Formation and stability of eisosomes in the filamentous fungus Ashbya gossypii

Shanon Seger, Ricarda Rischatsch and Peter Philippsen*

Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstraße 50-70, CH 4056 Basel, Switzerland

*Author for correspondence (peter.philippsen@unibas.ch)

Summary
One hallmark of the rapid expansion of the polar surface of fungal hyphae is the spatial separation of regions of exocytosis and endocytosis at hyphal tips, as recently shown for Ashbya gossypii and Aspergillus nidulans. To determine where cortex-associated eisosomes form with respect to these two regions, we monitored fluorescently marked eisosomes in A. gossypii. Each minute, 1.6±0.5 eisosomes form within the first 30 μm of each hypha and are exclusively subapical of the endocytosis region. This spatial separation of the processes of eisosome formation and endocytosis, and the much lower frequency of eisosome formation compared with that of endocytic vesicle production do not support a recently proposed role for eisosomes in endocytosis. Levels of mRNA encoding eisosome components are tenfold higher in spores than in hyphae, explaining the observed higher eisosome density at the cortex of germ bubbles.

Introduction
During cell surface expansion, the newly added plasma membrane undergoes several modifications, including formation of subcompartments. The Saccharomyces cerevisiae plasma membrane compartment of Can1, called the MCC, and its associated cytosolic macrocomplexes, called eisosomes, have been extensively studied during recent years (Grossmann et al., 2008; Luo et al., 2008; Malinska et al., 2004; Moreira et al., 2009; Stradalova et al., 2009; Walther et al., 2007; Walther et al., 2006). MCCs are assemblies comprising a subgroup of proton symporters, such as Can1 (Malinska et al., 2003), and the stabilizing membrane proteins Sur7 and Nce102 (Frohlich et al., 2009; Stradalova et al., 2009; Young et al., 2002). Eisosomes consist of non-membrane proteins, with Pil1 and Lsp1 as main components (Walther et al., 2006; Zhang et al., 2004).

In S. cerevisiae, eisosomes form in a wave in growing buds, starting at the bud neck; this apparently cell-cycle-dependent eisosome biogenesis is controlled at the level of transcription (Moreira et al., 2009). Deletions of several genes encoding eisosome components affect the biogenesis or stability of eisosomes, but not cell surface growth (Walther et al., 2006). These authors also suggested that eisosomes might mark sites of endocytosis.

In a recent study about Aspergillus nidulans eisosomes, PilA and PilB, homologues of Pil1 and Lsp1, and SurG, one of several Sur7-like proteins, were investigated (Vangelatos et al., 2010). Interestingly, they colocalized as patches in the periphery of conidiospores, with SurG additionally found at perinuclear locations. However, they had different destinations in hyphae. Only PilA remained at cortical patches in young and older hyphae. Deletions of the genes did not alter polar growth.

We started to investigate eisosomes in Ashbya gossypii for several reasons. The genome of this fungus is highly syntenic with the S. cerevisiae genome and carries homologues of most S. cerevisiae genes implicated in surface growth (Dietrich et al., 2004). Surface expansion of the hyphae is strictly polar and up to 40 times faster than in S. cerevisiae and, in contrast to S. cerevisiae, the zones of exocytosis and endocytosis are spatially separated in the tips of hyphae, as in A. nidulans (Araujo-Bazan et al., 2008; Knechtle et al., 2003; Kohli et al., 2008; Taheri-Talesh et al., 2008). Using the A. gossypii system, we tried to answer several questions that cannot be addressed in S. cerevisiae. Is a higher surface expansion rate coupled with increased eisosome biogenesis? Do eisosomes form within the zone of endocytosis or subapically thereof? Are eisosomes or specific eisosome components important to achieve efficient polar growth? And, finally, which components support the stability of eisosomes in hyphae?

