A Correlation between Mode of Growth and Regional Ultrastructure of the Plasma Membrane of *Schizosaccharomyces pombe* as Revealed by Freeze-fracturing before and after Filipin Treatment

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The ultrastructure of the plasma membrane of *Schizosaccharomyces pombe* was studied by freeze-fracture using invaginations of the plasma membrane as natural markers and filipin-induced deformations as artificial markers. In accord with the mode of growth of this organism, ultrastructural aspects of the plasma membrane were related to the following ring zones: the growing pole, adjacent regions, proximal regions, the new cell pole, and the middle in dividing cells. The growing pole and adjacent regions had no or only a few invaginations. Filipin induced numerous deformations in these regions. By contrast, the proximal regions of the plasma membrane had several invaginations and resisted filipin-induced deformation. Concomitantly with commitment to cytokinesis, both the invaginations and the resistance to filipin-induced deformation disappeared in the middle. The results presented here strongly suggest the existence of two states of the plasma membrane of *S. pombe*, a fact which correlates well with the mode of growth of this organism.

**INTRODUCTION**

*Schizosaccharomyces pombe* grows by elongation, usually only at the old cell pole (the growing pole) though it may also do so at the new cell pole (Mitchison, 1957; May 1962; Johnson, 1965). It divides in the middle by binary fission after cessation of elongation (Mitchison, 1957). Thus the cylindrical shaped plasma membrane of *S. pombe* can be separated into the following ring regions: the old cell pole, adjacent regions, proximal regions, the new cell pole, and the middle in dividing cells. Typically, the old cell pole and the division furrow are dynamic regions which rapidly change shape; regions adjacent to them may be involved in, influenced by, or newly formed at growth or cytokinesis, and may also be regarded as dynamic regions. The proximal regions and the new cell pole are mostly static regions. In *S. pombe*, the place where a cell will divide is not predetermined (Fantes & Nurse, 1977). Thus, the plasma membrane in the middle must change from a static to a dynamic region when it is committed to cytokinesis. This change has been detected ultrastructurally as the disappearance of the invaginations of the plasma membrane (Takeo, 1984). The invaginations are the most impressive structures of the plasma membrane of yeasts seen after freeze-fracturing (Moor & Mühlethaler, 1963). These facts support the idea that the dynamic and static regions of the plasma membrane of *S. pombe* may be distinguishable ultrastructurally.

The polyene antibiotic filipin specifically and stoichiometrically binds with cholesterol, ergosterol and the related 3-β-hydroxysterol, and induces morphological lesions in membranes, i.e. protrusions or pits of about 25 nm diameter (Norman *et al.*, 1972; Verkleij *et al.*, 1973). Because of these characteristics, filipin has frequently been used as a specific and *in situ* cytochemical probe for free sterol in membranes (Elias *et al.*, 1979; Severs & Robenek, 1983), although false negative responses may occur. Several regions of the plasma membrane that are
virtually devoid of filipin-induced deformations have been found to contain enough sterol to respond to filipin (Severs et al., 1981; McGookey et al., 1983; Pumplin & Bloch, 1983; Severs & Simons, 1983; Tamm & Tamm, 1983; Severs & Robenek, 1983). Although the mechanisms for the false-negative response have not always been clear, some factors must exist, such as closely-packed intramembrane particles (Severs et al., 1981), membrane-associated protein components (Feltkamp & Van der Waerden, 1982), and filamentous structures underlying the membrane (Gotow & Hashimoto, 1983), which inhibit filipin-induced deformation of the membrane in these regions.

Lack of labelling by filipin thus indicates either deprivation/low level of free sterol, or the existence of regional constraints which restrict the ability of filipin to deform the membrane. In either case, lack of label in the plasma membrane indicates regional specialization.

This paper describes a correlation between the mode of growth and the regional ultrastructure of the plasma membrane of S. pombe as revealed by freeze-fracture, using the invaginations as natural markers, and the filipin-induced deformations as artificial markers.

