RESEARCH ARTICLE

The impact of protein glycosylation on Flo11-dependent adherence in *Saccharomyces cerevisiae*

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Abstract

Fungal cell adhesion molecules are critical for the attachment of cells to each other and to surfaces and in pathogens contribute to virulence. Fungal adhesins are typically heavily glycosylated. The impact of protein glycosylation on the function and regulation of adhesion glycoproteins is not clear. We examined the role of protein glycosylation on the adherence properties of the major adhesion molecule Muc1/Flo11 in the budding yeast *Saccharomyces cerevisiae*. Using a conditional mutant required for an early step in protein glycosylation, *pmi40-101*, we show that the glycosylation of Flo11 is required for invasive growth and biofilm/mat formation. Underglycosylated Flo11 was not defective in cell-surface localization or binding to wild-type cells *in trans*. However, wild-type Flo11 was defective for binding to the surface of cells undergoing a glycosylation stress. Shed Flo11 and other shed glycoproteins (Msb2 and Hkr1) were extremely stable with half-lives on the order of days. The glycosylation of Flo11 contributed to its stability. Moreover, the overall balance between Flo11 production, shedding, and turnover favored accumulation of the shed protein over time. Our findings may be applicable to fungal adhesion molecules in other species including pathogens.

Introduction

Many fungal species undergo specific behaviors in response to environmental inputs, including growth in mats/biofilms of interconnected cells and cell differentiation to a filamentous/hyphal growth mode. In fungal pathogens, biofilm formation is an underlying cause for virulence and a major problem in patients whose immune system has been compromised (Li *et al.*, 2007). Infections caused by the opportunistic pathogen *Candida albicans* often begin with colonization of implanted medical devices (Douglas, 2003; Mukherjee *et al.*, 2005). Life-threatening complications may result from dispersal of planktonic cells from mats to cause systemic candidiasis. Biofilms can slow the penetration of drugs, which makes treatment of fungal infections challenging (Douglas, 2003). *Candida albicans* and other fungal pathogens also undergo filamentous growth, in which cells differentiate into tube-like structures, which also contributes to virulence (Lo *et al.*, 1997).

Cell adhesion molecules regulate biofilm formation and filamentous growth in fungal species (Lambrechts *et al.*, 1996; Lo & Dranginis, 1998; Guo *et al.*, 2000; Reynolds & Fink, 2001; Finkel & Mitchell, 2011). In *C. albicans*, the adhesion molecules Als1, Als2, Als4, Als5 (Ala1), Hwp1, and Eap1 regulate attachment to biotic and abiotic surfaces (Gaur & Klotz, 1997; Staab *et al.*, 1999; Fu *et al.*, 2002; Zhao *et al.*, 2005; Li *et al.*, 2007). These proteins associate with the cell wall, typically through a C-terminal glycosylphosphatidylinositol (GPI) anchor. Fungal adhesins are often heavily glycosylated as a result of posttranslational modification by the addition of oligosaccharides (Lehle *et al.*, 2006; Lommel & Strahl, 2009). Together with other glycoproteins in the cell wall, fungal adhesins can contribute to the regulation of cell wall integrity, cell-surface variegation, and host cell recognition (Leach & Brown, 2011).

In fungal pathogens, defects in protein glycosylation can compromise virulence. Fungal O- and N-linked glycoproteins promote binding to host cells and protect the fungal cell wall from exposure to the host's immune system (Netea *et al.*, 2006; Gow *et al.*, 2007). Mutations in genes encoding mannosyltransferases (*PMT1, PMT2, PMT4, PMT5,* and *PMT6*; Timpel *et al.*, 1998, 2000; Prill

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et al., 2005; Rouabhia *et al.*, 2005) and Golgi mannosyltransferases (*MNT1, MMT2, and MNN5*; Buurman *et al.*, 1998; Munro *et al.*, 2005; Bai *et al.*, 2006) have been shown to result in decreased virulence. Cells lacking *MNT1, MNT2,* and *MNS1* further show an enhanced rate of phagocytosis by macrophages (McKenzie *et al.*, 2010). However, the specific impact of protein glycosylation on the regulation or the properties of cell-surface glycoproteins has been relatively unexplored.

