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Review

Involvement of amyloid proteins in the formation of biofilms in the pathogenic yeast *Candida albicans*

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ABSTRACT

Candida species represent a major fungal threat for human health. Within the *Candida* genus, the yeast *Candida albicans* is the most frequently incriminated species during episodes of candidiasis or candidemia. Biofilm formation is used by *C. albicans* to produce a microbial community that is important in an infectious context. The cell wall, the most superficial cellular compartment, is of paramount importance regarding the establishment of biofilms. *C. albicans* cell wall contains proteins with amyloid properties that are necessary for biofilm formation due to their adhesion properties. This review focuses on these amyloid proteins during biofilm formation in the yeast *C. albicans*.

1. Introduction

Human fungal pathogens are frequently encountered in superficial infections of the skin and the nails. These infections affect nearly 1 billion people worldwide and do not pose a threat for patient survival [1]. However, in certain circumstances fungi can invade the bloodstream and cause fungemia [2]. Among all the fungi responsible of fungemia, the *Candida* genus is the most often incriminated [3]. Candidemia are bloodstream infections caused by *Candida* species, and 54% of them are linked to *Candida albicans* [3]. Although *C. albicans* is a normal resident of the genital and gastrointestinal tracts, it can move towards the blood compartment in immunocompromised patients or patients suffering from dysbiosis [4]. *C. albicans* is thus a tremendous burden on public health systems by being associated to high mortality rates (>40%), and high healthcare costs. Over the last decades the health situation has worsened and the number of patients suffering from candidemia has raised dramatically [5]. Misuse of antibiotics, increased resistance to antifungal drugs, unreliable diagnosis and invasive care procedures are all the reasons that account for the increase of *C. albicans*-based candidemia [6,7]. One of the major virulence traits in *C. albicans* is its ability to alternate between the yeast and the hyphae morphotypes [8]. The yeast form is more suitable for dissemination within the body via the bloodstream and adhesion to surfaces while the hyphal form is required to cross epithelial barriers and to establish biofilms [8]. Regulation of biofilm formation has been addressed in several studies; thus, the molecular mechanisms that govern biofilm formation at the transcriptional level within the nucleus are rather well understood [9]. However, the involvement of the cell wall in biofilm development is more nebulous. The purpose of this mini-review is to present the latest findings related to the cell wall during biofilm formation or maintenance, with a special focus on amyloid proteins.

2. Biofilm formation in *C. albicans*

A biofilm can be defined as a highly structured three-dimensional community of microbial cells that are adhered to biotic or abiotic surfaces and encased in an extracellular matrix [10,11]. Biofilms are of tremendous importance for *C. albicans* pathogenesis because, in addition to their adhesion properties, they decrease the efficiency of antifungal drugs as well as the recognition by the immune system [10,12]. In biofilms, *C. albicans* can be found either alone as a monomicrobial structure or associated to bacteria (e.g. *Staphylococcus aureus, Streptococcus gordonii, Pseudomonas aeruginosa*) in a polymicrobial community [10,13]. Biofilm formation in *C. albicans* consists of four successive phases, namely adherence, initiation, maturation and dispersion [14]. Adherence is