METHODS

Haploid strains of Schizosaccharomyces pombe [972 (h-) and 975 (h+)] from Dr U. Leupold, Institute of General Microbiology, University of Bern, Switzerland, were used. The organisms were usually grown in EMM (2) medium (Mitchison, 1970), with shaking at 25–26 °C, and harvested at the late exponential phase. Changes in invaginations of the plasma membrane were also observed in cells growing exponentially in 10G YNB medium (Takeo, 1984) as above, and on a potato/yeast/glucose agar medium at 27 °C (Takeo et al., 1973), and especially when stationary phase cells, which had abundant invaginations, were released into fresh media. Two types of stationary phase cells were obtained. Incubations in EMM (1) or (2) medium as above for 3–7 d yielded short viable cells which had a densely invaginated plasma membrane. Incubation for 4–6 d as above in 10G YNB, or 10G YNB supplemented with 5 mg ml⁻¹ of KH₂PO₄ (Johnson, 1967), yielded increased proportions of long viable cells which were relatively densely invaginated. These stationary phase cells were released into either EMM (2) or 10G YNB medium and incubated as above for 1.5–6 h.

Filipin was generously provided by the Upjohn Co. (Kalamazoo, MI, USA). Digitonin and tomatin were reagent grade and purchased from Nakarai Chemical Co., Kyoto, Japan, and Tokyo Chemical Industry Co., Tokyo, Japan, respectively. Filipin and tomatin were solubilized in a small amount of dimethyl sulphoxide (final concentration was less than 0.1% and 1%, w/v, respectively), and digitonin in tetrahydrofuran (1%, w/v). These chemicals were used in 0.07 M-phosphate buffer, pH 7, containing 2.5% (v/v) glutaraldehyde. Cells were treated with filipin (50 or 100 µg ml⁻¹) with shaking for periods of 10 min to overnight at room temperature (about 20 °C) after fixation at 0 °C for 30 min with 2.5% (v/v) glutaraldehyde in 0.07 M-phosphate buffer, pH 7. The filipin treatment was stopped by washing cells with 0.07 M-phosphate buffer, pH 7. Freeze-fracturing was essentially as described previously (Takeo et al., 1973). Digitonin-sterol complexes have been reported to be progressively removed from membranes (Elias et al., 1978). Thus all the processes of experiments with saponin treatment were done rapidly. Cells were grown on the potato/yeast/glucose agar at 27 °C for 1 d, fixed for 30 min by pouring the fixative over the cells, and suspended in digitonin solution (100 µg ml⁻¹) for 1 or 3 h, or tomatin solution (400 µg ml⁻¹) for 4 h, or tomatin solution (100 or 200 µg ml⁻¹) overnight. After being immersed for 10 min in 20% (v/v) glycerol solution containing 0.07 M-phosphate buffer, pH 7, the cells were immediately freeze-fractured.

RESULTS

During exponential growth of S. pombe, intramembrane particles were distributed randomly over the plasma membrane, though some were in paracrystalline arrays (see also Walther et al., 1984). A few long invaginations (0.5–2 µm long and 50 nm wide) were scattered over the bulk of the plasma membrane. The invaginations were specialized portions of the plasma membrane, since both on the P- and E-faces, the invaginations in exponential phase cells usually had a dearth of intramembrane particles (see also Takeo et al., 1976; Walther et al., 1984).

Changes in the invaginations during growth were most clearly observed when stationary phase cells were released into fresh media. Stationary phase cells grown in EMM (1) or (2) medium were short. The plasma membrane, including both cell poles, was densely invaginated (Fig. 1). After the release into fresh EMM (2) medium, a cell pole changed to the growing pole, which was ultrastructurally characterized by the absence of the invaginations. Fig. 2 shows a typical plasma membrane of an elongating cell. The membrane appeared as if it consisted of two
In all figures (1–9), *S. pombe* was grown to the exponential phase in EMM (2) medium unless otherwise described. The small arrows point to the invaginations of the plasma membrane. Bar markers represent 1 µm, except for Fig. 8(b) as indicated in the legend.

