Reassessment of the role of plasma membrane domains in the regulation of vesicular traffic in yeast

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Summary

The Saccharomyces cerevisiae plasma membrane has been proposed to contain two stably distributed domains. One of these domains, known as MCC (membrane compartment of Can1) or eisosomes, consists of furrow-like membrane invaginations and associated proteins. The other domain, called MCP (membrane compartment of Pma1), consists of the rest of the membrane area surrounding the MCC patches. The role of this plasma membrane domain organization in endocytosis is under debate. Here we show by live-cell imaging that vesicular traffic is restricted to the MCP and the distribution of endocytic and exocytic sites within the MCP is independent of the MCC patch positions. Photobleaching experiments indicated that Can1 and Tat2, two MCC-enriched permeases, exchange quickly between the two domains. Total internal reflection fluorescence and epi-fluorescence microscopy showed that the enrichment of Can1 at the MCC persisted after addition of its substrate, whereas the enrichment of Tat2 disappeared within 90 seconds. The rates of stimulated endocytosis of Can1 as well as Tat2 were similar in both wild-type cells and pil1Δ cells, which lack the MCC. Thus, our data suggest that the enrichment of certain plasma membrane proteins in the MCC does not regulate the rate of their endocytosis.

Key words: Endocytosis, Exocytosis, Eisosome, MCC, MCP

Introduction

The yeast plasma membrane contains two membrane domains, which have a stable distribution. One of the domains consists of patches distributed throughout the cell surface and the other is formed by the network-like membrane area surrounding the patches (Malinska et al., 2003; Young et al., 2002). A recent electron microscopy study showed that the patches defining the first domain correspond to furrow-like membrane invaginations which are ~300 nm long, ~150–250 nm deep and ~50 nm wide (Stradalova et al., 2009). This membrane domain has been called eisosomes (Walther et al., 2006) or MCC (membrane compartment of Can1) because the arginine permease Can1 is enriched in the patches (Grossmann et al., 2007). Later reports distinguished the MCC from the underlying cytosolic protein clusters or eisosomes (Fröhlich et al., 2009; Grossmann et al., 2008). For simplicity, we will use here the term MCC to collectively refer to the membrane furrows and all associated transmembrane and cytosolic proteins. In total 22 proteins (9 transmembrane proteins and 13 cytosolic proteins) have been shown to localize to this domain (Deng et al., 2009; Grossmann et al., 2008). A key protein in the maintenance of MCC organization is Pil1. Deletion of the PIL1 gene prevents the formation of the membrane furrows and the concentration of the associated proteins (Grossmann et al., 2008; Stradalova et al., 2009; Walther et al., 2006). The H+-ATPase Pma1 is excluded from the MCC and localizes to the remainder of the plasma membrane surrounding the MCC patches (Malinska et al., 2003). This domain was thus termed MCP (membrane compartment of Pma1) (Grossmann et al., 2007). The hexose transporter Hxt1 and the general amino acid permease Gap1 are evenly distributed to both MCC and MCP domains (Lauwers et al., 2007; Malinska et al., 2003).

The functional significance of the plasma membrane domain organization in yeast is not well understood. However, it has been suggested that the MCC has a direct role in regulating endocytosis (Grossmann et al., 2008; Walther et al., 2006). Walther and colleagues (Walther et al., 2006) proposed that eisosomes mark static sites of endocytosis, thus regulating the localization of endocytic events. However, Grossmann and co-workers (Grossmann et al., 2008) suggested that endocytosis takes place exclusively outside the MCC, in the MCP area, and that endocytosis of certain transmembrane proteins is regulated by sequestering them in the MCC where they are protected from endocytosis. Consequently, these suggested links between the MCC and endocytosis contradict each other.

We have used quantitative live-cell imaging to study the relationship between the domain organization of the yeast plasma membrane and vesicle trafficking processes in detail. We show that both endocytosis and exocytosis are excluded from the MCC and distributed within the MCP area independently of the MCC pattern. Using total internal reflection fluorescence (TIRF) microscopy and fluorescence recovery after photobleaching (FRAP) experiments, we show that the absence of vesicle traffic in the MCC is unlikely to be a mechanism that significantly protects transmembrane proteins enriched in the MCC from endocytosis. Consequently, our results suggest that the MCC and MCP membrane organization is not important for the regulation of vesicular traffic in yeast.