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the step during which round yeast cells stick to a surface, creating a basal layer whose physiological function is to anchor the biofilm to its substrate [11,14]. Then, the initiation step consists of cell proliferation and the emergence of pseudo-hyphae and hyphae from adherent cells, a process called filamentation. In the maturation phase, *C. albicans* cells continue to proliferate and to elongate to constitute an intricate and thick network of yeast cells, pseudo-hyphae and hyphae. In addition, this phase is characterized by the synthesis of an abundant extracellular matrix composed of carbohydrates, lipids and nucleic acids [11,14,15]. This extracellular matrix will encase the fungal cells and provide a physical barrier that protects the biofilm from physical insults from the environment. Lastly in the dispersion phase yeast cells leave the biofilm to colonize secondary infection sites in the human body [11,14]. The establishment of a biofilm is a complex biological process used by *C. albicans* to adapt to physical and chemical changes in its environment. As *C. albicans* is a normal resident of the human body, the environmental cues that trigger biofilm formation are linked to human physiology. Indeed, increasing temperature, presence of bacterial muramyl peptide or of N-acetyl glucosamine, CO_2 levels, nutrient starvation and surface contact are stimuli that are recognized by the sensing systems of *C. albicans* to initiate hyphal differentiation [16]. To date, numerous signaling pathways have been described in the literature. Among them, the cAMP-PKA and the mitogen-activated protein kinase (MAPK) are the 2 major signaling pathways that link the extracellular medium and the transcriptional response orchestrated by the yeast to trigger the filamentation [16–18]. Regarding the formation of biofilm in *C. albicans*, numerous transcriptional regulators have been linked to this biological process in the last decade [19]. Actually, genetic screens performed on a collection of *C. albicans* single deletion mutants identified a "core" of nine master regulators which are required to put in place biofilms in *in vitro* (polystyrene plates, silicone squares) and *in vivo* (rat catheter, rat denture) models [9,19]. The transcription factors Efg1, Bcr1, Ndt80, Tec1, Rfx2, Gal4, Flo8, Brg1 and Rob1 constitute this "core" of master regulators and they coordinate the expression of approximately 1000 genes [9,19]. The majority of the genes that belong to this transcriptional network still lack a molecular function, but some of them are correlated to biological processes required for biofilm formation such as lipid metabolism (*EHT1*), regulation of filamentation (*ORF19.4000*) or cell adhesion (*ALS1*, *HWP1*) [19,20]. Alongside the nine "core" transcription factors required for biofilm formation, other relevant regulators have been identified. The transcription factor Ume6, a downstream target of Efg1, can enhance biofilm formation by activating the transcription of Hgc1 (control of hyphal development) and Sun41 (cell wall integrity) [21]. Strikingly, numerous genes that are regulated by this network encode proteins found in the cell wall. Hence, the cell wall compartment is extremely important by allowing *C. albicans* to assemble in the form of a biofilm.

3. The cell wall of *C. albicans*

As a member of the fungi taxon, *C. albicans* owns a specialized cellular compartment called the cell wall. Because this organelle is the first point of contact between *C. albicans* and the host, it is crucial for fungus–host interactions [22]. Furthermore, the cell wall is essential for *C. albicans* development by assuming physiological roles such as morphogenesis, adherence, biofilm formation, immune system evasion, turgor, cell shape, cell cycle and resistance to physical insults and chemicals [23,24]. *C. albicans* cell wall exhibits a complex molecular architecture in the form of a multilayer structure. The innermost layer, which is in direct contact with the plasma membrane, is rich in chitin molecules and covered by a molecular assembly of β-(1,3)- and β-(1,6)-glucans. Molecules of chitin and β-glucans interact with each other through hydrogen bonds to create an intertwined fiber network called the inner cell wall [25]. The outer cell wall corresponds to the second layer of this cellular compartment and it consists exclusively of highly mannosylated glycoproteins [25]. These glycosylated proteins belong to several classes of proteins such as adhesins (adhesion), yapsins (protease activity for cell wall remodeling), hydrolases and deacetylases (cell wall plasticity) [25]. The cell wall is a highly dynamic organelle whose architecture is reshaped during the transition between yeast and hyphae morphotypes and according to variation of the environment [26,27]. Indeed, FITR spectroscopy experiments have revealed that *C. albicans* hyphae exhibits 10% more β-1,3-glucans and 20% less β-1,6-glucans than yeast-shaped cells [28], and their structure also varies in hyphae as compared to yeast cells [29]. Under the hyphal form, β-glucans adopt a cyclical structure where the β-1,3-linked polymer backbone is decorated with β-1,6-linked side chains [30]. The molecular mechanisms underlying the cell wall remodeling during the transition from the yeast form to the hyphal form are still largely unknown. However, the β-1,3-glucanosyltransferase Phr1 and the β-glucanase Mp65 are increasingly being thought to reshape the cell wall in response to environmental cues that trigger the filamentation process [31–33]. The glycoprotein pattern of the outer layer of the cell wall is also modified when cells trigger the hyphal growth to form biofilms. The reorganization of cell surface proteins is essential to sustain biological functions required for biofilm development such as immune system escape or adhesion [34]. Furthermore, adhesins from the Als family or the adhesin Eap1 are identified at the surface of *C. albicans* grown under the hyphal mode [35]. One striking feature of these adhesins is that they display amyloid properties [36].