Fig. 1. The E-face of the plasma membrane of *S. pombe*, in the stationary phase, grown in EMM (1) medium for 6 d. Note the thickness of the invaginations and the short cell size.

Fig. 2. The E-face of the plasma membrane. The cell was grown in EMM (1) medium for 6 d, put into fresh EMM (2) medium, and incubated for 5 h 12 min. Note the apparent contrast in two parts of the plasma membrane: one is densely invaginated like a stationary phase cell; the other is only sparsely invaginated.

Fig. 3. The P- (P) and E-faces (E) of the plasma membranes in the exponential phase. A dividing cell (D) has several invaginations scattered on the bulk of the plasma membrane except for regions near the division furrow (—). Four cell poles are also observed: one is invaginated (white arrow); the other three are uninvaginated (—).
parts: one was densely invaginated like a stationary phase cell (compare Fig. 2 with Fig. 1); the other apparently contained the growing pole and adjacent regions, which had no or only a few invaginations, and proximal regions which had several invaginations. After elongation, cells divided at or near the regions that had not been densely invaginated. Thus, the disappearance of the invaginations, just before cytokinesis, could be shown most clearly when long stationary phase cells were released into fresh media as described previously (Takeo, 1984). In view of the above findings, ultrastructural aspects of the invaginations were analysed in cells growing exponentially. The plasma membrane at the growing pole and adjacent regions had no or few invaginations (Fig. 3). During cytokinesis, the invaginations were hardly visible in the division furrow and regions about 1 μm wide adjacent to the furrow (Fig. 3). The other cell pole (the new cell pole), was often uninvaginated. Two factors which might be contributory are that invaginations at the new cell pole did not appear immediately after cytokinesis, and that some cells grow at both the old and new cell poles (Mitchison, 1957). By contrast, the proximal regions of the plasma membrane had several invaginations (Fig. 3). Thus, the plasma membrane regions that were changing dynamically at growth and cytokinesis were essentially uninvaginated, whereas the static regions were invaginated.

To show more clearly the existence of ultrastructural differences between the two functionally different regions, or rather states, of the plasma membrane of S. pombe, cells were observed after filipin treatment. Filipin induced protrusions of 15–30 nm diameter and pits on both the P- and E-faces of the plasma membrane (Figs 4–7). These deformations have been reported to be due to the specific response of free sterol to filipin (Elias et al., 1979; Sekiya & Nozawa, 1983). In addition, a high concentration of filipin (100 μg ml⁻¹) sometimes induced a cluster of deformations of a smaller diameter (15–20 nm) on the plasma membrane or rather in the wall. Artifacts due to decomposition products of filipin appear unlikely as an explanation for these structures since other yeasts studied did not show the clusters (unpublished observation).

After treatment with filipin (50 μg ml⁻¹) for 10 min, the plasma membrane at the growing pole and adjacent regions revealed numerous filipin-induced deformations. Dividing cells had several deformations in the middle. By contrast, deformations were virtually absent from the proximal regions of the plasma membrane. After treatment with filipin (50 μg ml⁻¹) for 1 h, all of the growing poles and adjacent regions, and the middle in the dividing cells, revealed numerous deformations. The other cell pole, too, often revealed numerous deformations. By contrast, proximal regions were usually only sparsely deformed (Fig. 4). Essentially the same pattern was observed after filipin treatment at 50 μg ml⁻¹ overnight, and at 100 μg ml⁻¹ for 1 h (Figs 5, 6). In the cytoplasm, several vacuolar membranes were deformed, although proximal regions of the plasma membrane in the same cell were only sparsely deformed (Fig. 7).

Generally speaking, invaginations existed in proximal regions where filipin caused little deformation of the membrane. However, there was no clear boundary between each region. Invaginations were sometimes observed near the growing pole. In such cases, filipin deformed near these invaginations, though the invaginations themselves appeared to remain undeformed. Filipin induced numerous deformations of the plasma membrane in the middle, which was as yet unfurrowed but was devoid of invaginations (Fig. 6). This indicates the simultaneous disappearance of the invaginations and of the resistance to filipin-induced deformation, just before cytokinesis (Takeo, 1984). Both events appear to be involved in the commitment of the plasma membrane to cytokinesis.

