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Abstract

Candida albicans is an opportunistic pathogen. It adheres to mammalian cells through a variety of adhesins that interact with hosts ligands. The spatial organization of these adhesins on the cellular interface is however poorly understood, mainly because of the lack of an instrument able to track single molecules on single cells. In this context, the Atomic Force Microscope (AFM) makes it possible to analyze the force signature of single proteins on single cells. The present study is dedicated to the mapping of the adhesive properties of *C. albicans* cells. We observed that the adhesins at the cell surface were organized in nanodomains composed of free or aggregated mannoproteins. This was demonstrated by the use of functionalized AFM tips and synthetic amyloid forming/disrupting peptides. This direct visualization of amyloids nanodomains will help in understanding the virulence factors of *C. albicans*.

Background

The yeast *Candida albicans* has emerged as a major public health problem these last two decades. This opportunistic pathogen causes a wide range of infections from surface infections, to mucosal and blood-stream infections[1]. Whereas mucosal infections are common and occur in healthy organisms, blood-stream infections are observed only in immunocompromised patients and are life-threatening. This type of infections, also known as candidaemia, can develop into disseminated candidiasis when the infection spreads to internal organs, leading to high mortality rates[2]. In order to colonize and subsequently to disseminate in the blood stream *C. albicans* needs to adhere to different substrates. This first stage of infection[3] is mediated by adhesins that are found on the surface of the yeast cell wall. Many of these adhesins are mannoproteins, and among them, the adhesin family identified as having a major role in host cell attachment is the Als (Agglutinin-like Sequences) family[4].

The Als were initially reported as having homologies with the proteins responsible for auto-agglutination in the baker yeast *Saccharomyces cerevisiae*. Eight Als have been identified, they all are primarily involved in host-pathogen interactions[5]. It was found that there were amyloid-forming sequences in the Als adhesins of *Candida albicans*[6]. Amyloids are insoluble fibrillar protein aggregates whose core consists in crystalline arrays of identical sequence in many molecules of the amyloid protein[7,8]. Cells expressing the Als proteins can rapidly aggregate, and the aggregation has amyloid-like properties. Like amyloid formation, aggregation ability propagates through the adherent cell population and depends on conformational changes of the Als protein. This transition of the conformational state to an aggregative state of the proteins is characterized by the formation of hydrophobic nanodomains on the entire surface of the cell[9].

A few papers written by Lipke's team were dedicated to the direct visualization of these nanodomains using fluorescent dyes such as thioflavin T or 8-anilino-1-naphtalene-sulfonic acid

(ANS)[6,8,9]. Another technique that can be used to visualize these nanodomains is Atomic Force Microscopy (AFM). AFM has recently emerged as a valuable tool to study the surface of living cells[10], and especially pathogenic cells[11]. This technology has been used by Alsteens *et al*. to image the formation and propagation of nanodomains in living yeast cells[12] and also to unfold amyloid proteins from the yeasts surface using Single Molecule Force Spectroscopy[13–15]. To this end, the authors functionalized AFM tips with antibodies targeted against the Als protein directly or against an epitope tag present in the Als protein. These studies allowed the authors to localize the adhesive nanodomains caused by the aggregation of Als proteins at the surface of living yeast cells, and to unravel the structure of the Als proteins studied by stretching.

In our study, we used AFM as an imaging tool to visualize and localize adhesins nanodomains at the surface of living wild-type *Candida albicans* cells. Using recent developments in the AFM technology, we have imaged and quantified at the same time the nanomechanical properties, the adhesiveness (force and nature of the interaction), the size and the thickness of the nanodomains[16,17], at high resolution. The data collected showed that these nanodomains are localized differently at the surface of the cell, depending on the structures featured by the cells (bud scars, buds). We also showed that there were degrees of adhesiveness, depending on whether the amyloid proteins had totally aggregated (hydrophobic nanodomains) or not, and that these degrees of aggregation were directly correlated to the stiffness of the yeast cell wall. Finally, using force measurements and amyloid forming or inhibiting peptides, we showed that Als proteins (probably among others) were participating to these nanodomains.

Methods

Yeasts growth conditions

Candida albicans (from ABC Platform® Bugs Bank, Nancy, France) was stocked at -80°C, revivified on Yeast Peptone Dextrose agar (Difco, 242720-500g) and grown in Yeast Peptone Dextrose broth (Difco, 242820-500g) for 20 hours at 30°C under static conditions.