Results

Endocytic sites are localized within the MCP area and their distribution is independent of the MCC pattern

To analyze the spatial relationship between endocytic sites and the plasma membrane domains, we imaged cells that expressed Abp1–GFP, a marker for sites of clathrin-mediated endocytosis (Kaksonen et al., 2007) and the general amino acid permease Gap1 are evenly distributed to both MCC and MCP domains (Lauwers et al., 2007; Malinska et al., 2003).
et al., 2003) and Pil1–mCherry, a component of eisosomes, as a marker for the MCC (Walther et al., 2006; Grossmann et al., 2007). In this study, we consider the MCC area as the membrane areas labeled by Pil1–mCherry and the MCP area as the remainder of the plasma membrane devoid of Pil1 signal (Grossmann et al., 2007). To obtain optimal spatial separation of the plasma membrane domains, we focused to the bottom surfaces of cells. In small-budded cells, most of the endocytic events are concentrated in the bud, preventing the detection of individual endocytic sites by light microscopy. Furthermore, MCC patches are practically absent from small buds (Moreira et al., 2009). We therefore limited our analysis to large-budded or un budded cells. The acquired movies showed that endocytic events were excluded from the MCC (Fig. 1A; supplementary material Movie 1), which is in agreement with observations by Grossmann and colleagues (Grossmann et al., 2008).

To test whether MCC patches could regulate the localization of endocytic events within the MCP area, we measured the distance of the centroid positions of Abp1–GFP patches to the centroid position of the respective closest MCC patch. In total 383 endocytic events were detected in 12 cells. Distances smaller than 100 nm were absent, which confirms that none of the considered endocytic events colocalized with a MCC patch (Fig. 1B).

We then wanted to compare the distribution of endocytic events to a distribution of points whose positions are independent of the MCC pattern. We therefore calculated the distances from the center of each pixel within the cell surface area to the centroid position of the respective closest Pil1–mCherry patch (Fig. 1B,C). Interestingly, for distances longer than 100 nm, the distance distribution of endocytic sites closely followed the distribution of cell pixels, which shows that the number of endocytic events that took place within the MCP area at a certain distance to a MCC patch was tightly connected to the number of pixels in this MCP area, i.e. the area size. Our quantification thus indicates that endocytic events are distributed within the MCP independently of the MCC pattern. In other words, the likelihood that an endocytic site forms at a certain position within the MCP area is independent of the distance to the closest MCC patch.

To control for the accuracy of the positional measurements we imaged two proteins, Sla1 and Abp1, which transiently localize to endocytic sites (Kaksonen et al., 2003). We acquired two-color movies and determined the centroids of Sla1–GFP and Abp1–mCherry patches at the time points when they reached their respective maxima and measured their distances. 79% of the Sla1–mCherry patches at the time points when they reached their respective closest Pil1–mCherry patch (Fig. 1B, C).

We aimed to further test the exclusion of endocytic events from the MCC. It was recently shown that the protein kinases Pkh1 and Pkh2 (Pkh1/2) are involved in eisosome organization via phosphorylation of Pil1 and Lsp1, the main protein components of eisosomes, and that inhibition of Pkh1/2 kinase activity can lead to the formation of extended net-like eisosome structures, thus reducing the MCP area (Fröhlich et al., 2009; Luo et al., 2008; Walther et al., 2007). We therefore created pkh1Δ/pkh2Δ strain, that has PKH2 deleted and PKH1 replaced with a temperature-sensitive allele (pkh1L338G) (Friant et al., 2001; Inagaki et al., 1999), both at the permissive (25°C) and non-permissive temperature (37°C) (data not shown). Importantly, in the cells with stable net-like Pil1–mCherry structures, endocytosis was still excluded from the area marked by these structures (Fig. 1D; supplementary material Movie 2, left panel). We also visualized the localization of Can1–GFP after depletion of the Pkh1/2 kinases. In wild-type cells, the arginine permease Can1–GFP is enriched in the MCC patches (Malinska et al., 2003). Interestingly, neither the cells with net-like Pil1–mCherry structures, nor the cells with motile Pil1–mCherry clusters exhibited any clear enrichment of Can1–GFP into the area marked by the Pil1–mCherry structures. Instead, after the depletion, the Can1–GFP distribution appeared dispersed at the plasma membrane, similarly to what has been shown in pilla Δ cells (supplementary material Fig. S1, Movie 3) (Grossmann et al., 2007). Hence, the Pil1–mCherry structures observed after Pkh2 depletion are probably not fully functional eisosomes, but they still exclude endocytic events perhaps as a result of simple steric hindrance of vesicle coat assembly by the membrane-associated eisosome proteins.