4. Relevant amyloid proteins used by *C. albicans* to develop biofilm

Amyloids represent structured protein aggregates that are found in all kingdoms of life. More specifically, amyloids adopt an unbranched and elongated high molecular weight fibrillar form [37]. At the structural level, amyloids adopt a quaternary folding formed by the assembly of multiple copies from the same protein [38]. Each of the monomers is organized perpendicularly to the fibril axis by constituting a cross β-type molecular architecture [39,40]. For detailed description of the amyloid fibril structure see reviews from Eisenberg and Sawaya as well as Close et al. [39,40]. Aggregates formed by amyloid proteins have long been considered harmful for biological systems [41]. However, the concept of functional amyloid, where aggregated proteins play biological roles, has recently emerged [42]. A well described example in the literature concerns the protein Cdc19 from *S. cerevisiae*. In the presence of glucose, the pyruvate kinase Cdc19 is located in the cytoplasm to ensure the last enzymatic reaction of glycolysis [43]. Conversely, following glucose starvation, Cdc19 self assembles under the form of an amyloid-like aggregate before being sent within the P-bodies [43]. This intracellular traffic of Cdc19 avoids its degradation under stressful conditions and will improve the fitness of the cells during the following recovery phase [43]. *C. albicans* proteins with amyloid properties have also been described in the literature. The majority of these polypeptides are classified into the *ALS*-type family of adhesins [28,44]. All the Als proteins share a common organization [45]; indeed, they all own a N-terminal Ig-like domain, which gives them adhesive properties during attachment to surfaces (Fig. 1). Beside the Ig-like domain, the Thr-rich T domain holds the amyloid forming sequence that is required to trigger the assembly of the amyloid structures within the cell wall under specific conditions (Fig. 1). In the middle part of the proteins, the TR domain is