Sample preparation for AFM experiments

Yeast cells were concentrated by centrifugation, washed two times in acetate buffer (18 mM CH_3COONa, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH = 5.2), resuspended in acetate buffer, and immobilized on polydimethylsiloxane (PDMS) stamps prepared as described by Dague *et al*[18]. Briefly, freshly oxygen activated microstructured PDMS stamps were covered by a total of 100 µL of the solution of cells and allowed to stand for 15 minutes at room temperature. The cells were then deposited into the microstructures of the stamp by convective/capillary assembly. Images were recorded in acetate buffer in Quantitative Imaging™ mode with MLCT AUWH (Bruker) cantilevers (nominal spring constant of 0.01 N/m). The applied force was kept at 1.5 nN for imaging and at 0.5 nN for force spectroscopy experiments. The loading rate for imaging was of 2 500 000 pN/s (acquisition frequency of the force curves is of 25 Hz) and for force spectroscopy of 75 000 pN/s (acquisition frequency of the force curves is of 1.25 Hz). For imaging and force spectroscopy, we used an AFM Nanowizard III (JPK Instruments, Berlin, Germany). The cantilevers spring constants were determined by the thermal noise method[19]. For all the results presented in this study, silicon nitride AFM tips were bare, expect in the case of figure 4g (lower panel), where a functionalized AFM tip has been used.

AFM tips functionnalization

The functionalized tips were produced according to a french patent of the authors described later in sensors and actuators[20]. Briefly, AFM tips were functionalized with dendrimers presenting

CHO functions able to covalently link with NH_2 functions of proteins. These dendritips were then incubated with the lectin Concanavalin A (Sigma, L7647-100MG, 100µg/mL) for 1 hour, before being used for force spectroscopy experiments.

Results analysis

All results were analyzed using the Data Processing software from JPK Instruments. The stiffness values measured on cells were determined from the slope of the linear portion of the raw deflections versus piezo displacement curves, according to:

$$kcell = k\left(\frac{s}{1-s}\right)$$

with *s* the experimentally accessible slope of the compliance region reached for sufficient loading forces. In this model, the experimental setup can be represented by two linear springs, one is the AFM's cantilever, and the other is the cell envelope exhibiting an effective spring constant. It is then possible to calculate the effective spring constant *kcell* of the cell envelope from the observed slope s of the force curve and the known spring constant k of the cantilever[21].

Results

Candida albicans cells display localized adhesiveness

Thanks to our innovative method to immobilize cells into PDMS stamps[18], and using Quantitative Imaging™ mode[16], we were able to image and quantify the adhesive properties of single *C. albicans* cells at the same time. Figure 1a shows a budding yeast cell; on the corresponding adhesion image (Figure 1b), we can see that only the bud, and not the mother-cell, presents adhesives patches. This original result is surprising as non-budding cells are highly adhesive (see below). This result seems to indicate that the mother-cell cell wall changes during

the budding process. As for the cell in figure 1c, this cell displays two bud scars, a common feature at the surface of yeast cells, which are not adhesive whereas the rest of the cell is. This type of distribution of the adhesion on yeast cells has already been seen using immunofluorescence with antibodies targeted against surface proteins of *C. albicans*. Coleman *et al*. for example showed that the Als1 protein was expressed all over *C. albicans* cells, with the exception of bud scars[22]. The comparison of our results to these data suggests then that the adhesions probed by AFM might be due to surface proteins, such as Als1 in the case of the cell presenting bud scars, but perhaps also others adhesins.