**Exocytic sites are localized within the MCP area and their distribution is independent of the MCC pattern**

Endo- and exocytosis are spatially closely coupled in yeast cells. The majority of exocytic events take place in the bud to support polarized cell growth. Similarly, endocytic events are concentrated in the growing bud to support membrane recycling and polarity maintenance. To analyze the spatial distribution of exocytosis with regard to the MCC and MCP pattern, we imaged cells expressing Pil1–mCherry and Sec3–GFP or Exo70–GFP. Sec3 and Exo70 are components of an exocytic docking complex, the exocyst. Sec3 and Exo70 are thought to serve as landmarks for exocytic vesicle docking at the plasma membrane (Finger et al., 1998; He et al., 2007). In small-budded cells, exocytosis, similarly to endocytosis, is too concentrated in buds to resolve individual Sec3–GFP or Exo70–GFP patches by light microscopy. However, in large-budded or unbudded cells Sec3–GFP and Exo70–GFP formed transient diffraction-limited patches at the plasma membrane that are likely to be individual sites of exocytosis (supplementary material Movie 4). Similarly to endocytic sites, they formed exclusively in the MCP area of the plasma membrane (Fig. 2A). In analogy to the analysis of endocytic events described above, we measured the centroid positions of the Sec3–GFP and Pil1–mCherry patches and calculated the distance to the closest MCC patch for 165 exocytic events (Fig. 2B). Very small distances that would indicate colocalization with a MCC patch were absent from the distance distribution of exocytic sites. Similarly to the distribution of endocytic sites, the distribution of exocytic events resembled the distance distribution of cell pixels for distances longer than 100 nm. We then imaged cells that expressed Exo70–GFP and Pil1–mCherry and determined the distance to the closest MCC patch for 262 exocytic events in 16 cells. The distance distribution was very similar to that obtained with Sec3–GFP (Fig. 2C).

In summary, our analyses indicate that exocytosis, similarly to endocytosis, is restricted to the MCP area and that the likelihood of an exocytic event occurring at a certain position within the MCP is independent of the distance to the closest MCC patch.
Vesicular cargo exchanges between the MCC and MCP

Three plasma membrane permeases, Can1, Tat2 and Fur4, have been shown to concentrate in the MCC (Grossmann et al., 2007; Malinska et al., 2003; Malinska et al., 2004; Grossmann et al., 2008). These permeases are trafficked to the plasma membrane by exocytosis and they are removed in a regulated manner from the plasma membrane by endocytosis (Beck et al., 1999; Galan et al., 1996; Grossmann et al., 2008; Lin et al., 2008). As both trafficking events are confined to the MCP area, we tested whether the permeases can move between the domains. To study this, we imaged cells expressing Can1–GFP and Pil1–mCherry. Consistent with earlier studies, Can1–GFP was concentrated in most MCC patches in wild-type cells (Malinska et al., 2003). However, the permease was also present in the MCP area. To estimate the fraction of the total plasma membrane Can1 molecules located in the MCC, we analyzed confocal images of cells expressing Can1–GFP and Pil1–mCherry. The analysis showed that only about 28% of the total plasma membrane Can1 molecules are located in the MCC.

Fig. 1. Formation of endocytic sites in relation to MCC patches. (A) Surface views of a cell expressing Abp1–GFP and Pil1–mCherry. The first six columns show the cell at a different time. The last column shows respective maximum projections of all the six time points. Scale bar: 2 µm. (B) Distribution of distances between the patch centroid positions of the analyzed Abp1–GFP-labeled endocytic sites and the centroid position of the respective closest MCC patch marked by Pil1–mCherry. 383 endocytic events were analyzed in 12 cells (minimum distance, 104 nm; maximum distance, 947 nm; mean ± s.d., 388±159 nm). The black bars show the same distribution of distances between cell pixels and the respective closest MCC patch, as shown in B. (C) A pkhl1 Δpkhl2 cell expressing Sla1–GFP and Pil1–mCherry after 12 hours of depletion. The Sla1–GFP signal, marking endocytic events, is green in the overlay panel, the net-like Pil1–mCherry signal is red. The images are maximum projections of 90 movie frames (see supplementary material Movie 2). Scale bar: 2 µm.
MCC (Fig. 3, see the Materials and Methods for details). The MCC enrichment of Tat2–GFP is very similar to that of Can1 in surface view epifluorescence images (data not shown).