The *Saccharomyces cerevisiae* flocculin Flo11/Muc1 is homologous to adhesion molecules in pathogenic fungi (Nobile & Mitchell, 2006) and serves as a model adhesin for studies on fungal adhesion regulation (Lambrechts *et al.*, 1996; Lo & Dranginis, 1996; Bruckner & Mosch, 2011). Flo11 is the major adhesion molecule that regulates biofilm/mat formation and filamentous growth in yeast (Lambrechts *et al.*, 1996; Lo & Dranginis, 1998; Guo *et al.*, 2000; Reynolds & Fink, 2001). There are several members of the *FLO* gene family (*FLO1, FLO5, FLO8, FLO9, FLO10,* and *FLO11*). Each *FLO* gene encodes a protein with distinctive adherence properties, although most *FLO* genes are not expressed owing to transcriptional silencing at subtelomeric loci (Guo *et al.*, 2000). Among the Flo proteins, Flo11 specifically regulates cell–cell adhesion (Guo *et al.*, 2000). The *FLO11* gene is regulated by a large and complex promoter (Rupp *et al.*, 1999), and changes in the expression of *FLO11* create variation in the cell surface and a population of cells with dynamic adherence properties (Halme *et al.*, 2004; Bumgarner *et al.*, 2009; Octavio *et al.*, 2009). Like other fungal adhesion molecules, Flo11 contains mucin-like Ser-/Thr-/Pro-rich repeats that are thought to be heavily glycosylated and a C-terminal GPI anchor (Lambrechts *et al.*, 1996; Lo & Dranginis, 1996; Douglas *et al.*, 2007). Variation in the tandem repeats can lead to dramatic differences in adherence (Verstrepen *et al.*, 2005; Fidalgo *et al.*, 2006). Furthermore, the large glycosylated extracellular portion of Flo11 can be shed from cells, which further modulates cell adherence (Karunanithi *et al.*, 2010). Trafficking of Flo11 by way of the Vps proteins also contributes to its overall regulation (Sarode *et al.*, 2011). Recent studies have shown that Flo11 and other adhesion molecules contain β-amyloid forming sequences that may aid in protein aggregation and adherence (Otoo *et al.*, 2008; Ramsook *et al.*, 2010).

In this study, we examined the relationship between protein glycosylation, filamentous growth, and Flo11 protein regulation in *S. cerevisiae*. This idea was prompted by a correlation between Flo11 shedding/levels and protein glycosylation that was previously uncovered in a genome-wide screen (Karunanithi *et al.*, 2010). We sought to determine whether the glycosylation of Flo11 impacts its adhesion function. A panel of mutants that lacked protein glycosylation enzymes showed altered filamentous growth. The defects in filamentous growth that accompanied the glycosylation defects were in some cases associated with a decrease in Flo11 stability or the inability of Flo11 to bind strongly to an underglycosylated yeast cell surface. We also examined the relationship between protein glycosylation and the stability of the shed glycoproteins. Our findings validate the idea that protein glycosylation is important for fungal foraging and provide connections between protein glycosylation and the properties of fungal adhesion molecules.

Materials and methods

Microbiological assays

The disruption of genes that regulate protein glycosylation was performed in strain PC2043 (Karunanithi *et al.*, 2010), a ∑1278b background derivative, which contains an integrated and functional version of Flo11-HA (Table 1). Gene disruptions were made by PCR amplification of cassettes that contain auxotrophic markers (Longtine *et al.*, 1998) or antibiotic resistant markers (Goldstein & McCusker, 1999). To move the *pmi40-101* allele into PC2043, we first constructed a strain that contained the *NAT* resistance gene integrated 100 bps downstream of the *pmi40-101* allele (PC1032). Specifically, strain PC244 (Cullen *et al.*, 2000) was transformed with a PCR product containing the *NAT* resistance cassette (Goldstein & McCusker, 1999) flanked by sequences to direct integration downstream of the *PMI40* locus. Chromosomal DNA was prepared from strain PC1032 to use as a template to amplify *pmi40-101::NAT* to direct integration in PC2043. The resulting strain *pmi40::NAT* Flo11-HA was designated PC5014. Gene disruptions/mutations were confirmed by PCR analysis. The plate-washing assay was performed as described (Roberts & Fink, 1994). Mat formation was performed as described (Reynolds & Fink, 2001).

Immunoblot analysis

Immunoblots were performed as described (Cullen *et al.*, 2004). To detect shed Flo11-HA, cells were grown in YEPD liquid media for 16 h. Cells were separated by centrifugation, and supernatant and pellet fractions were examined by SDS-PAGE followed by immunoblot analysis. Epitope-tagged Flo11-HA from cell pellets was separated by SDS-PAGE (6% gel). Proteins were transferred to nitrocellulose membranes (protan BA85, VWR International Inc., Bridgeport, NJ) that were incubated in 10 mL of blocking solution made of 5% nonfat dry milk and TBST (10 mM Tris–HCl pH8, 150 mM NaCl

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and 0.05% Tween 20) for 1 h at 25 °C, followed by incubation with primary anti-HA antibodies (HA, Roche, 12CA5, #11583816001). Membranes were washed five times for 5 min each with TBST and incubated with horseradish peroxidase conjugated secondary antibodies (Bio-Rad, 170-6516) for 50 min at room temperature, then washed with TBST as previously described. ECL Plus Immunoblotting reagent (GE Lifesciences) was used for detection of secondary antibodies. Colony immunoblot analysis was performed as described (Karunanithi *et al.*, 2010).

Fluorescence microscopy

Immnofluorescence was performed as described (Guo *et al.*, 2000). Cells were isolated from mats grown on YEPD + 0.3% agar using a toothpick and fixed with 3.7% formaldehyde for 1 h. Blocking with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was performed for 1 h at 30 °C. After blocking, cells were collected and resuspended in PBS containing 2% BSA and monoclonal mouse anti-HA antibodies (1 : 1000; HA, Roche, 12CA5, #11583816001) for 1 h. Cells were washed twice with PBS + 2% BSA and incubated with PBS containing 2% BSA and Cy3-conjugated goat anti-mouse antibody (1 : 1000; Jackson Immunoresearch) for 1 h at 30 °C. Cells were then washed twice with PBS containing 2% BSA. For visualization by fluorescence microscopy, differential-interference-contrast and fluorescence microscopy using rhodamine filter sets were used in an axioplan 2 fluorescent microscope (Zeiss) with 5×, 10×, 20×, and 40× objectives, and a plan-apochromat 100×/1.4 (oil) objective (n.a. 0.17). Digital images were obtained with the Axiocam mrm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image analysis.