C. albicans cell wall adhesins are able to aggregate into nanodomains

As showed before[9,12], the proteins expressed at the surface of *C. albicans* cell wall are able to aggregate, and to form nanodomains. However, these nanodomains have not been yet characterized at the nanoscale, nor were imaged at high resolution. In fact, these nanodomains have specific adhesive properties that can be mapped using AFM in the Quantitative Imaging™ mode. High resolution (256 pixels²) adhesive images are presented in Figure 2. It shows adhesive nanodomains, at the surface of a living wild-type *C. albicans* cell. On the cell presented in this figure (Figure 2a), the corresponding adhesion image shows very distinct adhesive nanodomains that were probed with bare AFM tips. These nanodomains are homogeneously distributed all over the cell here, which does not present any morphological features such as buds or bud scars. When zooming into small areas on top of the cell (white squares on figure 2b), we could measure the area of each nanodomain. On this cell and on another one showed in Figure 3a, 60 nanodomains areas were measured; the values obtained plotted on figure 2e shows that nanodomains have an average area of 0.09 ± 0.03 µm². This corresponds to an average diameter of 170 nm, which confirms the nanoscale of these nanodomains. Some of the nanodomains are also higher than the

rest of the cell wall. When the whole cell is imaged, it is not visible; however, specific analysis of the Figure 3d and the graphic 3h representing the topography of the cell surface revealed nanodomains that had a different height compared to the rest of the cell. The cross-sections taken along the blue line showed the height of a nanodomain of 20 nm. Once again, this confirmed the nanoscale of the nanodomains at the surface of *C. albicans*.

Different nanodomains have different nanomechanical properties

Adhesion is measured as the rupture force recorded when retracting the tip from the surface, when approaching and pulling with the tip on the cell wall, thus AFM makes it possible to measure nanomechanical properties of living cells. Here we choose to use an analysis based on the Hooke model which considers the coupled cantilever / cell wall as a spring. The stiffness values measured on cells were determined from the slope of the linear portion of the raw deflections versus piezo displacement curves, according to:

$$kcell = k\left(\frac{s}{1-s}\right)$$

with *s* the experimentally accessible slope of the compliance region reached for sufficient loading forces. Indeed, the most interesting result in this study is the correlation that can be directly made between the adhesiveness of the nanodomain, and its stiffness. Nanodomains on figure 3e (adhesion map) circled in red were found on the stiffness image (figure 3f) circled in black; they correspond to the zones where the stiffness of the cell wall is increased, to 13.4 ± 0.3 nN/µm. As for less adhesive nanodomains, they do not present any difference in stiffness from the rest of the cell, and are 12.4 ± 0.2 nN/µm. The 3D-view of the adhesion, mapped with the stiffness (figure 3g) illustrates this clear correlation; the more adhesive the nanodomain is, the stiffer it is.

Another fascinating point is that for the more adhesive nanodomains, the retract force curves present typical hydrophobic adhesions[23–25], with adhesions occurring immediately when the tips is retracted from the surface. Force curves from the other, less adhesive, nanodomains presented retract adhesions resembling to proteins unfolding, occurring several nanometers after the tip withdrawal. Therefore it seems that the nanodomains are of 2 different natures. There is a class of nanodomains, hydrophobic, higher and stiff, and another class displaying proteins unfolding properties, as soft as the rest of the cell wall. What is the molecular nature of these 2 types of nanodomains, and are they correlated?

Understanding the adhesive properties of the 2 nanodomains classes

To answer the previous question, we monitored the retract force curves recorded on the nanodomains (Figure 4). We found the same correlation as in figure 3; the force curves recorded on an adhesive nanodomains presented hydrophobic retract adhesions, whereas the force curves recorded on a less adhesive nanodomain presented protein, unfolding like, profiles. In order to determine the nature of these last unfoldings, we probed the surface of *C. albicans* cells with an AFM tip functionalized with Concanavalin A (ConA), a protein that interacts with yeast mannoproteins, such as surface adhesins. The resulting force curves (figure 4g) showed retract adhesions displaying unfoldings of different lengths, but with a similar profile. We also observed condensed spikes with adhesion forces between 0 and 50 pN. This value was consistent with specific interactions between ConA and mannoproteins[26].

In a previous study conducted in 2009[14] by Alsteens *et al.*, adhesins (Als5) were unfolded from the surface of live *S. cerevisiae* cells overexpressing this protein. The retract force curves obtained in this study show high similarity with the ones we obtain here with functionalized AFM tips, with the presence of serin-threonin rich segments (condensed pikes on figure 4g). We can

therefore, based on this comparison with the data of the literature, conclude that the less adhesive nanodomains at the surface of live *C. albicans* are composed of free adhesins, and maybe of Als proteins. However, since all adhesins (like Als, Hwp1, Eap1, Rbt1 etc) are mannoproteins, we cannot, at this stage, make a statement on which adhesins are unfolded here.

