter, the Venus in pContV vector was replaced with mCerulean. The sso1 and sso2 cDNAs were cloned into this vector, and the resulting vectors were called pMV67 and pMV77, respectively. The expression vectors were transformed into T. reesei RutC-C30 (30), creating pMV63, pMV67, and PMV77 single transformants or pMV63/67 and pMV63/77 double transformants.

For imaging purposes, the strains were grown on plates on T. reesei minimal medium (31) for 5 days, and the inverted agar block method of sample preparation (32) was used. 10 or 30 μM FM4-64 (prepared from 1 M stock in Me₂SO; Invitrogen-Molecular Probes) was used in combination with fluorescent protein labeling. The stain was added to the agar block containing the mycelial sample 10 min before imaging. Confocal laser scanning microscopy was performed using a Radiance 2100 confocal system equipped with blue diode and argon-ion lasers (Bio-Rad Microscience) mounted on a Nikon TE2000U Eclipse inverted microscope.

mCerulean was imaged on its own with excitation at 405 nm and fluorescence detection at 470/90 nm; Venus was imaged on its own with excitation at 514 nm and fluorescence detection at 470/90 nm; and FM4-64 was imaged simultaneously by excitation with the 514 nm laser line with fluorescence detected at 530/50 nm (for Venus) and >620 nm (for FM4-64). A ×60 (1.2 NA) plan apo water immersion objective lens was used for imaging. The laser intensity and laser scanning were kept to a minimum to reduce phototoxic effects. Simultaneous, bright-field images were captured with a transmitted light detector. Kalman filtering (n = 1) was used to improve the signal-to-noise ratio of individual images. Images were captured with a laser scan speed of 50 lines/s at a resolution of 1,024 by 1,024 pixels. Confocal images were captured with Lasersharp 2000 software (version 5.1; Bio-Rad Microscience) and were further processed using Image J 1.34S software (W. Rasband, National Institutes of Health, Rockville, MD).

Two-photon Microscopy—All further imaging experiments were performed using a Zeiss LSM 510 Axiovert confocal laser scanning microscope, equipped with a pulsed excitation source (MIRA 900 Ti:Sapphire femtosecond pulsed laser, with a coupled VERDI 10-watt pump laser (Coherent, Ely, UK). The laser was tuned to provide a two-photon excitation (TPE) wavelength of 800 nm, which efficiently excited mCerulean, without any detectable excitation or emission from Venus in the absence of FRET from a donor. TPE data acquisition was performed using 1024 × 1024-pixel image sizes, with 4× frame averaging, using a Zeiss Plan NeoFLUAR ×63 (1.4 NA) oil immersion objective or a Zeiss C-Apochromat ×63 (1.2 NA) water immersion objective. Band pass and long pass emission filters were used, as detailed under “Results”, in conjunction with a Schott (New York, NY) BG39 IR filter to attenuate the TPE light.

Image data acquired at Nyquist sampling rates were deconvoluted using Huygens Pro software (Scientific Volume Imaging, Hilversum, The Netherlands), and the resulting three-dimensional models were analyzed using Imaris software (Bitplane AG, Zürich) on dual Opteron workstations. Co-localization was quantified as described previously by extracting all voxels in three dimensions containing both mCerulean and Venus data above background and reconstructing them into a new, co-localization channel. Co-localization maps were generated using the Manders approach, with automatic thresholding performed according to the method of Costes. Cell peripheries were determined using transmitted light imaging combined with confocal laser scanning microscopy data (33).

TCSPC-FLIM—A number of different approaches to analyzing FRET have been described, using fluorescence intensities in image data and requiring arithmetical corrections to subtract background and to remove undesired spectral bleed-through between the donor and the acceptor. We found that intensity based approaches were not sufficiently sensitive to quantify FRET efficiency between the two fluorescent proteins with useful spatial resolution; also, the limited dynamic range reported an all-or-nothing signal. Thus, to further quantify FRET between labeled SNCI and the two SSO proteins, we employed multidimensional time-correlated single photon counting (TCSPC)-FLIM, essentially as described previously (33, 34).