In vitro binding assay

In vitro binding of shed Flo11-HA was performed as described (Karunanithi *et al.*, 2010). Equal volumes of conditioned media from Flo11-HA tagged cells from wild-type (PC2043) or *pmi40-101* mutant cells were incubated with equal volumes of wild-type cells (PC538), or *pmi40-101* mutant cells grown with and without 50 mM mannose for 2 h at 30 °C. Cells were centrifuged, and the supernatant was used as the 'Flow-through' fraction. Pelleted cells were washed with water twice and then with buffer (50 mM Tris pH 9, 10 mM DTT) to remove unbound proteins. Fifty microlitres of conditioned media and eluates were spotted onto a nitrocellulose membrane followed by immunoblotting to detect Flo11-HA.

Stability assay for shed glycoproteins

To determine the stability of shed mucin glycoproteins, strains expressing Flo11-HA (PC2043), Msb2-HA (PC999) and Hkr1-HA (PC2740) were grown in 10 mL of YEPD medium to an OD of 4.0. Supernatants were prepared by centrifugation to remove cells. Supernatants were filter sterilized and maintained at 25 °C for the indicated times. Aliquots were removed at 3, 4, 5, and 6 days and stored at −80 °C. Extracts were spotted onto nitrocellulose membranes and proteins were detected by immunoblot analysis. To measure the turnover of mucins in cultures in which cells were present, the experiment was performed as mentioned above using additional sets of 10-mL cultures that were maintained at 25 °C.

Data analysis

To determine band and spot intensity, the ImageJ program (http://rsbweb.nih.gov/ij/) was used. Images were inverted, and background levels subtracted. Gene annotation and functional information was obtained using *Saccharomyces Genome Database* (http://www.yeastgenome.org/).

Results and discussion

A panel of glycosylation mutants shows a spectrum of phenotypes in invasive growth and Flo11 shedding

To examine the relationship between protein glycosylation and nutritional scavenging responses in yeast, nonessential genes that regulate protein glycosylation were disrupted in strains of the filamentous (∑1278b) background. Mutants lacking *ALG8* or *ALG9*, which encode glycosyl transferases involved in N-linked protein glycosylation (Runge & Robbins, 1986; Oriol *et al.*, 2002), or *VAN1*, a mannan polymerase (Jungmann & Munro, 1998), caused defects in invasive growth (Fig. 1a). A partial loss-of-function allele of *PMI40* also caused an invasive growth defect (see below). Deletion of *MNN10*, which encodes a Golgi mannosyltransferase (Ballou *et al.*, 1989; Hernandez *et al.*, 1989), caused a subtle defect in invasive growth (Fig. 1a). Deletion of *MNN11* did not cause an invasive growth defect (Fig. 1a). Loss of *PMT4*, a mannosyltranferase involved in O-linked glycosylation (Immervoll *et al.*, 1995), caused hyperinvasive growth (Fig. 1a). This phenotype might result from hyperactivation of the filamentous growth pathway (Yang *et al.*, 2009). Thus, defects in protein glycosylation cause a range of phenotypes, including reduced invasive growth in some mutants.

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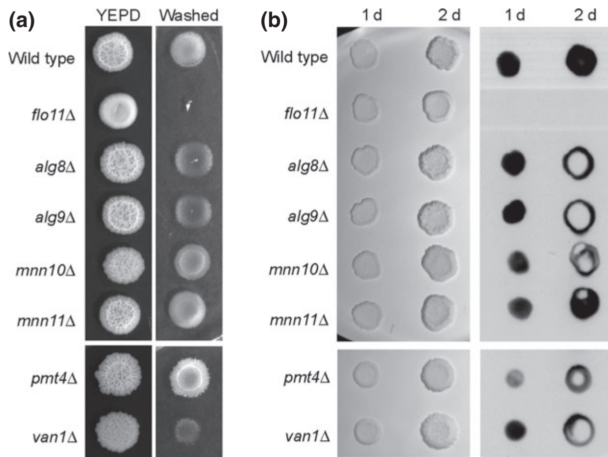

Fig. 1. Invasive growth and Flo11 shedding in protein glycosylation mutants. (a) To evaluate invasive growth, 5 μL of overnight cultures of wild-type (PC2043), *flo11Δ* (PC1029), *alg8Δ* (PC5396), *alg9Δ* (PC5399), *mnn10Δ* (PC5400), *mnn11Δ* (PC5401), *pmt4Δ* (PC5403) and *van1Δ* (PC5405) cells were grown in liquid YEPD and spotted onto YEPD agar plates. Colonies were grown for 2 days, and plates were photographed, washed in a stream of water to reveal the extent of agar invasion, and photographed again. (b) To evaluate Flo11-HA shedding, 5 μL of overnight cultures (same strains as in a) were spotted onto YEPD agar plates overlaid with a nitrocellulose membrane. Colonies were grown for 1 and 2 days, photographed (middle two panels), and rinsed off the nitrocellulose with water. Shed Flo11-HA was detected by immunoblot analysis (right two panels). The experiments were performed in duplicate and representative images are shown.

