Loss of mannosylphosphate from *Candida albicans* cell wall proteins results in enhanced resistance to the inhibitory effect of a cationic antimicrobial peptide via reduced peptide binding to the cell surface

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Abbreviations: CMAC, CellTracker Blue 7-amino-4-chloromethylcoumarin; CTG, Cell Tracker Green chloromethylfluorescein diacetate; DsS3(1-16), dermaseptin S3(1-16); PI, propidium iodide.

INTRODUCTION

Over the past 30 years there has been a significant increase in the number of life-threatening fungal infections (Edmond *et al.*, 1999; Enoch *et al.*, 2006). This rise can be explained by the increased use of invasive surgical procedures and the application of chemotherapeutic treatments, such as exposure to broad-spectrum antibiotics and immunosuppressants after organ transplantation and cancer chemotherapy. Furthermore, in many cases, these treatments are carried out on patients who are already immunocompromised, such that fungal pathogens are readily able to overwhelm host defence mechanisms. Infections caused by *Candida albicans* have an incidence of between 1.1 and 24 cases per 100 000 individuals, with an associated mortality rate of greater than 30% (Gudlaugsson *et al.*, 2003; Wisplinghoff *et al.*, 2004).
Also, the incidence of invasive aspergillosis has increased fourfold over a decade in Europe (Groll et al., 1996) and has a mortality rate in excess of 50%, even when antifungal drugs are administered (Lim et al., 2001). Importantly, many of the existing antifungal drugs in use have undesirable side effects, are ineffective against new or re-emerging fungi, or have led to the development of drug-resistant strains in patients undergoing treatment. Thus, there is a need for more research to develop new antifungal drugs.

A potential new source of antifungals are cationic antimicrobial peptides, which are produced by bacteria, mammals, fish, amphibians, insects and plants as a defence against invasive microbial pathogens (Hancock & Scott, 2000). Cationic peptides have a number of potential advantages as future therapeutics, including a broad spectrum of activity and rapid killing of microbes, and they are unaffected by classical antibiotic resistance mechanisms, show synergy with classic antibiotics, neutralize endotoxin and are active in animal models (Hancock & Scott, 2000).

It has long been perceived that the microbicidal action of cationic antimicrobial peptides is due principally to disruption of target cell membranes. However, increasing evidence indicates complex and diverse mechanisms of action, including intracellular targets (reviewed by Yeaman & Yount, 2003). In two recent studies, evidence is presented that the antifungal action of a cationic, α-helical antimicrobial peptide is principally due to the induction of programmed cell death (Morton et al., 2007b), which was attributed to interaction of the peptide with cellular DNA (Morton et al., 2007a). It is clear that in order to kill microorganisms by whatever mechanism, cationic peptides must interact with target cell membranes. In fact, differences in composition and charge between microbial and host cell membranes have been proposed to account for the selective toxicity of cationic peptides (Yeaman & Yount, 2003).

The majority of mechanistic studies on the inhibitory action of antifungal peptides have given little consideration to the precise role or influence of the outermost layers of fungal cells on the inhibitory action of cationic peptides. For example, yeast has a strong, thick cell wall that protects the cell from mechanical injury and osmotic stress, and maintains structural integrity (Lesage & Bussey, 2006). In Saccharomyces cerevisiae, the cell wall represents ~30% of the total cell dry weight and is composed primarily of polysaccharides (~85%) and proteins (~15%) (Nguyen et al., 1998). Thus, it represents a potential barrier that antimicrobial peptides must interact with and pass through before they can contact the plasma membrane.

In bacteria, there are many documented examples that demonstrate the importance of the cell wall in mediating the efficacy of antimicrobial peptides. For example, Staphylococcus aureus enhances the positive charge of the cell wall such that basic antimicrobial peptides such as protegrins are repelled (Peschel et al., 1999). The inhibitory action of the type B lantibiotic mersacidin is due to interference with the conversion of the peptidoglycan precursor lipid II into the cell wall polymer peptidoglycan in susceptible Gram-positive bacteria (Brötz et al., 1998).

Using electron microscopy, Friedrich et al. (2000) demonstrated that the inhibitory action of a number of structurally diverse cationic peptides can be partly explained by cell wall effects such as cell wall breaks, disintegration, thinning and abnormal septation. Notably, few studies have examined the role of the yeast cell wall in mediating the efficacy of antifungal peptides. One such study has shown that cells of S. cerevisiae can be sensitized to nisin, an antimicrobial peptide produced by lactococci that normally has no inhibitory effect on yeast cells, by deletion of the gene encoding cell wall protein 2 (CWP2) (van der Vaart et al., 1995). A double null mutant, lacking both Cwp1 and Cwp2, is hypersensitive to nisin and displays impaired cell wall structure (Dielbandhoesing et al., 1998). Similarly, treatment of yeast cells with compounds that lead to impaired formation of the layer of glycosylphosphatidylinositol (GPI)-dependent cell wall proteins results in increased sensitivity to the amphiphilic antimicrobial peptide MB-21 (Bom et al., 2001).