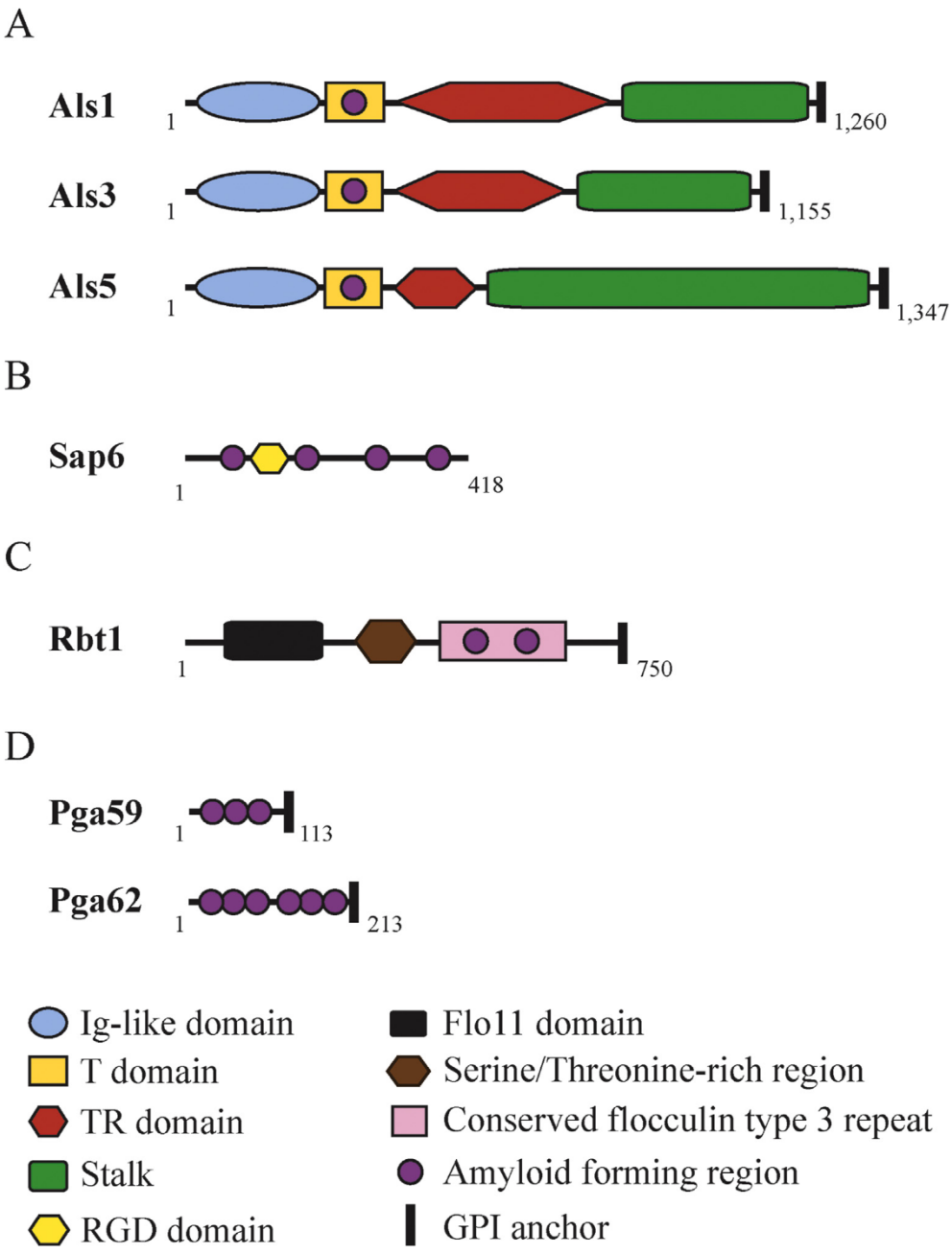

Fig. 1. Topological comparison of cell surface amyloid proteins in *C. albicans*. *A*, Typical domains of the Als proteins family: Ig-like domain (blue), T domain (orange), TR domain (red), Stalk (green) and GPI anchor (black). Amyloid-forming regions are represented as purple circles. *B*, the secreted aspartic protease Sap6 is shown with its four amyloid-forming regions (purple circles) and the integrin-binding (RGD) domain (yellow). *C*, Topological organization of the protein Rbt1. The Flo11 conserved domain is shown in black while the serine/threonine-rich region is colored in brown. The pink colored rectangle contains 2 amyloid-forming regions (purple circles) and corresponds to the conserved flocculin type 3 repeat. The GPI anchor is represented by a vertical black line. *D*, both Pga59 and Pga62 are represented with their respective amyloid-forming regions (purple circles) and GPI anchors (black lines). Protein length is indicated with numbers of amino acid residues under the sketch of each proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

characterized by the presence of tandem repeat sequences that allow hydrophobic interactions between adhesins on the one hand and surfaces on the other hand (Fig. 1). At their C-terminal domain, Als proteins have a highly glycosylated and Ser-Thr-rich region called the stalk, followed by a GPI anchor that, upon maturation, serves to link adhesins to the cell wall glucans through glycosidic bonds (Fig. 1). Among the Als proteins, only Als1, Als3 and Als5 have been shown to display adhesion activity [28,44]. When Als1, Als3 or Als5 adhesins are subjected to shear stresses, like those encountered in the blood flow within the human body, they have the ability to organize themselves into nanodomains at the cell surface [27,46–49]. Consequently, Als1, Als3 or Als5 are self-aggregated through the amyloid forming sequence supplied by each of the monomers. Thus, the amyloid structures are crafted by the assembly of adhesins to constitute the nanodomains, which will allow the creation of hydrophobic patches in specific area of the cell wall and promote cell adhesion on biotic or abiotic surfaces [27,47]. It is suggested that the adhesive properties of nanodomains