As for the second type of nanodomains, the hydrophobic ones, our hypothesis is that they are composed of the same proteins as the less adhesive ones. In fact, adhesins (like Als) display amyloids sequences located on a domain of the protein called T, that enable them to change their conformation[6] and to aggregate into amyloid nanodomains. And when this phenomena is started, it propagates to the whole cell[9]. We therefore made the hypothesis that the adhesive nanodomains are in fact amyloid nanodomains, made of Als proteins.

From adhesins to amyloid nanodomains: the role of Als proteins

To verify this hypothesis, and according to the literature on Als proteins, we synthesized a peptide exhibiting the same sequence as the one of the T domain of the Als1/3/5 proteins. We then put this peptide in the presence of the cells in order to trigger the amyloids formation. We also synthesized the same peptide, but with a mutation on one amino acid (V326N peptide), in order to obtain a peptide that inhibits the formation of the amyloid nanodomains[8]. Since Als3 is only expressed on the surface of hyphae, we will only be able to generate or destroy the amyloid formation of Als 1 and 5. The results presented in figure 5 showed a cell before and after adding the mutated peptide. We can clearly see on these adhesion images the loss of general adhesion and of two nanodomains at the center of the cell. It seems like the mutated peptide disrupted the amyloids at the surface of the cell. And the other way around, when cells were incubated with the amyloid forming peptide, we could observe the formation of the nanodomains at the surface of the cells, as it is showed on the adhesion images on local areas on top of *C. albicans* cells in

figure 5c, d and e. These results allow us to conclude that the proteins at the origin of the nanodomains are then mannoproteins, more specifically Als1 or 5, or both, that form amyloids.

Discussion

We show in this study that wild-type live *C. albicans* cells exhibit extraordinary adhesive properties. In the case of budding cells, placed in acetate buffer at 25°C for 2h, we observed that the mother cell is not adhesive and that only the bud presents adhesive nanodomains. On the contrary, we show that non budding cells are covered by adhesive nanodomains, in the same experimental conditions. This illustrates the amazing plasticity of this species[27,28] able to grow as a commensal or as a pathogen[29,30] , in all the parts of the intestinal track, but also on the vaginal mucosa, as unicellular budding cells or as filamentous hyphae. Moreover its cell wall is permanently remodeled as a reaction to its environment (temperature, pH, dissolved O_2, ions, interacting surface/cells/bacteria) what makes it challenging to reproduce the experimental conditions inducing a certain cell wall phenotype. We then demonstrate that the molecules at the origin of these adhesions could aggregate into nanodomains, which can be probed at high resolution using a suited AFM mode, QI™. These nanodomains are different in terms of level of adhesiveness, which is a property directly correlated to their stiffness and to the hydrophobic state or not of the molecule at the origin of these nanodomains. We then went further in the study, using functionalized AFM tips, and were able to determine that the less adhesive nanodomains were formed by mannoproteins that can interact specifically with Concanavalin A. These mannoproteins are able to aggregate to form the adhesive nanodomains because they have amyloid properties as we showed in figure5.

Amyloid aggregation is a primitive[31] and very stable[32] protein folding and a common structural motif. It is a cross β-sheet quaternary structure that usually auto-aggregates as fibrils. It

248 has been, first, associated with neurodegenerative diseases like Alzheimer, Parkinson, or
249 Creutzfeldt-Jakob diseases. However it is more and more unclear if the amyloids lesions are the
250 cause or a consequence of the disease. Amyloid aggregates are now described as functional
251 proteins assembly and can be found from bacteria to humans[33]. In microorganisms, amyloid has
252 been described as a functional coat[34]. It constits in curli (*E. coli*), chaperons (*Streptomyces)* or
253 hydrophobins (*Aspergillus etc)*; all of these proteins are implicated in adhesion to the host and in
254 the invasion, infection process. It is now well known that adhesins (and especially *Als*) of *C.*
255 *albicans* have amyloid-forming sequences[6,35] and that these proteins form domains involved in
256 cell aggregation or biofilm formation[36]. Nevertheless the characterization, structure and
257 properties of the amyloids adhesive nanodomains remain unclear.