The outermost layer of the C. albicans cell wall is enriched with mannoproteins containing both long-chain and highly branched N-linked mannosyl residues and shorter, linear chains of O-linked mannans that constitute 30–40% of the cell wall dry weight (Klis et al., 2001). Inside this outer layer, the underlying cell wall is composed of chitin, β-1,3- and β-1,6-glucan chains. The cell wall mannoproteins are thought to be involved in adhesion to host cells, virulence and cytokine production (Klis et al., 2002; Netea et al., 2008; Vecchiarelli et al., 1991). The N-linked mannan of C. albicans has an α-1,6-linked polymannose backbone with attached side chains consisting of α-1,2- and α-1,3-linked oligomannosides and β-1,2-linked mannoside residues. There is a mannosylphosphate-containing fraction that consists of between one and 14 β-1,2-linked mannoside residues attached to the side chains via phosphodiester bonds (Holbush et al., 2004; and references cited therein).

Additionally, it has been found that about 15% of the cell wall phosphomannan is attached to the O-linked mannan (Mora-Montes et al., 2007). In S. cerevisiae, one consequence of the loss of mannosylphosphate from the cell wall is a drastic reduction in surface negative charge, such that these cells are no longer able to bind the positively charged dye Alcian Blue (Ballou, 1990). Notably, the outer layer of mannoproteins is also believed to determine the porosity of the yeast cell wall (De Nobel et al., 1990). Thus, we reasoned that changes in the outer layer of the yeast cell wall may influence the ability of antimicrobial peptides to interact with and pass through the cell wall, and ultimately target the plasma membrane beneath.

**METHODS**

**Yeast strains and growth media.** Yeast strains used in this work are shown in Table 1. All strains were cultured in 100 ml flasks with malt
Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>NGY152</td>
<td>CAI4 + Clp10</td>
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<tr>
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<td>mnt1A + Clp10</td>
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<td>Munro et al. (2005)</td>
</tr>
<tr>
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<td>pmr1A + Clp10-PMR1</td>
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<td>H. M. Mora-Montes &amp; N. A. R. Gow, unpublished results</td>
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<td>H. M. Mora-Montes &amp; N. A. R. Gow, unpublished results</td>
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</table>

extract broth (MEB), pH 7 (1% glucose, 0.6% malt extract, 0.12% yeast extract) at 30 °C with shaking. For enumerating cell survival after exposure to antimicrobial peptides, yeasts were plated on YEPD agar (2% glucose, 2% agar, 1% bactopeptone, 1% yeast extract). Numbers of cells used in the assays described below were calculated using an OD600 versus viable cell numbers calibration curve that was generated for each yeast strain used.

Antifungal peptides. Dermaseptin S3(1-16)-NH2 [DssS3(1-16)] was synthesized according to the published sequence, ALWKNMLKG-KGLAGK (Mor et al., 1994), by Peptide Protein Research to >95% purity, and verified by HPLC and MS. Dermaseptin S3(1-16) was also synthesized with fluorescein tagged to the N-terminal lysine. Experiments were carried out using stock solutions in water.

Liquid culture assay of yeast growth inhibition. Deletion strain growth sensitivity assays were carried out in 48-well microtitre plates (Greiner Bio-one) using 300 μl MEB, pH 7. Sensitivity assays with or without the presence of glucosamine hydrochloride (Sigma) or glucosamine 6-phosphate (Sigma) were carried out in 96-well microtitre plates with 150 μl MEB, pH 7. DsS3(1-16) was added from stock solutions to the indicated concentrations. Wells were then inoculated with yeast cells from mid-exponential-phase cultures to give a starting concentration of 1.0 × 106 cells/ml. The fresh cultures were then incubated at 30 °C for 10 h. An initial viability reading was taken and cultures were then exposed to appropriate concentrations of DsS3(1-16). Culture viability was then measured every 30 min by serial dilution and plating on YEPD agar plates. Plates were incubated at 30 °C for 48 h prior to counting.

Assay of yeast cell viability. C. albicans cultures were incubated overnight in MEB, pH 7, at 30 °C with shaking. A 1 μl volume of yeast culture was diluted in 19 μl sterile MEB, pH 7, with or without 15 mM glucosamine hydrochloride or 15 mM glucosamine 6-phosphate, to give starting cell numbers of ~1.0 × 107 cells/ml. The fresh cultures were then incubated at 30 °C with shaking and OD600 readings were taken every 60 min until cell numbers reached between 1.0 × 105 and 1.0 × 106 cells/ml. An initial viability reading was taken and cultures were then exposed to appropriate concentrations of DsS3(1-16). Culture viability was then measured every 30 min by serial dilution and plating on YEPD agar plates. Plates were incubated at 30 °C for 48 h prior to counting.
Fluorescence microscopy. Cell Tracker Green 5-chloromethyl-fluorescein diacetate (CTG; Invitrogen) was used to label metabolically active cells (FITC filter; excitation λ = 490/520 nm; emission λ = 528/538 nm). Propidium iodide (PI; Invitrogen) was used to identify cells with compromised membranes [rhodamine–Texas Red–phycocerythrin (RD-TR-PE) filter; excitation λ = 490/520 nm; emission λ = 528/538 nm]. CellTracker Blue 7-aminocoumarin (CMAC; Invitrogen) was used to stain yeast vacuoles, exactly as described previously (Makrantoni et al., 2007). Images were captured on an Olympus IX70 DeltaVision microscope (Applied Precision). SoftWoRx Explorer 1.3 software (Applied Precision) was used for image processing and analysis.