TCSPC measurements were made under 820 nm TPE, using a non-descanned detector (Hamamatsu R3809U-50; Hamamatsu Photonics UK Ltd., Herts, UK) multichannel plate-photomultiplier tube (MCP-PMT) or a fast photomultiplier tube (H7422; Hamamatsu Photonics) coupled directly to the rear port of the Axiovert microscope and protected from room light and other sources of overload using a Uniblitz shutter (Rochester, NY). Dark count rates were 10²–10³ photons/s. The MCP-PMT was operated at 3 kV, and signal pulses were preamplified using a Becker & Hickl HFAC-26 26 dB, 1.6-GHz preamplifier. TCSPC recording used the “reversed start stop” approach, with accurate laser synchronization using a Becker & Hickl SPC-830 card together with a PHD-400 reference photodiode, routinely at 79.4 MHz. Band pass and long pass filters were used to enable spectral separation of donor and acceptor FRET- and sensitized-emissions. 3–6-mm Schott BG39 filters were positioned directly in front of the MCP-PMT. TCSPC recordings were acquired routinely for between 5 and 25 s, and mean photon counts were between 10⁶ and 10⁶ cps. Images were recorded 256 × 256 pixels from a 1024 × 1024-image scan with 256 time bins over a 12-ns period. FLIM data were acquired for between 5 and 60 s for a 1024 × 1024-image frame.

FLIM Data Analysis and FRET Calculations—Off-line FLIM data analysis used pixel-based fitting software (SPCImage, Becker & Hickl). The fluorescence was assumed to follow a
**RESULTS**

**Cloning and Sequence Analysis of the *T. reesei* SNAREs—**

Complementation of a *S. cerevisiae* SSOL disruptant strain that had a temperature-sensitive ssol2 mutation was used in attempts to isolate *T. reesei* genes corresponding to the *S. cerevisiae* t-SNARE genes SSOL and SSOL2. This strain was transformed with a *T. reesei* cDNA expression library, and several clones were obtained that could support growth of the strain at a restrictive temperature of 31 °C. Some of these transformants were able to grow at temperatures up to 37 °C. The cDNA library plasmids were isolated from these transformants and sequenced. Two individual clones carried a cDNA that was similar at the amino acid level to the *S. cerevisiae* v-SNAREs, Snc1p and Snc2p, which are involved in exocytosis. No t-SNARE genes encoding Sso proteins were found in this screen. The isolated gene was named snc1, and the corresponding protein was named SNC1 (GenBank™ accession number AY766705).

The *T. reesei* snc1 gene encodes a protein of 111 amino acids that is 52 and 57% identical to *S. cerevisiae* Snc1 and Snc2 proteins, respectively (see Fig. 1A for cladogram).

As the isolation of a *T. reesei* gene encoding t-SNARE protein was not successful using the yeast complementation approach and the *T. reesei* genome sequence had recently become available, a BLAST search was performed against the *T. reesei* genome with the sequences from the *Neurospora crassa* nsyn1 and nsyn2 (17). Homologues for both genes were identified, and a PCR-based approach was used to amplify the coding sequences of the genes from a *T. reesei* cDNA library. cDNAs were sequenced and named sso1 and sso2 (GenBank accession numbers EF121546 and EF583658). The *T. reesei* sso1 cDNA encodes a protein of 335 amino acids, and sso2 encodes a protein of 385 amino acids. The *T. reesei* SSOI protein is 40% identical to the *N. crassa* NSYN2 protein, and the SSOII protein is 42% identical to the *N. crassa* NSYN1 protein (see Fig. 1A for cladogram).

There are some features that are common to all SNARE proteins. Sequence analysis using the SOSUI system (43) revealed that all three cloned *T. reesei* SNAREs have one putative C-terminal transmembrane helix: in SNC1, comprising amino acids 88–108; in SSOL, comprising amino acids 309–330; and in SSOII, comprising amino acids 310–330. Alignment of *T. reesei* SNC1 with *S. cerevisiae* Ncs and *N. crassa* ORF NCU005662 showed that the most conserved region of these proteins also carries the synaptobrevin signature sequence (amino acids 19–75 in *T. reesei* SNC1). The t-SNARE proteins have less identity at the primary sequence level, with the highest conservation found within the predicted SNARE motifs. There is a t-SNARE coiled-coil homology domain profile at position 234–296 in the *T. reesei* SSOL protein and at 233–295 in the SSOII protein. Furthermore, there is a syntaxin signature at 75–170 of the SSOL and at 74–176 of the SSOII protein.