We then performed FRAP experiments to test whether the Can1–GFP molecules concentrated in MCC patches exchange with the MCP pool. To study Can1 dynamics in the MCP, we first bleached areas within the MCP area. Can1–GFP fluorescence recovered in the MCP with a half time of 73±5 seconds (n=8; Fig. 4A–C,G; supplementary material Movie 5). We then photobleached Can1–GFP patches that colocalized with Pil1–mCherry-labeled MCC patches. Owing to the size of our bleach spot (diameter ~600 nm), some of the surrounding MCP area was also bleached. The Can1–GFP signal measured in the MCC patch area recovered on average with a half time of 344±17 seconds (n=10; Fig. 4D–G; supplementary material Movie 5). Because the 488 nm wavelength used for photobleaching also photobleached the Pil1–mCherry signal, we showed that more than 10 minutes after the recovery of the Can1–GFP MCC signal, there was still almost no recovery of the Pil1–mCherry signal (Fig. 4D–F), which demonstrates the high stability of Pil1 in eisosomes (Walther et al., 2006). Thus, the FRAP experiments indicate that Can1 molecules continuously exchange between the MCC and the MCP in cells that are not induced to endocytose Can1. Similar recoveries were observed for Tat2–GFP (supplementary material Movie 6).

The fast recovery rates indicate that the Can1 and Tat2 molecules in the MCC exchange with the MCP pool on average in about 5 minutes. This suggests that the accumulation of these cargoes in the MCC is unlikely to provide significant protection against endocytic turnover.

Endocytosis of Can1 and Tat2 is regulated normally in the absence of the MCC

To test whether the plasma membrane domain organization regulates endocytosis of the MCC-enriched permeases as suggested by Grossmann and colleagues (Grossmann et al., 2008), we analyzed the endocytosis rates of Can1 and Tat2 in wild-type cells and cells where the MCC pattern was disrupted by PIL1 deletion. Few large clusters of the MCC proteins Lsp1–GFP and Can1–GFP were reported to form at the plasma membrane in some pil1/H9004 cells.

Fig. 2. Formation of exocytic sites in relation to the MCC patches. (A) Surface views of a cell expressing Sec3–GFP and Pil1–mCherry. The first six columns show the cell at a different time (supplementary material Movie 4). The last column shows respective maximum projections of all the six time points. Scale bar: 2 μm. (B) Distribution of distances between the patch centroid positions of the analyzed Sec3–GFP-labeled exocytic sites and the centroid position of the respective closest MCC patch marked by Pil1–mCherry (red bars). 165 exocytic events were analyzed (minimum distance, 71 nm; mean ± s.d., 364±154 nm). The black bars show the distribution of distances between the centers of the pixels within the cells and the centroid position of the respective closest MCC patch marked by Pil1–mCherry. 13,718 pixels were analyzed (minimum distance, 1 nm; mean ± s.d., 394±190 nm). In total eight cells were evaluated. (C) Distribution of distances between the patch centroid positions of the analyzed Exo70–GFP-labeled exocytic sites and the centroid position of the respective closest MCC patch marked by Pil1–mCherry. 262 exocytic events were analyzed (minimum distance, 77 nm; mean ± s.d., 407±176 nm). The black bars show the distribution of distances between the centers of the pixels within the cells and the centroid position of the respective closest MCC patch marked by Pil1–mCherry. 27,797 pixels were analyzed (minimum distance, 1.5 nm; mean ± s.d., 431±223 nm). In total, 16 cells were evaluated.

Fig. 3. Localization of Can1–GFP to both the MCC and the MCP. Representative example of confocal images that were used to calculate the fraction of the total plasma membrane Can1 molecules in the MCC area to ~28%. Note that Can1–GFP is enriched at MCC patches, but there is also a prominent signal in the MCP area. Scale bar: 2 μm.
(Grossmann et al., 2007; Walther et al., 2006). However, we observed these clusters only in cells from cultures that had reached a cell density of OD$_{600}$>0.8. At lower culture densities, the MCC patches were completely absent and we only observed diffuse cytosolic or plasma membrane distributions of Lsp1–GFP and Can1–GFP, respectively.

We used addition of arginine to induce endocytosis of Can1–GFP in cells from exponentially growing cultures (OD$_{600}$=0.4) and quantified the progress of endocytosis over time by determining the ratio of GFP fluorescence signal between the plasma membrane and the cell interior (Fig. 5A,B; supplementary material Fig. S2). Endocytosis of Can1–GFP was dependent on the arginine stimulus in both wild-type and pil1D cells, and the rate of endocytic uptake was also indistinguishable in both strains. Interestingly, we observed that when the cultures were allowed to continue growing, Can1–GFP endocytosis was initiated without addition of arginine when the culture reached a certain density (OD$_{600}$=0.8). Quantification of the rate of this spontaneous endocytosis in wild-type and pil1Δ cells did not reveal any significant differences between the strains (Fig. 5C). We additionally analyzed the rate of Can1 endocytosis in wild-type and pil1Δ cells after triggering endocytosis with cycloheximide (Grossmann et al., 2008; Lin et al., 2008). The rate of endocytosis was slower in this case, but again, we determined similar rates for wild-type and pil1Δ cells (supplementary material Fig. S3).