To further examine the relationship between filamentous growth and protein glycosylation, the properties of the major yeast flocculin, Flo11, were examined. Colony immunoblot analysis showed that shed Flo11 was present at lower levels in several glycosylation mutants (Fig. 1b). This was true for the *mnn11* mutant for the 1-day time period, the *pmt4* and *mnn10* mutants for the 1- and 2-days time periods, and for the *van1, alg8*, and *alg9* mutants for the 2-day time period (Fig. 1b). The reduced levels of shed Flo11 correlated with an invasive growth defect for the *alg8, alg9, mnn10,* and *van1* mutants. Particularly interesting was that for the *alg8, alg9, mnn10*, and *van1* mutants, shed Flo11 present at day 1 was reduced by day 2 (Fig. 1b), which suggests that underglycosylated Flo11 may be turned over more rapidly in these mutants than in wild-type cells. Based on these findings, we further explored the relationship between the glycosylation of Flo11 and its adherence function.

Flo11 is defective for binding to cells undergoing glycosylation stress

We focused on a mutant defective for an early step in protein glycosylation, *pmi40-101* (Cullen *et al.*, 2000), which is partially compromised for phosphomannose isomerase activity (Smith *et al.*, 1992). The *pmi40-101* mutant has a conditional glycosylation defect, which can be rescued by growth in media containing mannose (Smith *et al.*, 1992). The *pmi40-101* allele was introduced into the ∑1278b background and that mutant showed an agar invasion defect (Fig. 2a). Specifically, the *pmi40-101* mutant had a mild invasive growth defect in YEPD + Mannose and a severe defect YEPD – Mannose (Fig. 2a). The *pmi40-101* mutant was also defective for biofilm/mat formation (Fig. 2b). In medium lacking mannose, the *pmi40-101* mutant had a smooth mat morphology, and the mat failed to expand to the same degree as wild-type mats or *pmi40-101* mats grown in YEPD + Mannose (Fig. 2b). Therefore, like seen previously for several glycosylation mutants, the *pmi40-101* mutant showed a defect in filamentous growth.

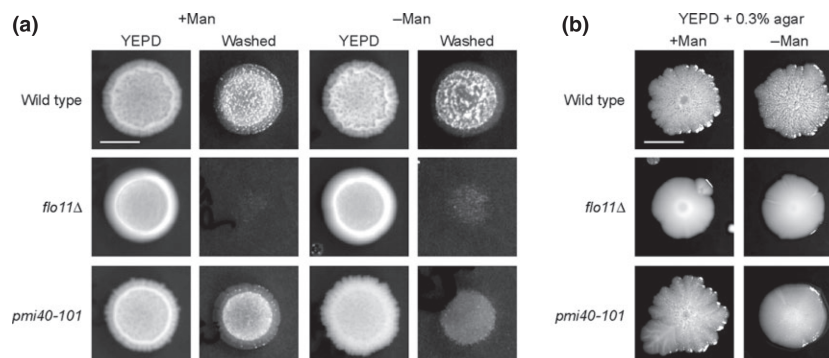

Fig. 2. Effect of the *pmi40-101* mutation on invasive growth and mat formation. (a) Plate-washing assay. Five microlitres of overnight cultures of wild-type (PC2043), *flo11Δ* (PC1029) and *pmi40-101* (PC5014) cells grown in YEPD media were spotted onto YEPD agar plates with (left) or without (right) 50 mM mannose. Colonies were grown for 3 days and washed off plates with water to reveal invaded cells. (b) Mat expansion. Three microlitres of overnight cultures were spotted onto YEPD plates (0.3% agar) with (left) or without (right) 50 mM mannose. The mats were grown for 4 days and photographed. The experiments were performed in duplicate and representative images are shown.

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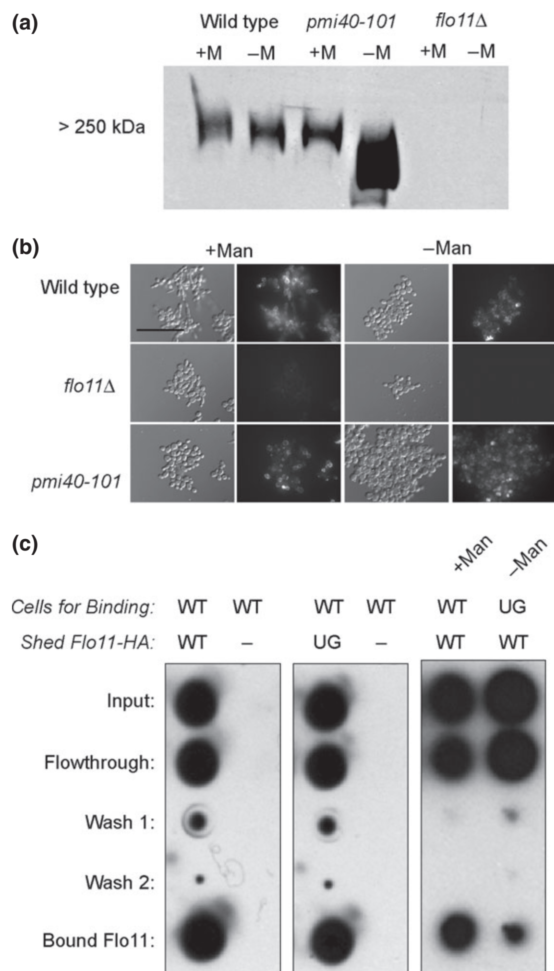

