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Responses of *Candida albicans* to the Human Antimicrobial Peptide LL-37

Pei-Wen Tsai[1], Yin-Lien Cheng[1], Wen-Ping Hsieh[3], and Chung-Yu Lan[1,2*]

[1]Institute of Molecular and Cellular Biology, [2]Department of Life Science, [3]Institute of Statistics, National Tsing Hua University, Hsinchu 30013, Taiwan

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Candida albicans **is a major fungal pathogen in humans. Antimicrobial peptides (AMPs) are critical components of the innate immune response in vertebrates and represent the first line of defense against microbial infection. LL-37 is the only member of the human family of cathelicidin AMPs and is commonly expressed by various tissues and cells, including surfaces of epithelia. The candidacidal effects of LL-37 have been well documented, but the mechanisms by which LL-37 kills *C. albicans* are not completely understood. In this study, we examined the effects of LL-37 on cell wall and cellular responses in *C. albicans*. Using transmission electron microscopy, carbohydrate analyses, and staining for β-1,3-glucan, changing of *C. albicans* cell wall integrity was detected upon LL-37 treatment. In addition, LL-37 also affected cell wall architecture of the pathogen. Finally, DNA microarray analysis and quantitative PCR demonstrated that sub-lethal concentrations of LL-37 modulated the expression of genes with a variety of functions, including transporters, regulators for biological processes, response to stress or chemical stimulus, and pathogenesis. Together, LL-37 induces complex responses in *C. albicans*, making LL-37 a promising candidate for use as a therapeutic agent against fungal infections.**

Keywords: LL-37, *C. albicans*, cell responses

Introduction

Fungi pathogens pose a significant threat to patients with suppressed immune systems and the overall incidence of fungal infections has increase dramatically over the past two decades (Pfaller and Diekema, 2007). For example, species of *Candida* are the second most common cause of urinary-tract infections and the fourth most frequently isolated pathogen in bloodstream infections, associated with considerable mortality (Edmond *et al.*, 1999; Wisplinghoff *et al.*, 2004). Among *Candida* species, *Candida albicans* is the most prevalent in humans and causes a wide range of diseases that are collectively referred to as candidiasis. *C. albicans* is a normal component of the body flora and colonizes a variety of anatomical sites including the skin, mucosal surfaces of the oral cavity and esophagus, and the gastrointestinal and genitourinary tracts. *C. albicans* can become highly invasive particularly in immunocompromised patients (Eggimann *et al.*, 2003). To date, only a limited number of antifungals are available and the persistent use of these drugs has caused the emergence of drug-resistant strains. Therefore, the need for developing new antifungals is urgent. Recently, the therapeutic application of antimicrobial peptides (AMPs) or their analogs has received a great deal of attention (Reddy *et al.*, 2004).

AMPs are important components of natural defenses against pathogens. A wide variety of AMPs are generated and secreted by various human tissues, forming the first wave of the innate immune response. There are at least three major families of AMPs: defensins, histatins, and cathelicidin. Defensins are small cationic peptides that consist of β-pleated sheets (Reddy *et al.*, 2004). Histatins are cationic histidine-rich peptides that are commonly found in human saliva.

LL-37 represents the only known member of the cathelicidin AMP family in humans and is found on the skin, as well as in neutrophils, salivary glands, and numerous epithelial linings (Kai-Larsen and Agerberth, 2008). Cathelicidin is first synthesized as an 18-kDa preproprotein named human CAP18, which contains an N-terminal signal peptide, a highly conserved cathelin prosequence, and a C-terminal antimicrobial domain. LL-37 is derived from the C-terminal domain of human CAP18 by proteinase 3–mediated proteolysis (Sorensen *et al.*, 2001; Burton and Steel, 2009). LL-37 consists of 37 amino acid residues that start with two leucines and form an amphiphilic α-helical structure (Sorensen *et al.*, 2001). LL-37 production is induced by a number of stimuli, including pro-inflammatory cytokines and the presence of an infectious agent (Kai-Larsen and Agerberth, 2008). LL-37 has a broad range of antimicrobial activities against both Gram-negative and Gram-positive bacteria, including the common wound pathogens Group A *Streptococcus* and *Pseudomonas aeruginosa* (Dorschner *et al.*, 2001; Kai-Larsen and Agerberth, 2008; Overhage *et al.*, 2008).