We then analyzed the endocytosis rate of Tat2–GFP in wild-type and pil1Δ cells. Endocytosis of Tat2–GFP was triggered by addition of tryptophan into the growth medium (Fig. 5D). As with Can1–GFP, Tat2–GFP endocytosis was dependent on the substrate trigger in both strains and we did not detect significant differences in the endocytosis rates between the strains (Fig. 5E). These results imply that the regulation of Can1 and Tat2 endocytosis is not significantly affected by the enrichment of the permeases into the MCC.

MCC enrichment of permeases does not correlate with their endocytosis

We then tested whether the MCC enrichment of Can1 and Tat2 is influenced by addition of their substrates. We triggered Can1–GFP endocytosis by addition of arginine and monitored its distribution...
on the plasma membrane. Notably, for up to 1 hour after the addition of arginine, we could still observe a clear enrichment of Can1 in the MCC by epifluorescence microscopy (Fig. 6A). Owing to the increasing intracellular signal and the decreasing plasma membrane signal, the enrichment of Can1–GFP in MCC patches became difficult to visualize with epifluorescence microscopy at later time points. However, using TIRF microscopy, we could show that the MCC enrichment of Can1–GFP persisted and was still evident even in cells that had already trafficked the vast majority of Can1–GFP molecules into the vacuole (Fig. 6B). The TIRF images in Fig. 6B illustrate that the Can1–GFP signal at the plasma membrane decreased significantly, correlating with the progress of endocytosis, whereas the enrichment in the MCC persisted. To quantify this observation, we determined the ratio of the Can1–GFP fluorescence signal in the MCC and the MCP from TIRF images at different time points after induction of endocytosis.

The average plasma membrane signal of Can1–GFP decreased as expected, whereas the ratio between the MCC and the cell interior (mean ± s.d., n ≥ 12) remained unchanged (Fig. 6C). These results imply that the Can1–GFP molecules are lost from both membrane domains at similar rates during endocytosis.

Interestingly, in contrast to Can1, the enrichment of Tat2–GFP in MCC patches was lost when its substrate tryptophan was added into the medium (Fig. 6D). To determine how fast the MCC enrichment is lost, we continuously monitored the surface of cells expressing Tat2–GFP and the MCC marker Lsp1–mCherry, and added tryptophan to these cells. The MCC enrichment was lost within ~90 seconds after addition of tryptophan (Fig. 6E; supplementary material Movie 7). Identical results were obtained by using Pil1–mCherry as a MCC marker (data not shown). The MCC enrichment was restored within 5 minutes after washing out the added tryptophan (data not shown). After the loss of MCC