Results and Discussion
A. gossypii has a reduced number of eisosome-associated components
Surprisingly, the A. gossypii genome lacks homologues of several S. cerevisiae genes reported to encode eisosome-associated components (Table 1). Homologues of the main components, Pil1 and Lsp1, are conserved, but genes encoding other potential eisosome components were apparently lost. Several functionally partially redundant components, such as Slm1 and Slm2 (Fadri et al., 2005), Pkh1 and Pkh2 (Casamayor et al., 1999; Luo et al., 2008; Walther et al., 2007), and Nce102 and Fhn1 (Loibl et al., 2005), Pkh1 and Pkh2 (Casamayor et al., 1999; Luo et al., 2008; Walther et al., 2007), and Nce102 and Fhn1 (Loibl et al., 2005), are encoded by only one gene in A. gossypii. Likewise only a single syntenic gene is present in the A. gossypii genome for the
reduced or restricted to a specific region in fast-growing hyphae. A peculiar pattern of transcription indicates that eisosomes might not be coupled to surface growth rates. A higher density of eisosomes is about 20 times faster than the average surface growth rate of yeast buds. Hyphae expressing a C-terminal (Ag) Pil1–Venus fusion showed a punctate pattern of eisosomes along the hypal cortex, starting in the tip region directly subapical of the ring of actin patches (Fig. 1A). As expected, a very similar pattern was observed with a C-terminal AgLsp1–mCherry fusion, whereas coexpression with AgPil1–Venus caused, for unknown reasons, formation of aberrant Lsp1–mCherry aggregates (supplementary material Fig. S1). We tested the apparent spatial separation of endocytosis sites, represented by the ring of actin patches, and eisosomes in hyphae lacking the endocytosis component AgAbp1. The formation of endocytic actin patches moved over 10 μm against the longitudinal axis of hyphae (Fig. 1B). Interestingly, over periods of several hours, the density of eisosomes at different stages of A. gossypii development (Fig. 1B). These differences in eisosome densities at different stages of A. gossypii development also indicate that eisosome formation is not coupled to surface growth rates. A higher density of eisosomes in conidia compared with in hyphae was also reported for A. nidulans (Vangelatos et al., 2010).

Localization and density of eisosomes in hyphae and germlings

We observed formation of eisosomes in hyphae growing with surface expansion rates of between 15 and 25 μm/minutes, which is about 20 times faster than the average surface growth rate of yeast buds. Hyphae expressing a C-terminal A. gossypii (Ag) Pil1–Venus fusion showed a punctate pattern of eisosomes along the hypal cortex, starting in the tip region directly subapical of the ring of actin patches (Fig. 1A). As expected, a very similar pattern was observed with a C-terminal AgLsp1–mCherry fusion, whereas coexpression with AgPil1–Venus caused, for unknown reasons, formation of aberrant Lsp1–mCherry aggregates (supplementary material Fig. S1). We tested the apparent spatial separation of endocytosis sites, represented by the ring of actin patches, and eisosomes in hyphae lacking the endocytosis component AgAbp1. The formation of endocytic actin patches moved over 10 μm further away from the tip, but the eisosomes did not move, indicating that endocytosis and eisosome biogenesis are not linked (Fig. 1A). A similar conclusion was drawn from FM4–64 uptake experiments with eisosome mutants of A. nidulans (Vangelatos et al., 2010).

The area of fluorescence of AgPil1–Venus foci is 0.3±0.12 μm² (n=100), similar to the reported size of yeast eisosomes (Walther et al., 2006). We also determined the surface density of eisosomes in hyphae and measured 0.16±0.02 AgPil1 foci/μm² (n=39 hyphae) and 0.17±0.03 AgLsp1 foci/μm² (n=32 hyphae), which is only half the density of 0.33±0.06 eisosomes/μm² reported for S. cerevisiae (Vangelatos et al., 2009). Because the expression of AgPIL1 and other eisosome-associated genes is higher at the onset of germination than in hyphae, we also determined the density of eisosomes at the cortex of isotropically growing germ bubbles and found 0.42±0.06 eisosomes/μm², which is higher than in S. cerevisiae cells (Fig. 1B). These differences in eisosome densities at different stages of A. gossypii development also indicate that eisosome formation is not coupled to surface growth rates. A higher density of eisosomes in conidia compared with in hyphae was also reported for A. nidulans (Vangelatos et al., 2010).