Prior to staining, yeast cultures were harvested at mid-exponential phase (OD600 0.6) and diluted with MEB, pH 7, to give 2 x 10^6 cells ml^-1. DsS3(1-16) was added at the desired concentration and incubated at 30 °C for 5 min. PI (3.75 mM stock in ethanol) was added to give a final concentration of 1.8 μM. CTG (10 mM stock in DMSO) was added to give a concentration of 10 μM. The 1 ml reaction mixture was then incubated for 25 min at 30 °C in the dark. Unbound dye was removed by centrifugation for 1 min at 12000 g. The resulting pellet was washed with double-distilled H2O, centrifuged at 12000 g and resuspended in 20 μl MEB, pH 7. Aliquots (2 μl) of the stained cells were fixed with 2 μl 1 % low-melting-point agarose (Biogene). Samples were placed on ice and protected from light until analysis as described above.

Experiments were also carried out with DsS3(1-16)–fluorescein. The labelled peptide was added to cells prepared as described above at appropriate concentrations and incubated at 30 °C in the dark for 30 min. The unbound peptide was removed by harvesting the cells for 8 min at 3000 g, washing the pellet gently in double-distilled H2O, and harvesting followed by resuspension in 20 μl MEB, pH 7. Samples were fixed and kept as described above.

DsS3(1-16) binding assay. Fluorescence emission spectra of DsS3(1-16)–fluorescein were measured on a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a xenon lamp. Excitation and emission wavelengths were 494 and 521 nm, respectively. Readings were taken in a Quartz SUPRASIL Micro cuvette (700 μl volume) (Perkin Elmer). Peptide bound to cells was calculated via a calibration curve with increasing concentrations of DsS3(1-16)–fluorescein in MEB, pH 7, against fluorescence intensity (in arbitrary units; a.u.). A 1 ml volume of cells (OD600 0.6) was taken and the desired concentration of DsS3(1-16)–fluorescein was added. Cells were then incubated in the dark for 30 min at 30 °C. The suspension was centrifuged at 10000 g for 2 min to remove the cells and bound peptide. Residual fluorescence in the supernatant was measured as described above.

RESULTS

Mutants of C. albicans defective in mannosylation of cell wall proteins show altered growth phenotypes in the presence of a cationic antifungal peptide

We have used a series of well-characterized C. albicans null mutants with alterations in the cell wall to investigate how changes in cell wall protein mannosylation affect the efficacy of a cationic, α-helical antimicrobial peptide. The peptide used was DsS3(1-16) (Mor et al., 1994), a truncated derivative of dermaseptin S3 from Phyllomedusa sauvagii with full antimicrobial activity (Mor & Nicolas, 1994). Fig. 1 illustrates where the various changes in cell wall protein mannosylation occur in the mutants described below. MNN4 encodes a putative positive regulator of mannosylphosphate transferase (Hobson et al., 2004; Odani et al., 1997). The mnn4Δ mutant lacks all phosphomannan and is unable to bind the cationic dye Alcian Blue (Hobson et al., 2004). PMR1 encodes a Golgi P-type ATPase that transports Mn^{2+}, an essential cofactor for Mn^{2+}-dependent mannosyltransferases, into the Golgi lumen (Bates et al., 2005; Durr et al., 1998). The pmr1Δ null mutant has severe truncations in both N-linked and O-linked mannans, and therefore an almost complete absence of phosphomannan (Bates et al., 2005). This modification leads to a thinner cell wall than that of control cells (Netea et al., 2006). OCH1 encodes an α,1,6-mannosyltransferase that initiates the elongation of the N-linked mannann outer chain (Bates et al., 2006; Lehle et al., 1995). Disruption of OCH1 results in the loss of outer, branched N-linked glycans, and transmission electron microscopy reveals a thicker, chitin-rich cell wall that lacks a fibrillar mannanprotein layer. Binding of Alcian Blue is reduced but not completely eliminated in the C. albicans och1Δ null mutant (Bates et al., 2006; Netea et al., 2006). MNT1 and MNT2 encode partially redundant α,1,2-mannosyltransferases involved in O-linked mannosylation. Double deletion of MNT1 and MNT2 results in the loss of four terminal O-linked α,1,2-mannosyl residues, but N-linked mannann is unaffected (Munro et al., 2005). This double null mutant displays only a 10 % loss of ability to bind Alcian blue (H. M. Mora-Montes & N. A. R. Gow, unpublished results). In contrast, MNT3 and MNT5 encode functionally redundant phosphomannosyltransferases involved in the modification of N-linked mannans but not O-linked mannans (H. M. Mora-Montes & N. A. R. Gow, unpublished results). A double mnt3Δ/mnt5A null mutant displays a reduction of 50 % in the binding of the cationic dye Alcian Blue (H. M. Mora-Montes & N. A. R. Gow, unpublished results).