Fig. 3. Analysis of the Flo11 protein in a *pmi40-101* mutant, and binding of shed Flo11 to cells with a glycosylation defect. (a) SDS-PAGE analysis. Wild-type (PC2043), *pmi40-101* (PC5014), and *flo11*Δ (PC1029) cells were grown for 16 h in YEPD medium with and without 50 mM mannose. Flo11-HA was detected by immunoblot. (b) Immunofluorescence analysis. Wild-type (PC2043), *flo11*Δ (PC1029) and *pmi40-101* (PC5014) cells were grown on 0.3% agar in YEPD media with (left) or without (right) 50 mM mannose for 2 days. Flo11-HA was detected by immunofluoroscence with Cy3-conjugated antibodies. Bar, 40 μm. (c) *In vitro* binding assay. Left and middle panels, Wild-type cells (PC538, *Cells for binding:* WT) were incubated in conditioned media from overnight cultures expressing Wild-type Flo11-HA (PC2043, *Shed Flo11:* WT), no Flo11 (PC1029, *Shed Flo11-HA:* –), or Flo11-HA shed from the *pmi40-101* strain (PC5014, *Shed Flo11-HA:* UG). Far right panel, *pmi40-101* cells (PC244) grown in 50 mM mannose (+Man, *Cells for Binding:* WT) or media lacking mannose (–Man, *Cells for Binding:* UG) were incubated with conditioned media prepared from wild-type cultures expressing Flo11-HA (PC2043, *Shed Flo11-HA*: WT). 'Input' represents starting amount of Flo11-HA; 'Flowthrough', the supernatant after centrifugation of the incubated cells; 'Wash 1', 'Wash 2' and 'Eluted' are supernatant fractions after cells were washed with water or elution buffer (50 mM Tris pH 9, 10 mM DTT). Flo11-HA was detected by immunoblot. Experiments were performed in duplicate and representative images are shown. Quantitation by ImageJ showed a > 3.3-fold difference in elution for the far right panel.

In principle, protein glycosylation might influence Flo11's adherence function in several different ways: (1) the glycosylation deficiency might result in a change in the sugar residues on the cell surface, which act as points of attachment for lectin-like adhesins; (2) glycosylation