Fig. 5. Endocytosis rates of Can1–GFP and Tat2–GFP are independent of their MCC enrichment. Endocytosis rates of Can1–GFP and Tat2–GFP were compared in wild-type cells and pil1Δ cells. (A) Representative Can1–GFP images of wild-type and pil1Δ cells before and 90 minutes after Can1 endocytosis had been induced by addition of arginine. Images recorded at further time points are shown in supplementary material Fig. S2. (B) The ratio of mean Can1–GFP fluorescence intensity between the plasma membrane and the cell interior (mean ± s.d., n ≥ 12) is shown for induced wild-type cells, induced pil1Δ cells, non-induced wild-type cells and non-induced pil1Δ cells. The times relate to the time of arginine addition (0 minutes). Arginine was added to cell cultures at a cell density of OD600 = 0.4. (C) Arginine was added to cell cultures at a cell density of ~OD600 = 0.65. The non-induced control cells started to endocytose Can1–GFP at cell densities of ~OD600 = 0.8. (D) Representative Tat2–GFP images of wild-type and pil1Δ cells before and ~180 minutes after Tat2 endocytosis was induced by addition of tryptophan. (E) The ratio of mean Tat2–GFP fluorescence intensity between the plasma membrane and the cell interior (mean ± s.d., n ≥ 12) is shown for induced wild-type cells, induced pil1Δ cells, non-induced wild-type cells and non-induced pil1Δ cells. The times relate to the time of tryptophan addition (0 minutes). Tryptophan was added to cell cultures at a cell density of ~OD600 = 0.4. Scale bars: 5 μm.
Fig. 6. MCC enrichment of Can1 persists, whereas MCC enrichment of Tat2 is lost within 90 seconds of addition of substrate. (A) Representative surface-view images of wild-type cells expressing Can1–GFP and Pil1–mCherry are shown before and 47 minutes after Can1 endocytosis had been induced by addition of arginine. Scale bar: 5 μm. (B) Cells expressing Can1–GFP and Pil1–mCherry were induced to endocytose Can1 by addition of arginine. In each row the Can1–GFP epifluorescence image at the cell center (Epi-Center) is followed by Can1–GFP surface image (Epi-Surface), Can1–GFP TIRF image (TIRF, all images in the column with same contrast settings), Can1–GFP TIRF image [TIRF(CA), images in the column have individually adjusted contrast], Pil1–mCherry surface image (Epi-Surface), overlay Can1–GFP TIRF image (green) and Pil1–mCherry surface image (red) (Overlay). The first row shows a cell before addition of arginine, the second row shows a cell 50 minutes after the addition of arginine, the third row shows a cell that had already endocytosed a significant portion of Can1–GFP (350 minutes after the addition of arginine), the bottom row shows a cell that had already endocytosed most Can1–GFP molecules (410 minutes after the addition of arginine). The MCC enrichment persisted, as can be seen in the TIRF and overlay images. Scale bar: 2 μm. (C) The mean fluorescence intensity at the plasma membrane of Can1–GFP-expressing cells was quantified from TIRF images (compare third column in 6B) at different times after Can1 endocytosis had been induced by addition of arginine (mean ± s.d., n (number of cells) ≥13). As a comparison, the ratio of mean Can1–GFP fluorescence signal between MCC patches and the corresponding local MCP areas that was quantified from the same TIRF images is shown [mean ± s.d., n (number of MCC patches) ≥18]. (D) Representative surface-view images of wild-type cells expressing Tat2–GFP and Lsp1–mCherry are shown before and 44 minutes after Tat2 endocytosis had been induced by addition of tryptophan. Scale bar: 5 μm. (E) Surface-view images of a wild-type cell expressing Tat2–GFP and Lsp1–mCherry are shown at different time points that refer to the time of addition of tryptophan to a concentration of 0.35 mM (see supplementary material Movie 7). Scale bar: 2 μm.
enrichment, the distribution of the Tat2–GFP signal was highly dynamic and not fully homogeneous (supplementary material Movie 7). However, distribution of the Tat2–GFP signal appeared uncorrelated with the MCC patches after addition of tryptophan.

In summary, the addition of substrates induces the uptake of both Can1 and Tat2, but affects their MCC enrichment in different ways, suggesting that the two processes are not functionally connected.

**Discussion**

Walther and co-workers (Walther et al., 2006) suggested that eisosomes mark sites of endocytosis. This proposal was mainly based on experiments done in pil1Δ cells. In these cells, the other main eisosome protein Lsp1 formed few large clusters, which were reported to colocalize with endocytic markers. However, our quantitative analysis in wild-type cells showed that endocytic events are excluded from the MCC (Fig. 1). This result is consistent with the observation of Grossmann and colleagues (Grossmann et al., 2008) that the endocytic proteins Rxs161 and Ede1 do not colocalize with a MCC marker. Furthermore, we showed that exocytosis is also excluded from the MCC. Hence, the MCP area is the domain of vesicular traffic at the yeast plasma membrane. Importantly, our distance distribution analyses showed that the likelihood that an endocytic or exocytic site forms at a certain position within the MCP area is independent of the distance to the closest MCC patch (Fig. 1B,C and Fig. 2B,C). Therefore, the MCC does not seem to have an influence, either positive or negative, on the selection of sites of vesicular traffic within the MCP area.

Grossmann and colleagues (Grossmann et al., 2008) suggested that MCC patches negatively regulate the endocytic uptake of MCC-enriched transmembrane transporters by sequestering them away from the MCP area where endocytosis takes place. So far, three plasma membrane permeases, Can1, Tat2 and Fur4, have been shown to be enriched in the MCC (Grossmann et al., 2007; Malinska et al., 2003; Malinska et al., 2004; Grossmann et al., 2008). In support of the negative regulation by the MCC, faster MCC-enriched transmembrane transporters by sequestering them away from the MCP area where endocytosis takes place. So far, three plasma membrane permeases, Can1, Tat2 and Fur4, have been shown to be enriched in the MCC (Grossmann et al., 2007; Malinska et al., 2003; Malinska et al., 2004; Grossmann et al., 2008). In support of the negative regulation by the MCC, faster Can1–GFP turnover rates were reported in cells lacking MCC patches than in wild-type cells (Grossmann et al., 2008).