In an initial screen, we exposed these C. albicans null mutants to increasing concentrations of DsS3(1-16) and compared their growth with that of the isogenic parent strain. The effect of exposure to this peptide on visible growth after 48 h incubation at 30 °C is shown in Fig. 2. Typically, the inhibitory concentration of DsS3(1-16) for the parent strain CAI-4 + Clp10 was between 6 and 8 μg ml^-1. Of the C. albicans null mutants, mnn4Δ and pmr1Δ were most resistant to DsS3(1-16) compared with the isogenic parent. mnn4Δ and pmr1Δ null mutants were inhibited by concentrations of >16 and 14 μg ml^-1 DsS3(1-16), respectively. Loss of OCH1 also resulted in enhanced resistance to the peptide but to a lesser extent than mnn4Δ and pmr1Δ. The inhibitory concentration of DsS3(1-16) for the och1Δ null mutant was between 8 and 10 μg ml^-1. Strains carrying a single disruption of either MNT1 or MNT2 (data not shown), and a double mnt1Δ/mnt2Δ null mutant, had no obvious peptide-induced phenotype, displaying sensitivity levels similar to those of
the isogenic parent. In contrast, loss of both *MNT3* and *MNT5* (*mnt3Δ/mnt5Δ*) induced a resistant phenotype upon treatment with DsS3(1-16) that was slightly less resistant than that observed upon deletion of *mnn4Δ* or *pmr1Δ*.

In all the cases, reintegrant control strains showed a sensitivity to DsS3(1-16) similar to that displayed by the isogenic parent strain.

**The extent of phosphomannan loss from *C. albicans* glycosylation mutants correlates with enhanced survival in the presence of DsS3(1-16)**

To characterize the glycosylation mutant phenotypes in more detail, we carried out a detailed study of the effect of exposure to DsS3(1-16) on viability measured by serial dilution and plate counts, and by fluorescent staining with CTG and PI using fluorescence microscopy. CTG is a fluorogenic esterase substrate that freely diffuses into cells, where it is converted into fluorescein, which is largely retained by cells with intact membranes but leaks rapidly from dead cells or cells with compromised membranes. Thus, CTG measures ‘viability’ by enzymic activity and cell membrane integrity (Haughland, 2005). PI is a membrane-impermeant probe normally excluded from intact cells. Disruption of the cellular membrane allows PI to enter the cell, where it binds to DNA, inducing fluorescence enhancement upon excitation at 490 nm. Uptake of PI has been used extensively to quantify the degree of membrane disruption and death in microbial populations (Haughland, 2005).

The effect on the viability of the glycosylation mutants of exposure to increasing concentrations of DsS3(1-16) is shown in Fig. 3. Confirming our previous observations, the *mnn4Δ, pmr1Δ* and *mnt3Δ/mnt5Δ* double null mutant strains displayed the greatest resistance to DsS3(1-16) compared with the isogenic parent (Fig. 3a). The *och1Δ* null mutant displayed an intermediate level of resistance, and the *mnt1Δ/mnt2Δ* null mutant was as sensitive to DsS3(1-16) as the parent strain.

Study of the dose response to DsS3(1-16) exposure via fluorescence microscopy confirmed these observations (Fig. 3b). Untreated populations of the parent strain, and all the glycosylation mutants tested, showed 100% staining with CTG, and no significant PI staining. This indicates that, under non-stress conditions, there was no evidence of loss of membrane integrity, or metabolic activity, in any of the glycosylation mutants compared with the parent strain. However, exposure of all strains to increasing concentrations of DsS3(1-16) resulted in a reduction in the proportion of cells in the population staining with CTG and a concomitant increase in the number of PI-positive cells. This is indicative of a loss of metabolic activity.
membrane disruption induced by exposure to DsS3(1-16) of resistance to the inhibition of esterase activity and compared with the parent strain (Fig. 3b). In fact, the order increasing doses of DsS3(1-16) was much less in membrane integrity and metabolic activity evident with mannan present at the cell surface. For example, the loss of DsS3(1-16) was dependent on the amount of phospho-
staining PI-positive and CTG-negative upon exposure to Notably, the proportion of cells within the population capable of metabolic activity. (esterase) and membrane integrity within the population, and correlates with DsS3(1-16)-dependent loss of viability (Fig. 3a). A small proportion of cells within treated populations also showed dual staining: PI-positive, indicating that membrane integrity was compromised, but still CTG-positive, signifying that these particular cells were still capable of metabolic activity.

Notably, the proportion of cells within the population staining PI-positive and CTG-negative upon exposure to DsS3(1-16) was dependent on the amount of phosphomannan present at the cell surface. For example, the loss of membrane integrity and metabolic activity evident with increasing doses of DsS3(1-16) was much less in mnn4Δ compared with the parent strain (Fig. 3b). In fact, the order of resistance to the inhibition of esterase activity and membrane disruption induced by exposure to DsS3(1-16) (most resistant first) was typically: mnn4Δ > pmr1Δ > mnt3Δ/mnt5Δ > och1Δ, followed by mnt1Δ/mnt2Δ and the parent strain CAI-4, which were most susceptible. Thus, there was good correlation between the degree of resistance to the inhibitory effects of DsS3(1-16) measured in this experiment and that observed for retardation of growth (Fig. 2) and loss of viability (Fig. 3a).