A three-dimensional model of the SNC1 protein was created using synaptobrevin 2 (PDB ID 1sfca) from rat (44) as the template and the models of the SSO proteins using the cytoplasmic domain of yeast Sox1 protein (PDB ID 1fioA) (45) as the template. The synaptobrevin II fragment from which the x-ray structure has been resolved comprises residues 1–96 (44), and
SNARE Interactions in Filamentous Fungi

The ability of the T. reesei SNCI to suppress the reduced ability of the Sncp-depleted S. cerevisiae strain to secrete invertase was also tested. Invertase activity at 24 °C was slightly higher in the Sncp-depleted strain that had been transformed with the T. reesei snc1 gene than in the same strain transformed with the expression vector alone or in the wild type strain (Fig. 2B). In cells grown at the restrictive temperature (30 °C), the invertase activities of both the Snc1/2p-depleted strain transformed with T. reesei snc1 and the wild type strain were higher than in cells kept at 24 °C. The invertase activity in the Sncp-depleted strain with the expression vector alone was lower than in cells grown at 24 °C. These results show that the T. reesei SNCI is able to complement both the growth defect and the defect in the invertase secretion of the Snc1/2p-depleted strain (Fig. 2B) and that the cloned T. reesei snc1 encodes a functional equivalent of the S. cerevisiae Sncp proteins.

The T. reesei snc1 gene was originally cloned as a suppressor of the S. cerevisiae SSO2 temperature-sensitive mutant in an SSOI deletion background. The plasmid that was obtained in the screening was reintroduced into the temperature-sensitive strain, and the suppression was tested at the restrictive temperatures. The suppression was also studied in a strain where both SSO genes had been deleted from the genome, and SSOI was under the control of the inducible GAL10 promoter in a plasmid. No suppression of the S. cerevisiae Sso protein depletion was observed with the T. reesei snc1 gene in either strain (data not shown), although the T. reesei SNCI was able to increase the temperature tolerance of the depletion strain used in the original screening from 31 to 33 °C, unlike any other protein in the screen.

The ability of the T. reesei SSOI proteins to complement the S. cerevisiae Sso protein deficiency was tested in the same strains as above. Both T. reesei SSOI proteins were unable to complement the growth defect, although the SSOI when
SNARE Interactions in Filamentous Fungi

A

SCGal-TRP-LEU

YPD

B

activity/mg cell dry weight (AU)

24°C  30°C

C

expression of the gene encoding the native SNARE protein. We found only moderate overexpression of the fusion constructs at mRNA level, suggesting that overexpression artifacts were unlikely to be a problem. In the Venus-SNCI transformants, the level of snc1 mRNA was on average 3.5-fold higher, and in the mCer-SSO transformants, the sso1 mRNA was on average 5.8-fold higher and the sso2 was on average 2-fold higher than in the parental strain.

SNARE Cluster Regulation—mCerulean-SSOI was shown to be restricted to puncta distributed over the cell plasma membrane of both apical and subapical compartments of old, non-growing hyphae in subperipheral regions of the colony (Figs. 3B and 4A). This is analogous to mammalian t-SNAREs, which form cholesterol-dependent clusters on neuroendocrine cell plasma membranes (46). The clusters observed here were ~250 nm in diameter (see below), smaller than the 400–700-nm SNARE clusters seen in mammalian cells (4, 47).

To determine whether the SSOI SNARE clusters were regulated by sterol concentration in a way similar to mammalian

4 R. R. Duncan, unpublished data.

41

FIGURE 2. Complementation of the S. cerevisiae Snc1p and Snc2p depletion by T. reesei SNCI. T. reesei snc1 cDNA was expressed in the JG8 T15:85 strain from a single copy vector. A, the strain transformed with the T. reesei snc1 construct (pMV20, lower row for each medium) and the strain transformed with the expression vector alone (pKKI, upper row for each medium) were grown at 30 °C on SC (-Trp-Leu) plates with 2% galactose (upper) and on YP with 2% glucose (YPD) (lower). B, the secreted invertase activity from a strain transformed with the T. reesei snc1 (pMV20, black), a strain transformed with the expression vector alone (pKKI, white), and a wild type strain (NY15, gray) at the permissive temperature (24 °C) and at the restrictive temperature (30 °C). The strain with the pMV20 construct secreted as much invertase as the wild type strain, whereas the strain transformed with the expression vector alone secreted significantly less invertase (** Mann-Whitney rank sum test, p < 0.02; mean ± S.E., n = 3). AU, arbitrary units. C, expression of the mCer-SSOI fusion protein in the Sso depletion strain.

expressed as a fusion with the cyan fluorescent protein, mCerulean in the same S. cerevisiae strain, showed a very clear plasma membrane localization (Fig. 2C), demonstrating efficient targeting to the cell surface as expected for a cell surface t-SNARE.