are required to form biofilms, because the lack of either Als1, Als3 or Als5 greatly reduced the ability of *C. albicans* to develop biofilm. In addition to adhesins, the secreted aspartic peptidase Sap6 is also suspected to have a function in the establishment of *C. albicans* biofilm [50]. Under its globular form, Sap6 displays a protease activity required for the virulence of the fungus. However, like Als proteins, Sap6 contains amyloid-forming sequences in its primary amino acids structure [50] (Fig. 1). It is thus suggested that Sap6 could adopt an amyloid shape that favors cell−cell adhesion during biofilm formation [50]. Rbt1 is another protein of *C. albicans* that contains amyloid forming sequences [51] (Fig. 1). In addition to the Flo11 domain (homotypic interactions) and the serine/threonine-rich region, Rbt1 owns the conserved flocculin type 3 repeat that includes 2 amyloid-forming regions with high β-aggregation potentials [51] (Fig. 1). Physiologically, Rbt1 is located within the cell wall of *C. albicans* and positively affects the formation of biofilm. Indeed, overexpression of the *RBT1* gene results in an increased biofilm biomass as compared to a strain that does not overexpress it. The results that emerge from this work on Rbt1 suggest that 2 amyloid-forming sequences with high β-aggregation potential are involved in cell−cell interactions by fostering hyphae aggregation. In addition to the last-mentioned proteins, Eap1 is a cell surface protein found in *C. albicans* that is able to self-aggregate and form an amyloid fiber *in vitro* [37]. Eap1 is an adhesin involved in the adhesion process of *C. albicans* on polystyrene as well as on epithelial cells [52]. However, to date, there is no evidence that Eap1 could form patches at the cell surface as described for the Als proteins [53]. Hence, the involvement of Eap1 as an amyloid in *C. albicans* biofilm formation is purely speculative. Except for Als proteins, Sap6, Rbt1 and Eap1, knowledge on the involvement of amyloid proteins during biofilm development is scarce. However, other cell wall-associated proteins, namely Pga59 and Pga62, exhibit interesting features and might also be involved in biofilm formation as amyloids. First, Pga59 and Pga62 are small proteins that contain 3 and 6 predicted amyloid-forming sequences, respectively (Fig. 1). The presence of these sequences strongly suggests that both Pga59 and Pga62 are able to organize themselves in the form of amyloid structures. Second, loss of *PGA59* and/or *PGA62* has a significant impact on cell wall integrity in *C. albicans*. Indeed, their concomitant inactivation results in an increased sensitivity to cell wall-perturbating agents [54]. Further analyses using mass spectrometry and electron microscopy also revealed that the absence of both Pga59 and Pga62 disturbs the cell wall composition and its architecture [10,54]. Thirdly, overexpression of *PGA59* has a positive impact on biofilm formation and cell adhesion [10]. Altogether, these results led us to assume that Pga59 and Pga62 could be functional amyloids in *C. albicans*. More precisely, the globular form of Pga59 would be involved in cell wall integrity and architecture, whereas the suspected amyloid form of Pga59 would contribute to a yet unknown adhesion mechanism during biofilm establishment. Overall, the role of amyloid proteins in *C. albicans* biofilm formation is increasingly evident. Indeed, adhesive properties of cell wall amyloid proteins (either through the Als nanodomains and/or by the action of Sap6 and Rbt1) are used to build up *C. albicans* biofilms. However, how amyloid proteins are used by *C. albicans* to develop biofilms is still poorly understood at the molecular level. The study of proteins such as members of the Als protein family, Sap6, as well as Pga59 and Pga62 if they prove to be genuine amyloid proteins, will allow us to deepen the knowledge linking amyloid proteins and biofilm formation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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