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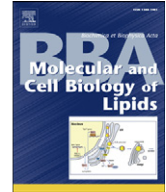

Reorganization of plasma membrane lipid domains during conidial germination

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Neurospora crassa, a filamentous fungus, in the unicellular conidial stage has ideal features to study sphingolipid (SL)-enriched domains, which are implicated in fundamental cellular processes ranging from antifungal resistance to apoptosis. Several changes in lipid metabolism and in the membrane composition of *N. crassa* occur during spore germination. However, the biophysical impact of those changes is unknown. Thus, a biophysical study of *N. crassa* plasma membrane, particularly SL-enriched domains, and their dynamics along conidial germination is prompted.

Two *N. crassa* strains, wild-type (WT) and slime, which is devoid of cell wall, were studied. Conidial growth of *N. crassa* WT from a dormancy state to an exponential phase was accompanied by membrane reorganization, namely an increase of membrane fluidity, occurring faster in a supplemented medium than in Vogel's minimal medium. Gel-like domains, likely enriched in SLs, were found in both *N. crassa* strains, but were particularly compact, rigid and abundant in the case of slime cells, even more than in budding yeast *Saccharomyces cerevisiae*. In *N. crassa*, our results suggest that the melting of SL-enriched domains occurs near growth temperature (30 °C) for WT, but at higher temperatures for slime. Regarding biophysical properties strongly affected by ergosterol, the plasma membrane of slime conidia lays in between those of *N. crassa* WT and *S. cerevisiae* cells. The differences in biophysical properties found in this work, and the relationships established between membrane lipid composition and dynamics, give new insights about the plasma membrane organization and structure of *N. crassa* strains during conidial growth.

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1. Introduction

Neurospora crassa, a haploid ascomycete, has been extensively used as a biological model for multicellular eukaryotes, namely in the study of developmental processes, apoptosis [1], aging [2], and in the molecular basis of the circadian rhythm observed in asexual spore formation, conidiation [3]. *N. crassa* cells have also been used for studies of resistance to antifungal drugs [4,5], where plasma membrane reorganization, in the presence of the antifungal drug, may have an important role [6–10]. Lipid domains are part of the current paradigm of biological membranes and their involvement in many fundamental cellular processes is currently acknowledged. However, their study in living eukaryotic cells and organisms is still a challenge [11]. Unlike in other fungi, such as *S. cerevisiae*, where the plasma membrane biophysical properties have been extensively studied [12–14], those regarding *N. crassa* remain practically unexplored [15]. Nonetheless, *N. crassa* is a highly suitable model organism to study membrane biophysical properties, namely the formation of SL-enriched membrane ordered domains. In fact, *N. crassa* plasma membrane contains both inositolphosphorylceramide-based phosphosphingolipids, such as the budding yeast *S. cerevisiae*, but also glycosphingolipids, similar to those found in pathogenic fungi and higher eukaryotes [16]. Moreover, *N. crassa* conidia display very low levels of sterols, including ergosterol [17], the major sterol in *N. crassa* mycelium and in other fungi such as *S. cerevisiae*. This is advantageous because large amounts of ergosterol induce the formation of a liquid ordered phase [18], with properties between the glycerophospholipid-enriched fluid and SL-enriched gel domains

Abbreviations: Di-4-ANEPPS, 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*- glycero-3-phosphocholine; FGSC, Fungal Genetics Stock Center; IPC, inositolphosphorylceramide; MMV, Vogel's Minimal Medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PhyCer, Phytoceramide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SC, Synthetic Complete Medium; SeM, Supplemented Vogel's Medium; SL, Sphingolipid; *t*-PnA, *trans*-parinaric acid; WT, wild-type.

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complicating the analysis of the latter [11]. Furthermore, recent results suggest that the distribution of SLs, rather than sterol, is highly heterogeneous in both yeast [12,19] and mammalian cells [20], and SLs are crucial for the formation of functional membrane domains imparting them the required biophysical features [21].

Detailed information concerning the plasma membrane organization during the germination and redifferentiation process as a *Neurospora* cell progresses from dormant conidia to a vegetative mycelia is also not available. Different reports present large variations in either the total lipid content per dry weight or in the content of major lipid classes, namely a reduction in the amount of triacylglycerols, and a net increase of membrane phospholipids found in conidia and mycelia. Also, small variations in phospholipid subclasses and/or unsaturation have been reported [17,22,23]. However, the biophysical impact of such changes has not been assessed.

Many *N. crassa* mutant cell strains present morphological changes and a quantitatively different cell wall carbohydrate composition [24,25].

Unlike the *N. crassa* WT, the wall-less mutant strain slime (FGCS 4761), when grown in liquid medium, shows variations in cell diameter while maintaining a spherical morphology. Slime and WT cells grow at comparable rates [26], being an interesting strain to address the relation between biophysical properties of the plasma membrane and morphological changes associated to the absence of cell wall.