In this study, we made several observations that do not support the idea that the MCC enrichment regulates the endocytosis of the permeases. First, our quantitative analyses of Can1 and Tat2 endocytosis rates did not show any significant differences between wild-type and pil1Δ cells, which lack MCC patches and have dispersed plasma membrane distributions of Can1 and Tat2. Notably, similarly to wild-type cells, endocytosis of the permeases in pil1Δ cells was still dependent on a specific trigger (a threshold culture density or addition of substrate) (Fig. 5). Consequently, a mechanism other than the enrichment in the MCC has to protect the permeases from endocytosis in the absence of endocytosis trigger. Furthermore, we estimated that only about 28% of the total plasma membrane Can1 molecules are located in the MCC in non-induced wild-type cells. Protection from endocytosis due to localization within the MCC is thus limited to the minority of the permease molecules. In addition, our FRAP experiments in non-induced cells showed that Can1 and Tat2 molecules exchange between the MCC and the MCP and stay in a MCC patch on average for only about 5 minutes. Consequently, despite the enrichment in MCC patches, the permease molecules frequently diffuse to the MCP area, where they would be available for endocytosis. Finally, we observed, that the release kinetics of the permeases from the MCC do not correlate with their endocytosis rates. For Can1 we showed by TIRF microscopy that its MCC enrichment was retained when endocytosis was induced by arginine. Instead, the plasma membrane signal for Can1–GFP decreased uniformly in both the MCC and the MCP. However, the MCC enrichment of Tat2 was lost within 90 seconds after addition of tryptophan, so that the permease became distributed more evenly between the two domains. The loss of MCC enrichment of Tat2 was thus much faster than the uptake of the Tat2 pool from the plasma membrane by endocytosis.

In summary, our results indicate that the enrichment of the permeases in the MCC does not have a major role in regulating their endocytosis. Therefore, we consider it more likely that endocytosis is regulated by mechanisms that are independent of the plasma membrane domain organization. Interestingly, for all three permeases, Can1, Tat2 and Fur4, ubiquitylation has been demonstrated to be a major regulator of their endocytic turnover (Beck et al., 1999; Galan et al., 1996; Lin et al., 2008; Nikko et al., 2008).

As a further test for the exclusion of endocytic events from the MCC, we created Pkh1/2 depletion strains. PIIl is known to be phosphorylated by Pkh1/2 (Luo et al., 2008; Walther et al., 2007). Inactivation of Pkh1/2 kinases has been reported to result in enlarged net-like eisosome structures, which were suggested to be due to a defect in either eisosome assembly (Luo et al., 2008) or disassembly (Fröhlich et al., 2009; Walther et al., 2007). We observed that most of the Pkh1/2-depleted cells exhibited normal size but highly motile Pil1–mCherry structures at the plasma membrane. Only a subpopulation of the cells (~20%) exhibited enlarged, stable net-like Pil1–mCherry structures (supplementary material Movie 2). Importantly, the enlarged net-like Pil1–mCherry structures always excluded endocytic events (Fig. 1D, supplementary material Movie 2). However, neither the motility nor the net-like Pil1–mCherry structures were able to enrich Can1–GFP signal, which was homogeneously distributed at the plasma membrane after depletion of Pkh1/2 (supplementary material Fig. S1, Movie 3), which is reminiscent of the normally homogenous plasma membrane distribution of Hxt1 and Gap2 (Lauwers et al., 2007; Malinska et al., 2003). Based on these observations, we hypothesize that the lack of Pkh1/2 activity leads to abnormal eisosomes that might be defective in bending the plasma membrane into furrows. This could explain the motility of the small Pil1 structures and the lack of permease enrichment. Our finding in Pkh1/2-depleted cells that the Pil1–mCherry structures still excluded endocytosis, even though they were not functional eisosomes as judged by the enrichment of Can1 permease, supports the idea that the local exclusion of vesicular traffic events in the MCC is due to a simple steric hindrance effect of the abundant membrane-bound eisosome proteins, which might prevent the assembly of the endocytic coat or the docking of an exocytic vesicle.

**Materials and Methods**

**Strains and media**

Yeast strains (supplementary material Table S1) were grown in standard rich medium (YPD) or in synthetic medium (SD) supplemented with adenine, uracil, histidine, leucine, lysine and methionine. All the yeast strains were cultured at 25°C and shaking at 220 r.p.m. EGFP or mCherry tags were integrated chromosomally at the C-termius of each gene, and gene deletions were generated by replacing the entire gene ORF with a selection cassette (Janke et al., 2004). Strains carrying several fusions or deletions were created by mating. The correct chromosomal integration was checked by PCR. Wild-type and mutant cells expressing EGFP- or mCherry-tagged proteins had growth properties indistinguishable from those of untagged cells.
Induction of Can1 and Tat2 endocytosis and depletion of Pkh2

Endocytosis of Can1 was triggered either by addition of arginine to a concentration of 5 mM or cycloheximide to a concentration of 50 μg/ml to yeast cultures of OD600=0.4 (unless other OD is stated). Endocytosis of Tat2 was triggered by addition of tryptophan to a concentration of 5 mM (unless other concentration is stated). For the depletion experiments with pkh1ΔΔpkh2 cells, in which PKH2 is controlled by a tetracycline-regulatable promoter (Bell et al., 1998), a liquid culture was diluted into a fresh medium of OD600=0.3 and split into two samples. To one sample, doxycycline was added to a concentration of 25 μg/ml. After 12 hours shaking at 30°C, the depleted and control cells were imaged.