Kinetics of eisosome formation in fast-growing hyphae

We first plotted the number of AgPil1–Venus foci seen in 5-μm-long surface segments against the longitudinal axis of hyphae (Fig. 1C). The number increased from a few eisosomes between 5 and 10 μm to a constant number at and beyond 30 μm. Each minute, 1.6±0.5 eisosomes form within the apical 30 μm, as determined from movies recording Z-stacks of seven hyphae (data not shown). Eisosomes once formed remain stably associated with the cortex, in a similar manner to eisosomes in S. cerevisiae (Walther et al., 2006). As shown in Fig. 1D, weakly fluorescent foci in the region close to the tip (arrow) increase in fluorescence during hyphal growth. Strongly fluorescent foci further away from the tip (arrow heads) do not change fluorescence intensity and are immobile. Fluorescence recovery after photobleaching (FRAP) experiments confirmed that mature eisosomes do not exchange subunits with presumptive cytoplasmic pools during two-minute intervals (Fig. 1E). Interestingly, over periods of several hours, the density of eisosomes in older hyphal segments slightly increases, whereas the average size of eisosomes decreases, indicating that eisosomes probably slowly reassemble (supplementary material Fig. S2).

Do eisosome components support polar growth?

Eisosome biogenesis in S. cerevisiae (Sc) occurs in the growing bud, not in the mother cell, and is controlled by ScPil1 in a cell-cycle-dependent manner (Moreira et al., 2009). In the absence of ScPil1, eisosome biogenesis is disrupted and the remaining