Expression of the SNARE Fusion Proteins in T. reesei—Each pMV63, pMV67, pMV77, pMV63/67, and pMV63/77 transformant containing a SNARE fusion protein also contained a copy
syntaxins, we treated the cells with a sublethal dose of itraconazole, an inhibitor of ergosterol synthesis (48). An itraconazole dose-response experiment was performed to determine the concentration of itraconazole that affected protein secretion but was sublethal. 50 μM itraconazole decreased the amount of active secreted endoglucanase to approximately half that from control samples without killing the hyphae. Growth was retarded in all the treated samples, and 100 μM was lethal (data not shown). Treatment of fungal cultures with 50 μM itraconazole for 16 h before imaging, to deplete ergosterol, had no affect upon SNARE clustering (Fig. 4, A and B). Prior to treatment, the mCerulean-SSO membrane clusters were measured to be 256 ± 8 nm (full width at half-maximum, FWHM, >100 clusters from four experiments), whereas after treatment, the cluster size remained the same, at 264 ± 12 nm (FWHM, 50 clusters from three experiments). These experiments were similar in design to those in mammalian systems using methyl-β-cyclodextrin, which is commonly used to deplete surface cholesterol and has been shown to inhibit mammalian exocytosis (49).

**FLIM/FRET Analyses**—Co-localization data are limited by the optical resolution of the imaging system; in our system, this is ~200 nm, which is insufficient to conclude whether two co-localized proteins interact. Quantifying the interaction between SSO proteins and the SNAREs, however, may help in the understanding of the functional significance of the proteins and define sites of ternary SNARE complex formation; no direct interaction occurs between a v-SNARE and a cognate syntaxin t-SNARE in the absence of a preassembled t-SNARE binary complex (4). To address this, we employed TCSPC-FLIM, using the same fluorescent strains as before. This approach has the advantage of very high statistical accuracy combined with low phototoxicity as low level two-photon excitation is used. FLIM can be used to report quenching of a donor fluorescence lifetime in a FRET system, thus revealing regions within a cell where two proteins interact (34, 50).

FLIM data were initially acquired from hyphae expressing mCerulean-SSO alone. These data fitted well to a monoexponential fluorescence decay function (Fig. 5, A–C), as reported previously for mCerulean (27). When such lifetime data were calculated for every pixel in an image, a “FLIM map” was generated, showing the fluorescence lifetime throughout an image. All data points from each FLIM map were plotted as a frequency distribution histogram, revealing the fluorescence lifetime of mCerulean-SSO to be uniform throughout an entire fungal hypha, with a mean fluorescence lifetime of 2.28 ± 0.04 ns (Fig. 5D; n = 5). However, in the presence of Venus-SNCI, donor fluorescence decay data could no longer be fitted to a monoexponential decay and instead fitted well to a biexponential model (Fig. 5, A and C). FLIM data acquired from a strain co-expressing both mCerulean-SSO and Venus-SNCI were treated in the same way. In this case, two fluorescence lifetimes were resolved within each pixel of the image, one identical to the non-FRET donor lifetime measured above. The additional fluorescence lifetime resolved arose from the proportion of donor fluorophore participating in energy transfer to an adjacent acceptor within each pixel of the digitized image. These data are presented in Fig. 5E as a weighted mean donor fluorescence lifetime composed of both the non-FRET and the FRET components. We were also able to resolve both the non-FRET and the FRET donor fluorescence lifetime contained within each pixel of the image (Fig. 5F). In Fig. 5F, the lifetime distributions represent the proportion of the FRET and non-FRET components of the donor, and the amplitude of each distribution represents...
the relative amount of each component. These parameters describe the fraction of bound SSOI (in complex with SNCI) versus unbound SSOI. Fluorescence lifetime variations throughout the hyphae result from relative differences in the amount of interacting SNAREs at different sites (i.e., variation within each pixel), not from differences in each lifetime (which in turn would suggest differences in complex conformation). These data revealed that SSOI and SNCI can form SNARE complexes on the plasma membrane of old, non-growing hyphae in both apical (data not shown) and subapical compartments (Fig. 5, E and F).

Spatially Regulated SNARE Complex Formation—To further understand how SNARE complex formation is regulated spatially in fungal cells, we performed similar experiments using SNCI and SSOII. We hypothesized that exocytosis at the hyphal tip may be mediated by a different SNARE complex when compared with that in subapical regions. Such a difference may represent important functional contrasts on the plasma membrane at different cellular sites.