**Loss of phosphomannan results in reduced sequestration and binding of DsS3(1-16)**

Previous studies have described how the extent of loss of negatively charged phosphomannan from the various C. albicans glycosylation mutants can be characterized by the extent of the reduction in binding of the cationic dye Alcian Blue. Therefore, the order of loss of phosphomannan, and thus surface negative charge, from the mutants tested was (greatest loss first): mnn4Δ > pmr1Δ > och1Δ > mnt3Δ/mnt5Δ > mnt1Δ/mnt2Δ and the parent CAI-4 (Bates et al., 2005, 2006; Hobson et al., 2004; H. M. Mora-Montes & N. A. R. Gow, unpublished data). Clearly, the order of loss of negative charge from the yeast cell surface correlates well with the order of induced resistance to the cationic peptide DsS3(1-16) described above. The only exception to this observation occurs when comparing och1Δ with the mnt3Δ/mnt5Δ double null mutant. Disruption of OCH1 results in an ~83 % reduction in Alcian blue binding compared with ~50 % for the mnt3Δ/mnt5Δ double null mutant, yet the mnt3Δ/mnt5Δ mutant displays greater resistance to DsS3(1-16) than och1Δ. Nonetheless, we reasoned that the loss of negatively charged phosphomannan from the cell surface could result in reduced binding of the cationic peptide and thus account for the apparent increase in resistance to the inhibitory effect of DsS3(1-16). To measure the degree of peptide binding, or sequestration, by the different glyco-
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sylation mutants that displayed a resistant phenotype to the cationic peptide DsS3(1-16), we employed a fluorimetric assay. Firstly, a calibration of DsS3(1-16)–fluorescein concentration versus fluorescence was carried out, and over the peptide concentration range employed in this experiment, this relationship was entirely linear (data not shown). Following this, equal population sizes of each mutant were treated with identical quantities of DsS3(1-16)–fluorescein, for an equal period of time, prior to removing the cells by centrifugation and retention of the supernatant. Measurement of the amount of residual fluorescence remaining in the supernatant allowed the calculation of the quantity of DsS3(1-16)–fluorescein that had been sequestered, or had bound to the cells (Fig. 4).

At a concentration of 5 μg ml⁻¹ DsS3(1-16)–fluorescein, over 80 % of the peptide was bound by cells of the parent strain CAI-4. In contrast, an equal quantity of mnn4Δ cells bound only approximately 45 % of the available peptide. Similarly, pmr1Δ, och1Δ and mnt3Δ/mnt5Δ bound approximately 56, 64 and 66 % of the peptide, respectively (Fig. 4). A similar order of peptide binding was also

![Fig. 2. C. albicans glycosylation mutants display variable resistance to the inhibitory effect of a cationic antimicrobial peptide. Visible growth of the parent strain (CAI-4), isogenic glycosylation mutants, mnt1Δ/mnt2Δ, och1Δ, mnt3Δ/mnt5Δ, pmr1Δ and mnn4Δ, and their reintegrant controls, in MEB, pH 7, after incubation at 30 °C for 48 h in 48-well microtitre plates in the presence of increasing concentrations of DsS3(1-16). Experiments were performed in triplicate, and representative results are shown.](http://mic.sgmjournals.org)
Fig. 3. *C. albicans* glycosylation mutants display varied tolerance to the fungicidal effects of DsS3(1-16). (a) Effect on viability of exposing CAI-4 (○), mnt1Δ/mnt2Δ (■), och1Δ (◇), mnt3Δ/mnt5Δ (▴), pmr1Δ (○) and mnn4Δ (▲) to increasing concentrations of DsS3(1-16) (0–15 µg ml⁻¹) in MEB, pH 7, for 30 min. The mean ± SD of triplicate experiments is shown. (b) Effect of exposing cultures of CAI-4, mnt1Δ/mnt2Δ, och1Δ, mnt3Δ/mnt5Δ, pmr1Δ and mnn4Δ to increasing concentrations of DsS3(1-16) (0–15 µg ml⁻¹) in MEB, pH 7, for 30 min on the proportion of cells within the population displaying PI (white bars), CTG (black bars), or both PI and CTG (grey bars) fluorescence. For each *C. albicans* strain tested, at each dose of DsS3(1-16), the fluorescence of over 300 cells was counted. A representative result of duplicate experiments is shown.

Fig. 4. The extent of DsS3(1-16)–fluorescein binding to *C. albicans* is reduced in glycosylation mutants that display resistance to the inhibitory action of the peptide. Equal numbers of CAI-4 (black bars), mnt3Δ/mnt5Δ (white bars), och1Δ (grey bars), pmr1Δ (hatched bars) and mnn4Δ (stippled bars) cells were exposed to increasing concentrations of DsS3(1-16)–fluorescein (5–20 µg ml⁻¹) in MEB, pH 7, for 30 min and harvested, and the amount of peptide remaining in the supernatant was measured fluorimetrically. Using a DsS3(1-16) concentration versus fluorescence calibration curve (not shown), the amount of peptide sequestered or bound by each strain was then calculated. The mean ± SD of triplicate experiments is shown.
observed at the higher doses of DsS3(1-16)–fluorescein tested, particularly 15 and 20 μg ml⁻¹, respectively. For all strains tested, as the dose of DsS3(1-16)–fluorescein increased, the fraction bound reduced. This was because as the concentration of exogenous peptide increases the proportion binding to the cell surface becomes a smaller fraction of the overall quantity of exogenous peptide.