258 In this work we measured for the first time the nanoscale size of amyloid domains
259 (average area of 0.09 μm^2) at the surface of live *C. albicans* cells. The domains are of 2 different
260 classes. Some present the characteristic of individual proteins whereas the others are
261 hydrophobic, stiffer than the rest of the cell (13.4 ± 0.3 nN/µm compared to 12.4 ± 0.2 nN/µm),
262 and are slightly protruding. It means that there is a state modification from soluble proteins into
263 insoluble proteins, which is a characteristic of amyloid structures. This transformation is
264 dependent on the proteins concentration and can only occur when the protein density exceeds a
265 threshold. The roles of the two classes of domains are probably different. On one hand we could
266 hypothesize that the hydrophobic nanodomains were involved in the cell adhesion to abiotic
267 hydrophobic surfaces or to cell membrane as it is known that membrane binding is an inherent
268 property of amyloid aggregates[37,38]. Amyloid aggregation is also a way to store proteins, in a
269 limited space and to sort them when required. This has been demonstrated for hormones in
270 secretory granules[39,40]. Thus *C. albicans* may store some adhesins for the subsequent invasion
271 phases. On the other hand the protein like domains may be responsible for specific adhesion to

fibronectin and other extra cellular proteins of the matrix. It seems rational that several adhesins, brought together, would be more efficient in a binding process than a single adhesion. This finding has to be added to *C. albicans* plasticity[28] and participate to explain its remarkable adaptation and pathogenicity.

However, there are still many things to explore on the cell wall of *C. albicans*, and future work will be dedicated to exploring the changes appearing on the mother cell during the budding process.

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Figure Caption

Figure 1. Localization of the adhesive properties of *C. albicans* cells. (a) Height image (z-range = 1.5 µm) of a budding *C. albicans* cell in a PDMS stamp, and (b) adhesion image corresponding to the height image. On (a), MC stands for Mother cell, BC stands for Budding cell, and the red dotted line represents the demarcation between the two different cells. (c) Height image (z-range = 3.5 µm) of a single *C. albicans* cell exhibiting two bud scars, and (d) adhesion image corresponding to the height image.

Figure 2. Imaging of the adhesive domains of *C. albicans* cells in acetate buffer, at 25°C for 2 hours. (a) Height image (z-range = 2.5 µm) of a single *C. albicans* cell in a polydimethylsiloxane (PDMS) stamp, and (b) adhesion images corresponding to the height images. (c, d) Adhesion images of small areas on top of the cell, represented by the white squares in b. (e) distribution of the areas values of the domains in c and d.

Figure 3. Nanomechanics of the adhesive domains of *C. albicans* cells. (a) Height image (z-range = 2.5 µm) of a *C. albicans* cell in a PDMS stamp, (b) corresponding adhesion image, and (c) corresponding stiffness image. (d) Height image (z-range = 100 nm) of a small area on top of the cell, represented by the white square on (a), (e) corresponding adhesion image and (f) corresponding stiffness image. Note that the adhesive nanodomains circled in red on (e) are also

found on the stiffness image (black circles on f). (g) is a 3D-image of the adhesion mapped with the stiffness. (h) cross-section taken along the blue line on (d), and (i), distribution of the stiffness values corresponding to the yeast cell wall and the less adhesive domains (blue columns) or to the most adhesive domains (yellow columns).

Figure 4. Adhesion force curves of *C. albicans* adhesive domains. (a) Height image (z-range = 4.0 µm) of a *C. albicans* cell in a PDMS stamp. (b) Adhesion image of a small area on top of the cell, represented by the white square on (a). (c and d) representative force curves obtained on the zones indicated by the arrows on (c). (e) Height image (z-range = 2.5 µm) of a *C. albicans* cell in a PDMS stamp and (f) corresponding adhesion image recorded with a bare tip. (g) representative force curves obtained in the zone delimited by a white square on (f) with the Con A tip.

Figure 5. Imaging of the adhesive domains of *C. albicans* cells treated with Als1, 3, 5p amyloid disrupting peptide (V326N peptide) or Als1, 3, 5p amyloid forming peptide (Als peptide). (a) Adhesion image of a single *C. albicans* cell in a PDMS stamp and (b) corresponding adhesion image after adding V326N peptide. (c, d and e) Adhesion images of small areas on top of a *C. albicans* cell after adding the Als peptide.