The mCerulean-SSOII fusion protein was observed in growing apical hyphal compartments at the colony margin (Fig. 6B). Co-localization analysis confirmed that SNCI and SSOII co-localize in clusters at the hyphal tip in apical colony regions. FLIM/FRET analysis of the same protein pair confirmed that SSOII and SNCI form SNARE complexes in these regions (Fig. 6, A and B), in contrast to SSOI and SNCI (Figs. 3 and 5).

DISCUSSION

In this report, we have described three T. reesei SNARE genes, snc1, sso1, and sso2, that encode a v-SNARE and two t-SNARE proteins, respectively. Only a few SNARE-encoding genes from filamentous fungi have been cloned: yup1 that encodes a putative t-SNARE protein involved in endocytosis from Ustilago maydis (51), two plasma membrane syntaxin-like SNAREs, nsn1 and nsn2, from N. crassa (17), and a vacuolar syntaxin Aovam3 from Aspergillus oryzae (52). Nevertheless, a total of 18 different SNARE helices have been identified in genomes of Schizosaccharomyces pombe, Candida albicans, N. crassa, Aspergillus fumigatus, and Phanerochaete chrysoporum with 30–70% identities to S. cerevisiae SNARE domains (53). Thus, there seems to be conservation in the SNARE domains in different fungi, and therefore, it is likely that the SNARE assembly involves similar structure formation in different fungal species.

The T. reesei snc1 gene was cloned using a complementation screen of a S. cerevisiae SSOI deletant strain that carried an sso2 temperature-sensitive mutation. Despite several attempts, the suppression could not be repeated in this strain or in Sso1p-Sso2p deletion strains (data not shown). This may indicate that the T. reesei SNCI protein is able to form inefficient, transient SNARE complexes with the S. cerevisiae Sso and Sec9 proteins resulting in transient suppression of the Sso2 temperature-sensitive mutant. Complementation of the S. cerevisiae Snc1-Snc2 protein depletion verified that the T. reesei SNCI protein is a functional homologue of the Snc proteins. Although members of the synaptobrevin/VAMP family of proteins are structurally and functionally well conserved (54), the mammalian VAMP1 and VAMP2 proteins cannot complement the S. cerevisiae Snc1-Snc2 deletion (55). These mammalian proteins have much lower sequence conservation with S. cerevisiae Snc1p and Snc2p than T. reesei SNCI.

The T. reesei sso1 and sso2 genes were identified from the genome based on sequence similarity to a syntaxin homologue of N. crassa, nsn2. The phylogenetic similarity of the filamentous fungal proteins to S. cerevisiae Sso proteins is distant, so it was not surprising that neither the T. reesei nor the N. crassa proteins (17) are able to complement the Sso protein depletion in S. cerevisiae. T. reesei SSOI has distinct plasma membrane localization with no concentration gradient, as shown previously for the endogenous S. cerevisiae Sso proteins (8) when expressed in yeast, making it most likely that it is a cell surface t-SNARE protein. The fact that the T. reesei SNCI protein can complement yeast deletion mutants, but that SSOI and SSOII cannot, is an interesting observation; the reason for this is unclear.

Previous studies have shown that mammalian exocytotic t-SNARE clusters on the cell surface define the docking and fusion sites for dense-cored granules (56, 57). In mammalian cells, the t-SNAREs are localized in lipid rafts that are enriched in sphingolipids and cholesterol. Furthermore, it has been suggested that lipid rafts regulate SNARE function and exocytosis (58). In yeast, it has been shown that lipid rafts are involved in the delivery of proteins to the plasma membrane (59). As indicated above, SSOI is concentrated in clusters on the cell surface of T. reesei with similar characteristics to mammalian SNARE clusters. Fungal cells do not contain cholesterol; their functional sterol is ergosterol (60). In contrast to previous work in mammalian systems, where the t-SNARE clusters are sterol-dependent (47), our results show no differences in the SNARE clustering in samples treated with itraconazole to deplete ergo-
**SNARE Interactions in Filamentous Fungi**