Notably, there was a good correlation between the degree of DsS3(1-16) binding to the cells and the previously observed order of growth retardation (Fig. 2), loss of viability (Fig. 3a) and resistance to the inhibitory effects of DsS3(1-16) measured by fluorescence microscopy (Fig. 3b). Thus, in order that cationic antifungal peptides can assert their maximum inhibitory effect on yeast cells it is crucial that they bind to the negatively charged phosphomannan present on the outermost layer of the cell wall.

The presence of exogenous phosphate influences the extent of inhibition induced by exposure to DsS3(1-16)

To identify whether DsS3(1-16) binds to negatively charged phosphate, we studied the effect of exogenous glucosamine 6-phosphate, and of glucosamine hydrochloride as a negative control, on the antifungal efficacy of DsS3(1-16). Glucosamine is a naturally occurring amino sugar and is a precursor of N-acetylglucosamine, the building block of the yeast cell wall polymer chitin. Thus, in the absence of access to phosphomannan itself, we considered glucosamine to be a suitable molecule to study whether or not attached phosphate affects the inhibitory efficacy of DsS3(1-16).

Addition of up to 15 mM glucosamine hydrochloride or glucosamine 6-phosphate to the culture medium had no adverse effect on growth or viable numbers of C. albicans CAI-4 (data not shown). However, in a visible growth assay, the presence of 5, 10 or 15 mM glucosamine 6-phosphate significantly reversed the normal inhibition of yeast growth that occurs upon exposure to increasing concentrations of DsS3(1-16) (Fig. 5a). This effect was observed on all strains tested, including the mnn4Δ null mutant that displayed the greatest resistance to DsS3(1-16). The inhibitory effect of glucosamine 6-phosphate on the efficacy of DsS3(1-16) could be attributed to the presence of phosphate, because addition of identical concentrations of glucosamine hydrochloride (at the same pH of 7) had little detrimental effect on the efficacy of the peptide. Following this, we studied the effect of glucosamine 6-phosphate on the viability of C. albicans CAI-4 and the mnn4Δ null mutant were exposed to 20 and 40 μg ml⁻¹ DsS3(1-16), respectively, and viability was monitored over a period of 150 min. The mutant strain was exposed to a higher concentration of DsS3(1-16) due to the resistant phenotype. After 30 min exposure to the peptide there was an initial drop in viability, after which no additional loss was observed (Fig. 5b). Confirming our previous observations,
inclusion of 15 mM glucosamine 6-phosphate retarded the fungicidal effect of DsS3(1-16) upon both yeast strains (Fig. 5b). The presence of the phosphate-lacking control compound glucosamine hydrochloride had no significant effect on the inhibitory effect of DsS3(1-16) upon CAI-4 or the mnn4Δ null mutant. A similar pattern of results was observed when the pmr1Δ null mutant was exposed to DsS3(1-16), with or without the presence of glucosamine hydrochloride or glucosamine 6-phosphate (data not shown).

To study the inhibitory action of DsS3(1-16) in more detail, we exposed cells to DsS3(1-16) tagged with fluorescein and observed their appearance via fluorescence microscopy. Over a period of 30 min of exposure to DsS3(1-16)–fluorescein we observed a series of phases of peptide interaction with cells in the population (Fig. 6a). After addition of DsS3(1-16)–fluorescein, a small fraction of cells within the population displayed clear, localized staining around the outside of the cell, indicating that the peptide was binding to the outer surface of the yeast cell (Fig. 6a, part 1). This outer surface staining was a transient phase, occurring immediately prior to peptide entry into the cytosol. Following exposure for 30 min, a large proportion of cells appeared to concentrate the peptide inside the vacuole (Fig. 6a, part 2). Clear staining of the vacuole and not the cytosol was confirmed by co-localization of DsS3(1-16)–fluorescein

Fig. 6. Microscopy study of the interaction of DsS3(1-16)–fluorescein with C. albicans. (a) Exposure to DsS3(1-16)–fluorescein (15 μg ml⁻¹) for 30 min revealed different stages of peptide interaction with cells. 1, Phase-contrast and fluorescence image showing early, transient interaction with the cell surface; 2, image showing fluorescence concentrated inside vacuoles, as demonstrated by co-localization with CMAC vacuolar stain; 3, image showing some cells in the population with complete intracellular fluorescence, with areas within the cytosol where the degree of peptide accumulation is highly variable. Other cells showed no fluorescence at all; bar, 5 μm. (b) The effect of 15 mM glucosamine hydrochloride (Gluc-HCl) or glucosamine 6-phosphate (Gluc 6-phos) on entry of DsS3(1-16)–fluorescein into C. albicans CAI-4, mnn4Δ or pmr1Δ. For each yeast strain, the proportion of cells displaying different phases of peptide interaction was calculated by counting between 200 and 300 cells in several fields from duplicate experiments. A representative result from one of these experiments is shown.
with CMAC, a specific vacuolar stain. Finally, after 30 min exposure to DsS3(1-16), a significant fraction of cells were stained throughout the entire cytosol and clearly displayed areas where the peptide was concentrated, such as vacuoles, but also had areas within the cell where staining was largely absent (Fig. 6a, part 3). Although a significant proportion of cells were fluorescent throughout the entire cytoplasm; nevertheless, within the same cell population, many cells were not stained at all and clearly had not taken up a significant amount of DsS3(1-16)–fluorescein (Fig. 6a, part 3). Together, these results confirmed that DsS3(1-16) binds to the outer surface of the yeast cell and support our previous conclusion that the presence of phosphomannan at the outer cell surface of yeast cells is a major determinant of the antifungal potency of DsS3(1-16). Notably, the results also revealed that the binding of the peptide to the cell surface was a transient event that was followed by apparent concentration of DsS3(1-16) in the vacuole or dissemination throughout the entire cytosol. The occurrence of different phases of peptide uptake was heterogeneous within the population. For example, even after 30 min exposure, some cells were completely stained by DsS3(1-16)–fluorescein, whilst others displayed no staining whatsoever. This implies that the physiological state of cells, possibly their position within the cell cycle or metabolic state, could influence the degree of susceptibility to the inhibitory action of the peptide.