Table 1. Eisosome-associated genes in A. gossypii

<table>
<thead>
<tr>
<th>S. cerevisiae gene</th>
<th>A. gossypii gene</th>
<th>Protein sizes in amino acids</th>
<th>Protein sizes in amino acids</th>
<th>Percentage identity between homologues (%)</th>
<th>Expression levels during germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScPIL1</td>
<td>AgPIL1</td>
<td>339</td>
<td>303</td>
<td>77</td>
<td>0 h: 12900 5 h: 4050 9 h: 1330</td>
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<tr>
<td>ScLSP1</td>
<td>AgLSP1</td>
<td>341</td>
<td>306</td>
<td>76</td>
<td>0 h: 3400 5 h: 1050 9 h: 340</td>
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<tr>
<td>ScSLM1 / ScSLM2</td>
<td>AgSLM1 / 2</td>
<td>686 / 656</td>
<td>736</td>
<td>45 / 13</td>
<td>0 h: 600 5 h: 330 9 h: 260</td>
</tr>
<tr>
<td>ScPKH1 / ScPKH2</td>
<td>AgPKH1 / 2</td>
<td>766 / 1081</td>
<td>1033</td>
<td>42 / 22</td>
<td>0 h: 120 5 h: 180 9 h: 180</td>
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<tr>
<td>YGR130c</td>
<td>AFRS310c</td>
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<td>623</td>
<td>21</td>
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<tr>
<td>YMR031c / YKL050c</td>
<td>ABR081c</td>
<td>843 / 922</td>
<td>842</td>
<td>26 / 23</td>
<td>0 h: 4250 5 h: 1020 9 h: 170</td>
</tr>
<tr>
<td>YMR086c / YKL105c</td>
<td>ABL037c</td>
<td>960 / 1132</td>
<td>657</td>
<td>24 / 16</td>
<td>0 h: 830 5 h: 470 9 h: 375</td>
</tr>
<tr>
<td>ScYCP4</td>
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<td>247</td>
<td>–</td>
<td>–</td>
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<tr>
<td>ScPST2</td>
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<td>–</td>
<td>–</td>
<td>0 h: – 5 h: – 9 h: –</td>
</tr>
<tr>
<td>ScRFS1</td>
<td>Lost</td>
<td>210</td>
<td>–</td>
<td>–</td>
<td>0 h: – 5 h: – 9 h: –</td>
</tr>
<tr>
<td>ScNCE102 / ScFHN1</td>
<td>Lost</td>
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<td>169</td>
<td>48 / 47</td>
<td>0 h: 13500 5 h: 6100 9 h: 1420</td>
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<tr>
<td>ScSUR7</td>
<td>AgSUR7</td>
<td>302</td>
<td>299</td>
<td>36</td>
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<tr>
<td>ScPUN1</td>
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<td>–</td>
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</tr>
<tr>
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<td>309 / 301</td>
<td>429</td>
<td>33 / 30</td>
<td>0 h: 4700 5 h: 1640 9 h: 260</td>
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</table>
Fig. 1. Formation of stable eisosomes subapically of the endocytic ring. (A) DIC and fluorescence microscopy of AgPil1–Venus and AgPil1–Venus abp1Δ strains stained with rhodamine–phalloidin; AgPil1 foci are coloured green and actin patches red. Venus and rhodamine–phalloidin images are maximum projections of 12 Z planes separated by 0.5 μm each. Bended lines mark the tip front, brackets the endocytic ring and arrowheads the first eisosomes appearing close to the growing tip. (B) Single focal planes of germ bubbles of the AgPIL1–Venus strain stained with rhodamine–phalloidin 5–6 hours after incubation of spores at 30°C. Eososome foci are coloured green and actin patches red. (C) Average number of AgPil1–Venus foci in 5-μm-long segments of ten hyphae starting from the tip. Data were collected from top view images. (D) Formation of AgPil1–Venus foci in growing hyphae. Three selected frames of a movie are shown focusing on the hyphal surface. Arrows mark one eisosome, which gradually increases its fluorescence. Arrowheads point to the stable distribution of eisosome foci. Numbers represent minutes. (E) FRAP analysis of AgPil1–Venus-labelled eisosomes. Images were acquired every 12 seconds. In the four representative images, the red box is the bleached area with three eisosomes, the fluorescence of which was monitored in the graph (lines 1, 2 and 3). The fluorescence of the non-bleached eisosome in the blue box was monitored as control (blue line in the graph). During the bleaching, only the red box was exposed and the intensity of the blue box could not be measured. Hyphae expressing a GFP fusion of the polarisome component AgSpa2 were similarly bleached and the fluorescence started recovering after 20 seconds (supplementary material Fig. S3). Scale bars: 5 μm.
eisosome components form aggregates at the cell periphery, but growth is not affected (Grossmann et al., 2008; Walther et al., 2006). Similarly, PilA is not important for hyphal growth in A. nidulans (Vangelatos et al., 2010). In A. gossypii, however, deletion of AgPIL1 dramatically affects polar growth (Fig. 2A). Hyphal tips show abnormal shapes, polar surface expansion is reduced by up to 80%, large AgLsp1–mCherry aggregates form instead of cortex-associated wild-type-like eisosome foci, and actin patches do not accumulate as an endocytic ring in the tip region (Fig. 2). Whether the observed decrease in polar growth efficiency is a direct consequence of the disruption of eisosome biogenesis or is caused by the lack of another AgPil1 function is not known at the moment.

AgLsp1 is highly similar in amino acid sequence to AgPil1, but the lack of AgLsp1 does not lead to observably altered hyphal growth, eisosome distribution or actin patch accumulation in the tip region (Fig. 2). Also, ScLsp1, the homologue in S. cerevisiae, does not play a significant role in growth or eisosome formation (Moreira et al., 2009). The transmembrane protein ScNce102 colocalizes with eisosomes and is important to maintain eisosome density, most likely by controlling the plasma membrane organization through sphingolipid signaling (Frohlich et al., 2009). The S. cerevisiae genome carries a duplicated copy of the ScNCE102 gene, called ScFHN1, which, when overexpressed, can complement the phenotype of a ScNCE102 deletion (Loibl et al., 1994). The proteins encoded by ABL037c, YMR086w and YKL105c revealed four highly conserved domains (I-IV) of 15-44 amino acids (Fig. 3A). We monitored the effect of the complete deletion of ABL037c and deletions of those regions encoding domain IV or domains III plus IV on the formation and/or stability of eisosomes (Fig. 2). Clustal W multiple sequence alignment (Thompson et al., 1994) of the proteins encoded by ABL037c, YMR086w and YKL105c revealed four highly conserved domains (I-IV) of 15-44 amino acids (Fig. 3A). We monitored the effect of the complete deletion of ABL037c and deletions of those regions encoding domain IV or domains III plus IV on the formation and/or stability of eisosomes. In the complete deletion strain, eisosomes form in the tip segments as in the wild type, but then up to 90% of the newly formed eisosomes disassemble concomitant with the formation of small and large aggregates (Fig. 3B,C). This phenotype is also seen when domains III plus IV are deleted, but not with the deletion of domain IV, indicating an essential role for domain III in the stabilizing function. The sequence of this domain does not resemble that of any other functionally characterized domain.