This work aimed at answering pressing questions concerning *N. crassa* plasma membrane behavior: Are there SL-enriched domains in the membrane of *N. crassa*? How does the absence of sterols impact its biophysical properties? Is the conidial growth from a dormancy state to the exponential phase (mycelial stage) accompanied by membrane reorganization? Is this dependent on growth conditions? How different are the membrane biophysical properties in a cell wall-less strain? The present work describes the first detailed study, using fluorescence spectroscopy, of the plasma membrane biophysical properties in *N. crassa*. The plasma membrane was labeled with each of the following probes: *trans*-parinaric acid (*t*-PnA), one of the few probes known to partition preferentially into gel domains (SL-enriched), where it displays increased fluorescence quantum yield; 1,6-diphenyl-1,3,5-hexatriene (DPH), a commonly used membrane probe known to report membrane global order/fluidity through its steady-state fluorescence anisotropy [12,27,28]; 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium (di-4-ANEPPS), which is a probe particularly responsive to changes in hydration patterns and membrane dipole potential due to its electrochromic properties; additionally, its emission spectrum presents a strong blue-shift with increasing concentrations of both ergosterol and cholesterol [18]. For *t*-PnA and DPH, the chromophore is buried in the hydrophobic core of the lipid bilayer, providing direct information on acyl chain packing, whereas the more superficial location of di-4-ANEPPS makes it useful for characterizing properties at the membrane/water interfacial region.

The application of multiple membrane probes and several photophysical parameters to *N. crassa* WT and slime cell wall-less conidia, under different growth conditions, along with a comparison with *S. cerevisiae* cells, allowed a thorough characterization of *N. crassa* plasma membrane biophysical properties. This work sets the basis for further studies with *N. crassa* to deepen our understanding of the biological roles of SL-enriched ordered domains and their (re)organization either during different physiological responses or during challenges by antifungal agents and other stress situations.

2. Materials and methods

2.1. Materials

Yeast extract, bactopeptone, yeast nitrogen base, and agar were from Difco (Detroit, MI, USA).

Na_2ATP (Adenosine 5′-triphosphate disodium salt), PMSF (phenylmethylsulfonyl fluoride), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and D-Sorbitol were obtained from Sigma-Aldrich and Na_2EDTA (Disodium ethylenediaminetetraacetate) from Carlo Erba.

DPH and di-4-ANEPPS were obtained from Invitrogen (Madrid, Spain). The probe *t*-PnA was purchased from Santa Cruz Biotech. (Santa Cruz, CA.). Ludox® (colloidal silica diluted to 50% in water) was purchased from Sigma-Aldrich (St. Louis, MO). Solvents/co-solvents such as ethanol, methanol and glycerol were spectroscopic grade and purchased from Merck and Scharlau. All other reagents were of the highest purity available. All the fluorescence probes were quantified spectrophotometrically [18,27].

2.2. N. crassa and S. cerevisiae strains, growth conditions and replicates

The *N. crassa* WT strain (FGSC 2489) and the cell wall-less slime mutant (FGSC 4761) were obtained from the Fungal Genetics Stock Center [29]. Standard procedures were employed for growth and handling of *N. crassa* WT in Vogel's Minimal Medium (MMV) [30]. For *N. crassa* slime mutant, Supplemented Vogel's Medium (SeM) containing 2% mannitol (w/v), 0.75% yeast extract (w/v) and 0.75 % nutrient broth (w/v) was used.

Each biological replicate of *N. crassa* WT cells was grown in solid MMV for 7 days at 30 °C in a different culture flask, to obtain biologically independent conidial suspensions. To this end, 25 mL of sterile water were added to the culture flask which was re-caped and shaken to dislodge the conidia. The conidia obtained were then filtered through a funnel with a four-layered cheesecloth into a sterile flask. An aliquot was observed under an optical microscope and cells were counted with a hemocytometer to ensure that the spectroscopic determinations were always made with the same number of conidia per mL of suspension.

The initial procedure was slightly different for the slime strain. Independent cultures were grown in liquid SeM during 3 days at 30 °C and 150 rpm, after which an aliquot was observed and cells counted as above. Each suspension was kept in 1 mL aliquots in glycerol and stored at −80 °C, and before each experiment was activated on a 30 °C water bath during 30 min.

The *S. cerevisiae* strain used in this work was BY4741 (WT; genotype *MAT*a; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0) obtained from EUROSCARF (Frankfurt, Germany). Independent *S. cerevisiae* cultures were prepared from solid plates, as follows: yeast cells were inoculated in synthetic complete medium (SC) containing 6.8 % (w/v) yeast nitrogen base, 2% (w/v) glucose, and the amino acids as indicated in [31]. *S. cerevisiae* cells were grown overnight in SC medium at 30 °C with shaking at 160 rpm and reinoculated into fresh SC medium at ~1.23×10^6 cell/mL (absorbance at 600 nm $A_{600} = 0.042$). After incubation for 5 h, cells in exponential phase were harvested at ~6.1×10^6 cell/mL ($A_{600} = 0.209$).

Results are presented as mean ± standard deviation (S.D.) obtained from independent cultures, and statistical significance was determined using Student's *t*-test. Mean values were considered significantly different for *p* values below 0.05.

2.3. Establishing N. crassa growth profile

WT or slime *N. crassa* at ~1.5×10^6 cell/mL were grown in liquid minimal medium or SeM for 14–16 h at 30 °C. Growth profile was monitored by following the absorbance of the liquid cultures at 690 nm (A_{690}) to avoid interference of culture media and cellular components (namely, carotenes). Morphological alterations were inspected under a transmission microscope Axiovert 100 A with an Adaptor Tube Soligor® for Canon A640 - PowerShot Digital Camera, used to record the observations made with a magnification of 100×, taking 50 μL aliquots at different time points. The images shown are representative of each culture condition/strain.

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