Time-correlated single photon counting TCSPC-FLIM reveals SSOI-SNCI protein-protein interactions in living hyphae. TCSPC-FLIM was applied to living hyphae and revealed a significant decrease in the donor (mCerulean-SSOI) fluorescence lifetime only in the presence of co-localized acceptor molecules (Venus-SNCI). A representative single-pixel fluorescence transient decays recorded from mCerulean-SSOI expressing hyphae. The non-FRET data (dark circles) were recorded from a sample not expressing the acceptor and had a time constant of decay of 2288 ± 128 ps (mean ± S.D., n = 15 separate recordings from different cells). The time constant was significantly reduced in the presence of acceptor (open circles) to 1200 ± 98 ps. The non-FRET data fit well to a single-exponential decay function (B, upper). Reduced chi-squared residual analysis revealed no advantage in introducing a second exponential into the fitting algorithm (B, lower). TCSPC decay data from the same donor in the presence of Venus-SNCI acceptor no longer fit satisfactorily to a monoexponential decay function, indicating the appearance of a second lifetime in pixels containing co-localized donor and acceptor (C, lower). These exponential fitting calculations were performed for every pixel in a 1024 × 1024 image and resulted in FLIM maps. D, left, shows an intensity image from a representative field of hyphae expressing mCerulean-SSOI. The FLIM map (D, middle) shows a uniform, single exponential and a fluorescence lifetime of 2288 ps in each pixel. In this image, false color represents the fluorescence lifetime (from 0 to 2500 ps blue) in a continuous color gradient. Brightness in this image represents intensity. All the FLIM maps presented have an identical color scale for clarity. These representative data are plotted in a frequency distribution histogram in D (right). When hyphae co-expressing mCerulean-SSOI (donor) and Venus-SNCI (acceptor) were examined in a similar way, high levels of co-localization were observed with a similar distribution as before (E, left). The donor lifetime, however, was significantly reduced from 2288 ± 128 ps (n = 15) to 1200 ± 98 ps (weighted mean of two lifetimes, n = eight separate experiments; E, middle). These weighted mean data are plotted in E (right), and appear as a broad distribution, indicating the presence of more than one component. Both fluorescence lifetimes from each pixel were analyzed separately, revealing the long component to be identical to the donor-alone fluorescence lifetime previously observed (~2288 ps). A second, quenched donor lifetime was present in every pixel containing co-localized Cerulean-SSOI and Venus-SNCI, representing the component of donor molecules participating in energy transfer and thus interacting with Venus-SNCI. These data are presented as a FRET-FLIM map as before in panel F. Data from the separately resolved FRET and non-FRET components are plotted as a frequency distribution histogram in F (right). These data are not normalized, and the relative amplitude of each component represents the relative proportion of bound versus unbound donor molecules in the entire image. Scale bar: 10 μm.

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On the other hand, the localization of the *T. reesei* SSOII protein only to the plasma membrane of older, non-growing hyphae indicates that there is spatial regulation of SNARE complex formation between functionally distinct SNAREs at different sites on the cell surface membrane. Also, it indicates that there is more than one pathway for exocytosis in filamentous fungi, employing different surface SNARE proteins at distinct sites in the fungal colony. The lack of SNARE complex formation between SSOI and SNCI in apical compartments of growing hyphae suggests that SSOI is not involved in constitutive exocytosis at the hyphal tip. Further support for this hypothesis comes from a study done in another filamentous fungus, *A. niger*, in which disruption of srgA was not lethal (19). This protein is an ortholog of *S. cerevisiae* Sec4 protein that is a secretory vesicle-associated Rab GTPase and essential for exocytosis and cell viability. Based on the data, the existence of two separate secretory pathways, constitutive and induced, in *A. niger* was suggested (19).
In hyphae from older, subperipheral regions of the colony, the plasma membrane co-localization of the v- and t-SNARE proteins was very consistent. The differences in the localization of the SNARE complexes between SNCI and SSOSI or SSOII was unexpected as it is generally believed that the secretion in fungal hyphae takes place primarily from growing hyphal tips (11, 12, 62). However, there is indirect evidence that some proteins may be secreted from older hyphal regions (11, 13, 63, 64). In addition, it has been suggested that there are at least three separate secretory vesicle populations in yeast cells (37, 65, 66). However, all these vesicle populations are directed to the growing bud.

Our findings demonstrate surprising differences in SNARE complex localization, targeting, and regulation between fungal and mammalian cells and even between filamentous fungi and budding yeast. We support the hypothesis that there may be more than one pathway for exocytosis in filamentous fungi, employing different surface SNARE proteins at spatially distinct sites both in the plasma membrane and in the fungal colony.

Acknowledgments—Riitta Nurmi is acknowledged for excellent technical assistance. We thank Dr. Sirkka Keränen, Dr. Hans Ronne, Dr. Jeffrey Gerst, and Dr. Peter Novick for providing S. cerevisiae strains and Dr. Natalie Curach for the plasmid pContV. Thanks to Dr. Colin Rickman for assistance with SwissProt analyses and Dr. Marilyn Wiebe for critical reading of the manuscript.

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