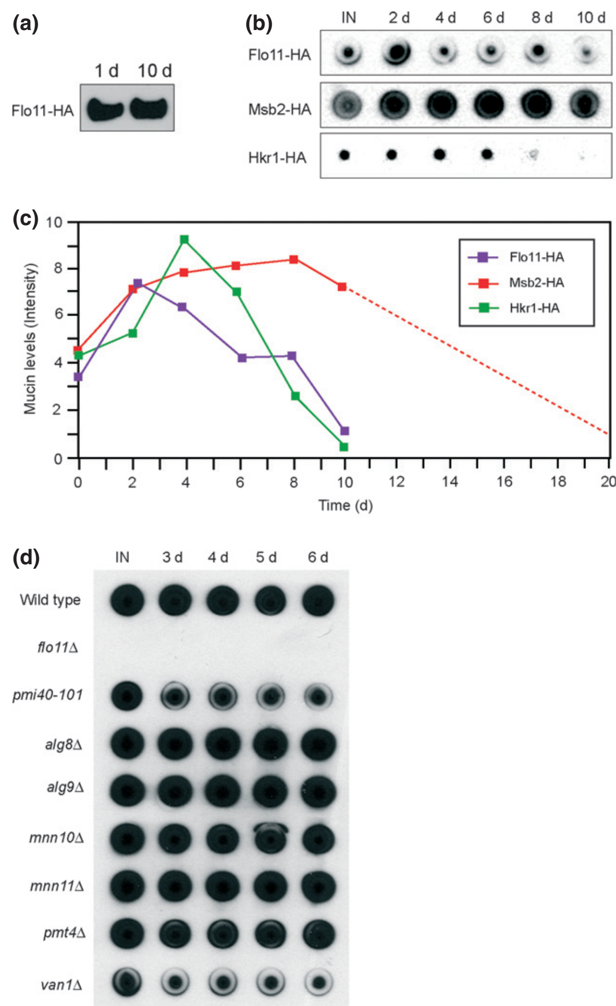

Fig. 4. Stability of shed mucin-like glycoproteins and the role of glycosylation on Flo11 stability. (a) SDS-PAGE analysis. Wild-type cells expressing Flo11-HA (PC2043) were grown in YEPD for 1 day, and the clarified supernatant was maintained for 10 days. Shed Flo11-HA in the supernatant was detected by immunoblot analysis. ImageJ analysis of several exposures showed a 40% decrease in the amount of Flo11-HA by the 10-day time point. (b) The levels of shed Flo11-HA (PC2043), Msb2-HA (PC999), and Hkr1-HA (PC2740) were examined. 'Input (IN)' refers to supernatants taken from the initial incubation. Fifty microlitres of supernatant were collected to evaluate Flo11-HA and 200 μL of supernatant were collected to evaluate the levels of Msb2-HA and Hkr1-HA. (c) Graph showing quantitation of the levels of shed mucins. (d) The levels of shed Flo11-HA from wild-type (PC2043), *pmi40-101* (PC5014), *alg8*Δ (PC5396), *alg9*Δ (PC5399), *mnn10*Δ (PC5400), *mnn11*Δ (PC5401), *pmt4*Δ (PC5403), *van1*Δ (PC5405) mutants were also examined.

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deficiency might affect the expression, secretion, localization, or stability of adhesins; and/or (3) glycosylation deficiency might directly affect the function of adhesins. To begin to address these different possibilities, the Flo11 protein was examined in glycosylation mutants. In the *pmi40-101* mutant, the Flo11 protein was produced at higher levels than in wild-type cells (Fig. 3a). This may result from elevated *FLO11* gene expression, because the filamentous growth pathway, which regulates *FLO11* expression, is activated in this mutant (Cullen *et al.*, 2000). Immunoblot analysis showed that in the *pmi40-101* mutant, Flo11 migrated as a product of a smaller size (Fig. 3a), which indicates that Flo11 is partially underglycosylated in this mutant. Immunofluorescence microscopy showed that underglycosylated Flo11 localized to the cell surface to the same degree as wild-type Flo11 (Fig. 3b). The same was true for other glycosylation mutants (data not shown). Taken together, the data indicate that Flo11 is underglycosylated in the *pmi40-101* mutant, but this change in modification does not reduce the overall abundance of the protein or its cell-surface localization.

We previously showed that shed Flo11 can bind to cells *in trans* (Karunanithi *et al.*, 2010). An *in vitro* binding assay showed that underglycosylated Flo11 that was shed from the *pmi40-101* mutant (underglycosylated) was capable of binding to wild-type cells to the same degree as Flo11 shed from wild-type cells (Fig. 3c, left and middle panels). Thus, full glycosylation of Flo11 is not required for its cell adherence function. An alternative possibility is that the Flo11 protein might be defective for binding to cells experiencing a protein glycosylation defect. This turned out to be the case. Specifically, Flo11 that was shed from wild-type cells was defective for binding to *pmi40-101* cells grown in medium lacking mannose, as compared to the *pmi40-101* cells grown in medium supplemented with mannose (Fig. 3c, right panel). Flo11 may bind poorly to cells experiencing a glycosylation defect because the cell wall and/or cell surface is compromised.

The glycosylation of Flo11 impacts the stability of the protein

We also examined the stability of shed Flo11. Shed Flo11 was separated from cells and measured in clarified supernatants over time. The levels of shed Flo11 were reduced by less than twofold after a 10-day time period (Fig. 4a). Spot immunoblot analysis was used to further examine the stability of shed Flo11 over a time course (Fig. 4b). Quantitation of the immunoblots showed that shed Flo11 levels had a half-life of 10 days (Fig. 4c). Yeast encodes other large cell-surface glycoproteins, including the signaling mucin-like proteins Msb2 (Cullen *et al.*, 2004) and Hkr1 (Tatebayashi *et al.*, 2007). The extracellular domains of Msb2 (Vadaie *et al.*, 2008) and Hkr1 (Pitoniak *et al.*, 2009) are shed from cells. Like Flo11, the shed portions of Msb2 and Hkr1 were extremely stable (Fig. 4b). Quantitation of shed Msb2 estimated a half-life of 18 days (Fig. 4c), and shed Hkr1 had a half-life of 8 days (Fig. 4c). For all three proteins, an increase in protein levels was observed at days 2 and 3, prior to turnover, which might possibly be due to enhanced detection of the proteins' epitopes as a result of de-glycosylation. Most yeast proteins are turned over rapidly inside cells, with an