Next, we studied how the addition of exogenous glucosamine 6-phosphate affected the uptake of DsS3(1-16) to explain why phosphate reduces the efficacy of the peptide. Thus, we exposed cells to DsS3(1-16)–fluorescein in the presence of glucosamine hydrochloride or glucosamine 6-phosphate and observed their appearance as described above (Fig. 6b). In all strains tested, over a 30 min exposure to the peptide, the presence of glucosamine 6-phosphate clearly reduced the proportion of cells in the population that showed complete cytosolic staining. This implies that the binding and entry of the peptide into the cytosol is significantly reduced due to the exogenous glucosamine 6-phosphate sequestering the peptide and reducing the amount of peptide able to bind to the phosphomannan present at the yeast cell surface. However, exposure to the phosphate-negative control glucosamine hydrochloride also reduced uptake of the peptide, particularly by mnn4Δ cells, but to a much lesser extent than glucosamine 6-phosphate with parental and pmr1Δ cells (Fig. 6b). Confirming our previous observations that showed reduced peptide binding (Fig. 4), both mnn4Δ and pmr1Δ cells had less peptide present inside the cells, reflecting the resistant phenotype displayed by these mutants. In addition, the microscopy studies revealed that exposure to glucosamine hydrochloride or glucosamine 6-phosphate did not alter the morphology of any of the strains tested, as there was no measurable change in the small proportion of cells displaying formation of hypha.

**DISCUSSION**

Throughout this study we observed a consistent pattern of decreased binding and susceptibility to growth inhibition by DsS3(1-16) in *C. albicans* mutants that are known to exhibit variable reductions in their content of negatively charged phosphomannan. For example, loss of MNN4, which is required for mannosyl phosphate transfer and attachment of β-1,2-mannose residues to the N-mannan acid-labile side chains, conferred the greatest resistance to DsS3(1-16) of all the glycosylation mutants tested, and resulted in the largest reduction in peptide binding. Disruption of MNN4 resulted in total loss of N-linked and O-linked phosphomannan, which is reflected in complete inability to bind the cationic dye Alcian Blue (Hobson *et al.*, 2004). Notably, there is a good correlation between the extent of resistance to DsS3(1-16) induced within the glycosylation mutants, the degree of phosphomannan present at the cell surface and the extent of peptide binding. For example, after mnn4Δ, the highest level of resistance to DsS3(1-16) was displayed by the pmr1Δ null mutant, that also exhibited a parallel decrease in the amount of peptide binding to these cells. Disruption of PMR1 led to a gross defect in glycosylation, with both N- and O-linked mannosylation severely reduced, and almost complete absence of phosphomannan (Bates *et al.*, 2005). However, some phosphomannan remains, ~5% of that present within the parent strain as measured by Alcian Blue binding (Bates *et al.*, 2005), perhaps explaining why pmr1Δ did not display the same degree of resistance to DsS3(1-16) as mnn4Δ, in which Alcian Blue binding was virtually abolished (Hobson *et al.*, 2004). Following mnn4Δ and pmr1Δ, the och1Δ and mnt3Δ/mnt5Δ null mutants demonstrated the highest levels of resistance to DsS3(1-16), with similar decreases in the extent of peptide binding. Och1 is an α-1,6-mannosyltransferase that initiates N-linked outer chain elongation via the addition of a single α-1,6-linked mannose residue to the Man8GlcNAc2 core already attached to the mannoprotein. This then allows mannan polymerase complexes to extend the α-1,6-mannose backbone, which is then branched due to the action of further mannosyltransferases (Bates *et al.*, 2006). Disruption of OCH1 resulted in the loss of outer, branched N-linked glycans that contain the majority of the phosphomannan fraction. Thus, this null mutant displayed an 83% reduction in Alcian Blue binding (Bates *et al.*, 2006) that correlates with the intermediate level of peptide binding and resistance to DsS3(1-16) that we observed with the och1Δ null mutant when compared with the mnn4Δ and pmr1Δ stains.