Because the gene ABL037c encodes a protein important for stabilizing eisosomes but not for eisosome biogenesis, we propose the name AgSEG1 for ‘stabilizer of eisosome in Gossypii’. Interestingly, eisosomes in the complete deletion strain are stable in young mycelia with up to four branches. Eisosome instability concomitant with formation of aggregates was only observed in fast-growing hyphae. These Pil1–Venus-marked aggregates are mobile (Fig. 3D). So far, only non-mobile cortex-associated...
eisosome aggregates had been described in different *S. cerevisiae* deletions.

**Materials and Methods**

**Growth conditions and gene targeting**

*A. gossypii* strains were grown and transformed by established methods (Ayad-Durieux et al., 2000; Wendland et al., 2000). For amplification of gene-targeting cassettes, the Expand High Fidelity System (Roche Diagnostics, Mannheim, Germany) was used. Transformants were selected by resistance to G418 or ClonNat (Werner Bioagents, Jena, Germany). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland) and are listed in supplementary material Table S1. Plasmids used as PCR templates are described in supplementary material Table S2. All *A. gossypii* strains were verified and are listed in supplementary material Table S3.

**C-terminal fusions with Venus and mCherry**

Genomic DNA clones carrying the target gene in pRS416 (Dietrich et al., 2004) were co-transformed with the respective PCR cassette into *S. cerevisiae* strain DHD5 (Arvanitidis and Heinisch, 1994). Correctly targeted plasmids were verified by PCR, transformed and amplified in *Escherichia coli*, and used for gene targeting in *A. gossypii* after restriction nuclease digestion, as listed in supplementary material Table S3.
Microscopy

The actin cytoskeleton was stained using rhodamine-phalloidin or Alexa-Fluo-488 (Knechtle et al., 2003). A Zeiss Axiosplan2 microscope was used (Knechtle et al., 2003) equipped with a PolyChrome V monochromator (Till Photonics, Gräfelfing, Germany) as illumination source and a CoolSNAP HQ camera (Photometrics, Tucson, AZ). Mycelium from the border of three-day-old A. gossypii colonies was inoculated on glass slides covered with 700 µL of half-concentrated Ashbya Full Medium (AFM) in 1% agarose. Slides were observed after 2 hours at 30°C, except where otherwise stated. Images were acquired with Meta Morph 6.2.6 software (Molecular Devices, Downingtown, PA). For movies, fluorescence and differential interference contrast (DIC) images were acquired every 5 minutes. For fluorescence images, Z-stacks of up to 12 focal planes, 0.5 µm apart, were processed with the ‘Nearest Neighbour’ 2D deconvolution algorithm and then converted to maximal intensity projection. Overlays of images and measurements were performed using ImageJ 1.44a.

FRAP experiments were performed using an inverted Leica SP5 confocal microscope controlled by the Leica SP5 software. Cells were observed with a 63× glycerol objective. Mycelium was inoculated on a glass slide covered with half-concentrated AFM in 1.7% agarose and sealed with another cover slide on the top. Images were taken every 12 seconds with an average from four lines. Two images were taken pre-bleaching, six images for bleaching with the ‘zoom in’ function and twelve images post-bleaching. Fluorescence intensities of bleached and non-bleached eisosome foci were plotted as a function of time.

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References