Live-cell imaging

For microscopy, cells were grown in SD at 25°C until early log phase. Cells were adhered to concanavalin-A-coated (0.1 μg/ml) circular coverslips (25 mm diameter, Menzel-Gläser) and imaged in SD in a custom-made holder. Imaging was done at room temperature using an Olympus IX81 microscope equipped with a 100× NA 1.45 objective, Orca-ER camera (Hamamatsu), and electronic shutters and filterwheels (Sutter Instrument). TIRF microscopy was carried out using a 60×/1.49 objective and a 488 nm solid-state laser (Coherent). The FRAP experiments were done with a custom-built set-up that focuses the 488 nm laser beam at the sample plane. The CCD camera, the filter wheels and the shutters were controlled by Metamorph software (Molecular Devices).

Confocal imaging was done with a Zeiss LSM 710 microscope using a 63×/1.4 objective. GFP fluorescence was excited with the 488 nm line of the argon laser and mCherry fluorescence was excited with the 488 nm line of the argon laser and mCherry fluorescence was excited with a 561 nm solid-state laser.

Image analysis

Image analysis was carried out using custom-written, MATLAB programs. Images were background subtracted and all movies were corrected for photobleaching. To segment the endocytic and exocytic sites and MCC patches from the background, the images were first smoothed by 3×3 pixel averaging. Global thresholding was used to identify the cell area, followed by adaptive thresholding with a 7×7 pixel window to segment the endocytic and exocytic sites and the MCC patches within the cell area. The centroids of the segmented patches were determined and the centroids from consecutive frames were connected into tracks. The automatic segmentation and tracking was confirmed by visual inspection of the movies. The position of an endocytic or exocytic event was considered to be the centroid from the frame at which the fluorescence intensity of the marker was the brightest. The position of an endocytic or exocytic event was then compared with the centroid positions of the Pil1–mCherry patches in the corresponding frame to determine the distance to the closest MCC patch. To get the fluorescence distribution of Sla1–GFP patches to Abp1–mCherry patches the position of each Sla1–GFP patch was compared with all Abp1–mCherry patches found in the same frame, or in one of the following five frames of the time-lapse movies. For the distribution of distances between the centers of cell pixels and the respective closest MCC patch, the cell area in focus was marked by hand for each cell. The center of each pixel within this cell area was then compared with the centroid positions of the Pil1–mCherry patches in the first movie frame to determine the shortest distance. A small shift in the alignment of the channels due to chromatic aberration was corrected based on images of immobilized microbeads that fluoresce at both green and red wavelengths.

To calculate the mean fluorescence intensity ratios between the MCC and the MCP for Pil1–mCherry, the cell area outside the MCC area was considered the MCP area. The mean fluorescence intensity values were corrected for the autofluorescence background determined by imaging cells that only expressed Pil1–mCherry or Can1–GFP. The mean fluorescence intensity ratio between the MCC and the MCP was 5.3 for Pil1–mCherry and only 1.37 for Can1–GFP. On average, the MCC area was 20.42% of the total cell area. The 20.42% is probably an overestimation caused by optical blurring of the small MCC patches as a result of the resolution limit of light microscopy. To further correct for potential additional background signal due to scattering, we imaged Pil1–GFP. We assumed that 100% of Pil1–GFP would localize to the MCC patches and considered the signal measured in the MCP as background. The ratio between the MCC and the MCP for Pil1–GFP was 5.3, as in case of Pil1–mCherry. We then used this ratio to correct the Can1–GFP signal to calculate the final intensity ratio of Can1–GFP distribution between the MCC and the MCP to 1.85. Note that this correction might lead to overestimation of the concentration of mCherry. We then used this ratio to correct the Can1–GFP signal to calculate the mean fluorescence intensity ratio between the MCC and the MCP for Pil1–GFP was 5.3, as in case of Pil1–mCherry epifluorescence images. Based on the centroid positions of the MCC patches, the ratio was calculated with a custom-written MATLAB program.

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