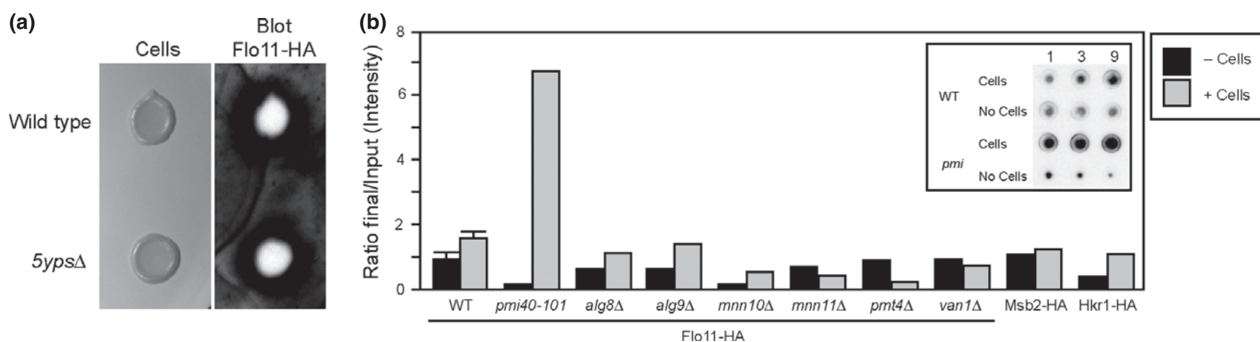

Fig. 5. The turnover of shed Flo11 and other mucin-like glycoproteins. (a) Five microlitres from overnight cultures of wild-type cells (PC2212) and *5ypsΔ* (PC2213) cells grown in YEPD media were spotted onto YEPD agar medium overlaid with a nitrocellulose membrane that was soaked in shed Flo11-HA. Shed Flo11-HA was prepared from an overnight liquid culture of wild-type (PC2043) cells grown in YEPD; cells were separated by centrifugation and the supernatant extract was filter sterilized. Colonies were grown for 2 days and washed off the membrane. Flo11-HA was detected by immunblot. Inset: Immunoblot of shed Flo11-HA was examined in wild-type (PC2043) and the *pmi40-101* (PC5014) mutant, either from supernatants in which cells were not separated (+ Cells) or that had been separated from cells (- Cells) grown in YEPD or their filter-sterilized cultures, respectively, detected by immunoblot. (b) Quantitation of levels of shed Flo11-HA from WT (PC2043), *pmi40-101* (PC5014), *alg8Δ* (PC5396), *alg9Δ* (PC5399), *mnn10Δ* (PC5400), *mnn11Δ* (PC5401), *pmt4Δ* (PC5403), *van1Δ* (PC5405), Msb2-HA (PC999) and Hkr1-HA (PC2740) strains grown in YEPD and their filter-sterilized cultures. Input, day 0. For the inset, pmi refers to the pmi40-101 mutant.

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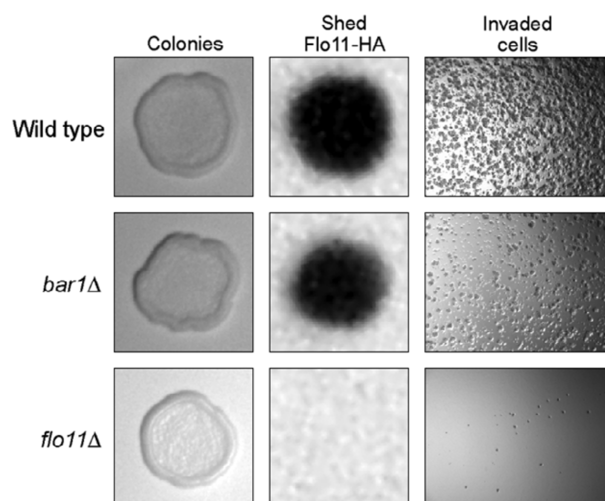

Fig. 6. Bar1 contributes to Flo11 shedding and Flo11-dependent adhesion responses. To evaluate shedding of Flo11-HA in a *bar1Δ* mutant, wild-type (PC2043), *bar1Δ* (PC3052) and *flo11Δ* (PC1029) cells from overnight cultures in YEPD were spotted (5 μL) onto YEPD agar plates overlaid with a nitrocellulose membrane (far left panels). Colonies were grown for 1 day and washed off the filters. Shed Flo11-HA was detected by immunoblot analysis (2nd panel from left). Agar invasion was assessed by washing 1-day-old colonies off the YEPD agar plate and photographing at 40× (3rd panel from left).

average half-life of 22.5 min (Belle *et al.*, 2006). Proteins from other eukaryotes have similar turnover rates (Yen *et al.*, 2008). The low turnover of shed glycoproteins makes them among the most stable proteins in the yeast proteome (Belle *et al.*, 2006).

The stability of yeast glycoproteins might result from the fact that the extensive glycosylation of these proteins protects them from degradation, which is true for many different proteins (Ng *et al.*, 2000). We tested whether the shed underglycosylated Flo11 was less stable than the wild-type protein. Flo11 that was shed from wild-type cells was more stable than Flo11 prepared from the *pmi40-101* mutant (Fig. 4d). A subset of glycosylation mutants showed a similar pattern (Fig. 4d, *van1* and to some degree *pmt4* and *mnn10*); other mutants did not (Fig. 4d, *alg8, alg9,* and *mnn11*). Therefore, the glycosylation of the Flo11 protein contributes to its stability. The specific glycosylation modification of Flo11, such as that induced by Van1, Pmt4, and Mnn10 may be more important for Flo11 stability than modifications induced by Alg8, Alg9, and Mnn11. Other interpretations are also possible, including the fact that other cell wall and cell-surface proteins are differentially glycosylated in these mutants.