The mnt3Δ/mnt5Δ double null mutant displayed slightly less resistance to the inhibitory effect of DsS3(1-16) than the mnn4Δ and pmr1Δ null mutants. MNT3 and MNT5 encode phosphomannosyltransferases that are functionally redundant (H. M. Mora-Montes & N. A. R. Gow, unpublished results). Together, they participate in N-mannan outer chain modification, responsible for about 50% of the total phosphomannan attached to these.
proteins (Mora-Montes and Gow, unpublished data). This may explain the intermediate phenotype displayed by this null mutant, in terms of resistance to DsS3(1-16).

The glycosylation mutants discussed above mainly affected N-linked mannosylation, but what effect did an exclusive reduction in O-linked mannosylation (Munro et al., 2005). The Mnt1 and Mnt2 mannosyltransferases add the second and third mannose residues to O-linked glycan. Disruption of both MNT1 and MNT2 resulted in the specific truncation of O-linked glycans but had no effect on N-linked mannan (Munro et al., 2005). These cells showed a 10% reduction in the binding of Acanth Blue (H. M. Mora-Montes & N. A. R. Gow, unpublished observations). Notably, this loss of O-linked mannan had no measurable effect on sensitivity to DsS3(1-16), as the resistance phenotype of mnt1Δ/mnt2Δ cells was identical to that of the isogenic parent.

In addition to the mutant studies, we showed that exogenous phosphate was able to retard the inhibitory efficacy of DsS3(1-16). Therefore, available evidence indicates that specific loss of negatively charged N-linked phosphomannan from the cell wall proteins of C. albicans induces resistance to a cationic antimicrobial peptide by reducing the amount of peptide able to bind to the cell surface and thus access the underlying plasma membrane. Disruption of MNN4 did not induce a weakened cell wall phenotype, as there was no increased sensitivity to compounds known to disrupt cell wall integrity, such as Calcofluor White and Congo red (Hobson et al., 2004). However, the pmr1Δ, och1Δ, mnt3Δ/mnt5Δ and mnt1Δ/mnt2Δ strains were all hypersensitive to these same cell wall-perturbing compounds, because the mutations result in loss of cell wall integrity such that the cells become more sensitive to stress (Bates et al., 2005, 2006; Munro et al., 2005; H. M. Mora-Montes & N. A. R. Gow, unpublished data). Thus, there was no close correlation between the degree of cell wall damage generated by the mutations that were studied and sensitivity to DsS3(1-16). For example, pmr1Δ and och1Δ are more resistant to the peptide than the parental strain, despite having a significantly damaged cell wall. Furthermore, the mnt1Δ/mnt2Δ strain has also been shown to have reduced cell wall integrity and yet has no discernible DsS3(1-16)-dependent phenotype compared with the parent strain. Therefore, the inhibitory action of DsS3(1-16) is dependent on the quantity of N-linked phosphomannan present at the surface of the cell wall, but is independent of overall cell wall integrity as measured by sensitivity to cell wall-perturbing agents. This implies that β-glucan and chitin are not a significant obstacle to the efficacy of the peptide. Instead, the crucial factor that determines the degree of sensitivity of C. albicans to DsS3(1-16) is the presence of phosphomannan.

Supporting our findings, the antifungal activity of osmotin, a basic 24 kDa protein expressed by many plant species in response to fungal infection, has also been shown to be partially dependent on the presence of cell wall phosphomannan in S. cerevisiae (Ibeas et al., 2000). Strains carrying disrupted MNN2, MNN4 or MNN6 are found to lack phosphomannan and are defective in binding osmotin to the cell wall. Whilst osmotin cannot be described as a classic antimicrobial peptide due to its large size, the Ibeas et al. (2000) study, in conjunction with our finding of the crucial role of phosphomannan in mediating tolerance to an amphibian-derived cationic peptide, implies that these essential components of the innate immune system of plants, and now amphibians, have evolved to recognize and bind to molecular targets present on pathogens, thus influencing their specificity and efficacy. Another example of this is the recognition of and binding to chitin present in the fungal cell wall of horseshoe crab tachystatin peptides (Osaki et al., 1999).

Netaa et al. (2006) concluded that C. albicans is recognized by mammalian monocytes or macrophages via three systems, each of which senses different pathogen-associated molecular patterns present within the yeast cell wall: N-linked mannans, O-linked mannans and β-glucans. Recognition of these components via different receptors results in the induction of pro- and anti-inflammatory cytokines, and represents a crucial mechanism that allows the innate immune system to combat Candida infections. Activated monocytes and macrophages use a range of antimicrobial peptides to attack and kill micro-organisms, such as the human cathelicidin LL-37, which like DsS3(1-16) is also a linear cationic α-helical peptide (Bowdish et al., 2005). Intriguingly, our results imply not only that the defence cells of the innate immune system have evolved to recognize molecular patterns on the surface of the yeast cell, but also that the antimicrobial peptides that are induced as a consequence of this recognition have evolved to recognize the same, or similar, molecular patterns in order to maximize their efficacy and specificity for pathogens.

ACKNOWLEDGEMENTS

M. H. and P. J. C. were supported by a Biotechnology and Biological Sciences Research Council-funded studentship (grant no. BBS/S/K/2004/11251A). N. A. R. G. and H. M. M.-M are supported by the Wellcome Trust (grant no. 080088).

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A cationic peptide binds yeast mannosylphosphate


Edited by: J. Pla