To know whether the distribution pattern of filipin-induced deformations of the S. pombe plasma membrane faithfully reflected that of free ergosterol, cells were treated with digitonin and tomatin, cytochemical probes for free sterol (Elias et al., 1978, 1979). Both of them induced two types of crystal-like deformations, tubular structures of at least 50 nm diameter or flat structures of 100–200 nm width (Figs 8, 9), and patches characterized by the absence of the intramembrane particles from a membrane that otherwise appeared normal (Fig. 8b). Tubular or flat structures represent saponin–ergosterol complexes, since similar structures have already been reported in cholesterol-containing membranes as saponin–cholesterol complexes (Elias et al., 1978, 1979; Severs et al., 1981). The flat structures were often observed, which may reflect an unusual characteristic of the plasma membrane of S. pombe or of membranes containing ergosterol, since these structures have only occasionally been observed in membranes containing
The P-faces of the plasma membrane after treatment with filipin at 50 µg ml⁻¹ for 1 h (Fig. 4), at 100 µg ml⁻¹ for 1 h (Fig. 5), and at 50 µg ml⁻¹ overnight (Fig. 6). Essentially the same pattern of filipin-induced deformations in different regions of the plasma membrane is observed in Figs 4–6, except for the differences due to the different stages in the cell cycle, i.e. the cell was harvested during elongation in Fig. 4, in an early stage of cytokinesis in Fig. 6, and in a late stage of cytokinesis in Fig. 5. The growing pole and adjacent regions (G), and cell division-associated regions (D) are virtually devoid of the invaginations and reveal numerous filipin-induced deformations (→→). The proximal regions have several invaginations and resist filipin-induced deformation. Note the disappearance of both the invaginations and the resistance to filipin-induced deformation before furrowing (D in Fig. 6). A new cell pole (N in Fig. 4) is uninvaginated and reveals numerous filipin-induced deformations (→→).

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cholesterol (Elias et al., 1978, 1979; Severs et al., 1981). Some tubular structures were removed into the cytoplasm and the wall, as described with cholesterol–digitonin complexes (Elias et al., 1978). The removal was milder in tomatin than in digitonin complexes. The absence of the intramembrane particles in small areas (Fig. 8b) indicates a lesion, in spite of the otherwise normal appearance, since the particles were removed after fixation with glutaraldehyde.

The relative response of the different regions of the plasma membrane to the saponins differed from the pattern obtained with filipin; both digitonin and tomatin also induced mild to severe deformations in the proximal regions.
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Fig. 7. The E-face of the plasma membrane after treatment with filipin (100 μg ml⁻¹). The cells were grown for 3 d in the EMM (2) medium, put into the fresh medium, and incubated for 5 h 19 min. A vacuolar membrane (white arrow) as well as the growing pole and adjacent regions reveal numerous filipin-induced deformations (---), although the proximal regions of the plasma membrane revealed few deformations. Similar results were also obtained with the exponential phase cells.

Figs 8, 9. The P-face of the plasma membrane after treatment with digitonin (100 μg ml⁻¹) for 1 h (Fig. 8) or tomatin (200 μg ml⁻¹) overnight (Fig. 9). Three types of deformations are apparent: tubular (T), and flat (F) crystal-like structures, and patches devoid of the intramembrane particles (white arrows in Fig. 8b). Some of the tubular or flat structures also exist on the invaginations (long arrow in Fig. 8b). Some of the tubular structures have been removed from the plasma membrane to the wall (long arrows in Figs 8a, 9). In Fig. 9, deformations may be a little denser in a cell pole and adjacent regions than the other regions, but this is dubious in Fig. 8(a). Fig. 8(b) (bar marker 0.2 μm) shows a portion of Fig. 8(a) at a higher magnification.
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Owing to the following as yet unsolved complications, the question remains whether or not the ergosterol level is higher at the growing pole and the division furrow than in the proximal regions. (1) Though more severe deformations were often observed at the cell pole(s), in the division furrow, and/or adjacent regions, this was not always true. (2) Under all conditions studied, some cells were severely deformed, while in others deformation was mild. (3) Tubular structures showed a clear tendency to accumulate (Figs 8, 9). The accumulation occurred in virtually any region of the plasma membrane. Thus, the emergence of a tubular structure appeared to render the adjacent regions of the plasma membrane more susceptible to saponins. This may partially explain the wide range of deformation density among cells as above. (4) Tubular or flat deformations also occurred just on the invaginations (Figs 8, 9). Thus, the saponin treatments did not help to distinguish the growing pole, the adjacent regions and the regions involved in early stages of cytokinesis, from the static or proximal regions, whereas the invaginations and the filipin-induced deformation both correlated in characterizing these regions.