Regulation of shed glycoprotein turnover in biofilm/mats

Although stable, the extracellular portion of shed Flo11 and other yeast glycoproteins are turned over at some rate. This conclusion is supported by the fact that shed Flo11 that is deposited at colony centers becomes reduced during colonial growth over time (Fig. 1b) and by obser-

Table 1. Yeast strains

Strain*	Genotype	Source
PC1291†	*MAT***a** *ste4 lys2 GAL-STE4 his3::FUS1-HIS3 FUS1-lacZ*	Cullen *et al.* (2000)
PC244	*MAT***a** *ste4 lys2 GAL-STE4 his3::FUS1-HIS3 FUS1-lacZ pmi40-101*	Cullen *et al.* (2000)
PC2043	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA*	Karunanithi *et al.* (2010)
PC538	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52*	Cullen *et al.* (2004)
PC999	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA*	Cullen *et al.* (2004)
PC2740	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 HKR1-HA*	Pitoniak *et al.* (2009)
PC1029	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KanMX6*	Karunanithi *et al.* (2010)
PC2212‡	*Wild type*	Krysan *et al.* (2005)
PC2213‡	*5ypsΔ*	Krysan *et al.* (2005)
PC1032†	*MAT***a** *ste4 lys2 GAL-STE4 his3::FUS1-HIS3 FUS1-lacZ pmi40-101::NAT*	This study
PC5014	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA pmi40-101::NAT*	This study
PC5396	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA alg8:KlURA3*	This study
PC5399	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA alg9:KlURA3*	This study
PC5400	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA mnn10:KlURA3*	This study
PC5401	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA mnn11:KlURA3*	This study
PC5403	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA pmt4:KlURA3*	This study
PC3052	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA bar1:kKURA3*	This study
PC5405	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA van1:KlURA3*	This study

*Strains were derived from the ∑ 1278b background unless otherwise stated.
†Strains were derived from the 246-1-1 background.
‡Strains were derived from the W303 background.

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vations showing that shed Flo11 in biofilms/mats has a highly patterned appearance that results in part from elimination of Flo11 along portions of the mats (Chavel *et al.*, 2010; Karunanithi *et al.*, 2010). The fact that shed Flo11 is turned over (albeit at a low rate) has implications into its overall function and regulation. For example, shed mucins may be recycled by cells to recoup the nutritional cost of producing and shedding these large glycoproteins. To better understand the turnover of shed Flo11, the ability of colonies to degrade Flo11 was examined by coating surfaces with shed Flo11-HA. Over time, Flo11 on nitrocellulose membranes became reduced underneath growing colonies, which confirms that Flo11 is turned over *in trans* (Fig. 5a). Presumably a shed or cell-surface protease(s) is required for turnover of shed Flo11. Using the above-mentioned assay, several protease mutants were examined for differences in turnover of shed Flo11. Turnover of shed Flo11-HA was not reduced by a mutant lacking all five yapsin aspartyl proteases (Fig. 5a, *5ypsΔ*; Krysan *et al.*, 2005) or by a mutant lacking the secreted protease Bar1 (data not shown; Sprague *et al.*, 1981). Possibly, a protease that has yet to be identified may regulate the turnover of shed Flo11 and other yeast glycoproteins. Alternatively, cell lysis may release internal proteases from the colony.

The overall level of shed Flo11 depends on a balance between its production (at the level of transcription, translation, and shedding) and turnover. The levels of shed Flo11 were determined in cells grown in liquid culture over time. In general, shed Flo11 accumulated over time in liquid cultures (Fig. 5b). Flo11 and other glycoproteins likewise accumulates over time in biofilms/mats (Karunanithi *et al.*, 2010). The levels of shed Flo11 also increased over time in most glycosylation mutants tested (Fig. 5b). In some mutants, this may be due to a compensatory mechanism, as has been shown for *pmi40-101* (Cullen *et al.*, 2000) and *pmt4* (Yang *et al.*, 2009). Some mutants (*mnn11, pmt4,* and *van1*) did not show an increase in Flo11 levels. Other mucin-like glycoproteins Msb2 and Hrk1 also showed a net accumulation over time (Fig. 5b). Therefore, although mucin glycoproteins are turned over at some rate, they are also replenished, which in general results in their net accumulation over time.

Our analysis also revealed a minor role for Bar1 in regulating Flo11 shedding and invasive growth (Fig. 6). This may be due to direct modification of Flo11 by Bar1 or the modification of another protein that impacts Flo11 function or regulation.

In summary, we report two new connections between protein glycosylation and the Flo11 adhesion molecule: (1) underglycosylated Flo11 is less stable than wild-type Flo11; and (2) Flo11 binds poorly to cells experiencing a glycosylation defect. We further show that shed Flo11 and other mucin-like glycoproteins are extremely stable proteins under normal growth conditions, with turnover rates on the time scale of days. These shed glycoproteins may be turned over by shed or cell-surface associated proteases. Over time, the balance between turnover and production leads to an overall accumulation of mucin-like glycoproteins in biofilms/mats and colonies.

Our studies agree with the general idea that protein glycosylation is critical for fungal behavioral responses. By tracing the glycosylation defect to the potential stability of an adhesion molecule and its failure to bind to cells experiencing glycosylation stress, we extend the understanding of fungal adhesion regulation in ways that might aid studies in fungal pathogens.

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