DISCUSSION

There are three possible factors which influence filipin-induced deformation: accessibility of filipin, the level of ergosterol, and constraints of the membrane, which can potentially cause a false-negative response to filipin. The wall of a yeast cell has been reported to be increasingly impermeable to the large polyene antibiotic amphotericin B with culture age (Gale et al., 1975, 1980). This also appears to be the case with filipin, a small polyene antibiotic, since this agent penetrated the cytoplasm of prefixed cells with more difficulty in stationary phase cells than in exponential phase cells (unpublished observation). At present, it is unknown whether local differences in filipin permeability exist in the wall of an exponential phase cell of S. pombe. One hypothesis to account for these observations is as follows. Owing to selective elongation at the pole (Mitchison, 1957; May, 1962; Johnson, 1965), the walls at the growing pole and adjacent regions contain newly synthesized material through which filipin may permeate; the wall covering the proximal regions of the plasma membrane is older and may be less permeable to filipin; and the wall in the middle is 'softened' before division. If so, short treatment with filipin may selectively deform regions of the plasma membrane which underlie the 'soft' wall. The deformation pattern observed after treatment with filipin for 10 min at 50 µg ml\(^{-1}\) may reflect the accessibility of filipin, i.e. the local permeability differences of the wall. However, the accessibility of filipin can not explain the characteristic deformation pattern of the plasma membrane of S. pombe, observed after treatment with filipin, at 50 µg ml\(^{-1}\) for 1 h or overnight, or at 100 µg ml\(^{-1}\) for 1 h. Under these conditions several vacuolar membranes were deformed, which indicates that filipin could gain access to any region of the plasma membrane at least by way of the cytoplasm. Thus, regional differences in filipin-induced deformations must reflect differences in the plasma membrane of S. pombe.

To distinguish true from false-negative responses to filipin (Severs & Robenek, 1983; Severs & Simons, 1983), digitonin or tomatin was applied. Owing to the complications described in Results, it remains questionable whether or not the ergosterol level is higher at the growing pole and the division furrow than in the proximal regions of the plasma membrane. Though more severe deformations were often observed at the cell pole(s) or regions near the division furrow, digitonin and tomatin also induced mild to severe deformations in the proximal regions of the plasma membrane. Filipin appears to be slightly more sensitive than, or at least as sensitive as, digitonin and tomatin in detecting low ergosterol concentrations (Elias et al., 1979). Thus, the fact that the proximal regions of the plasma membrane did not undergo filipin-induced deformations suggested a false-negative response. Alternatively, the proximal regions of the plasma membrane might be deprived of ergosterol, but during saponin treatment, some ergosterol might move to the proximal regions of the plasma membrane. Although the causes of the resistance to filipin-induced deformation in the proximal regions of the plasma membrane are unknown, these appear to be deeply involved in the control mechanism of growth of S. pombe.
Results described in the present paper strongly suggest the existence of two different states, D and S, in the plasma membrane of *S. pombe*. Regions of the plasma membrane in the D state are either dynamically changing (the growing pole and cell division-associated regions), or newly formed. Structurally, they are hardly invaginated and they are easily deformed by filipin. The plasma membrane regions in the S state are static and are found in the proximal regions. Structurally they are invaginated and they resist filipin-induced deformation.

REFERENCES