There are two major mechanisms of AMP-mediated killing of microbes: the formation of transmembrane pores and intracellular damage (Yeaman and Yount, 2003; Brogden, 2005; Jenssen *et al.*, 2006). An amphipathic structure is a key characteristic of AMPs, as it allows them to permeabilize membranes. LL-37, for example, is believed to exert its antimicrobial effect by the formation of membrane pores and leading to membrane disruption (Turner *et al.*, 1998; Henzler Wildman *et al.*, 2003; Brogden, 2005; Jenssen *et al.*, 2006;

*For correspondence. E-mail: cylan@life.nthu.edu.tw; Tel.: +886-3-574-2473; Fax: +886-3-571-5934

Burton and Steel, 2009). To *C. albicans*, LL-37 associates with the cell wall and cell membrane of *C. albicans* and severely affects membrane morphology (den Hertog *et al.*, 2005). Moreover, LL-37 treatment causes the efflux of both small and large molecules such as ATP and proteins (den Hertog *et al.*, 2006). In addition to its candidacidal activity, LL-37 inhibits *C. albicans* adhesion by interacting with cell wall components (Tsai *et al.*, 2011a, 2011b). Although the interaction between LL-37 and *C. albicans* has been demonstrated, the mechanisms by which LL-37 affects *C. albicans* are still unknown.

The cell wall of *C. albicans* interacts with host cells, maintains *C. albicans* morphology, and contributes to *C. albicans* pathogenesis (Nather and Munro, 2008). The cell wall is composed of three major polysaccharides: β-glucan, mannan, and chitin. As major structural components of the cell wall, β-glucans account for 50–60% of the cell wall by weight, containing both β-1,3- and β-1,6-glucans (Ruiz-Herrera *et al.*, 2006). Mannan conjugates with proteins to form mannoproteins, which localize to the outermost part of the cell wall and represent ~40% of total cell wall proteins. Mannoproteins generally mask the glucans on the cell wall surface (Boxx *et al.*, 2010). Chitin is a β-1,4-*N*-acetylglucosamine homopolymer that links to a network of glucans via β-1,3 and β-1,6 bonds, thereby strengthening the cell wall. Chitin, however, accounts for only 1–2% of the cell wall in *C. albicans* (Klis *et al.*, 2001; Masuoka, 2004).

Composition of the cell wall can be quite dynamic, as it changes during cell division, undergoing a morphological transition, or in response to environmental stresses. *C. albicans* constantly remodels the cell wall by breaking and reforming chemical bonds within and between polysaccharides to maintain integrity of the cell wall structure (Nather and Munro, 2008). This cell wall remodeling depends on glycosyltransferases, transglycosylases, and glycoside hydrolases, which are associated with cell wall proteins such as glucanases and chitinases (Adams, 2004; Hurtado-Guerrero *et al.*, 2009).

In this work, we highlight the complex processes involved in LL-37-mediated *C. albicans* killing, and provide new insights for using AMPs in prevention and treatment of fungal infections.

Materials and Methods

Peptides and reagents

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), and histatin 5 (Hst 5) (DSHAKRHHGYKRKFHEKHHSHRGY) were synthesized by MDBio, Inc. (Taiwan). High-performance liquid chromatography and mass spectroscopy revealed that the peptides were >97% pure. Reagents used in this study were purchased from Sigma-Aldrich (USA), unless indicated otherwise.

C. albicans strains, media, and growth conditions

C. albicans SC5314 strain was used in this study. Cells were stored at -80°C and plated on YPD agar (1% yeast extract, 2% Bacto Peptone, 2% glucose, and 1.5% agar) before each experiment. A single colony was inoculated into YPD broth and grown at 30°C overnight (~16 h). The overnight culture was sub-cultured in YPD broth [with an initial optical density at 600 nm (OD_{600}) of 0.25] and grown at 30°C with shaking (180 rpm) to an OD_{600} of 1.0. For LL-37 treatment, cells were washed twice with phosphate-buffered saline (PBS), collected by centrifugation, and suspended in Gibco RPMI-1640 medium (Invitrogen, USA). Different concentrations of LL-37 (0–40 μg/ml) were added to cell suspensions and incubated at 37°C with slow shaking (100 rpm) in 5% CO_2 for 30 min.

Spot assay for cellular susceptibility to AMPs

Cells were resuspended to a concentration of 1×10^7 cells/ml. After being treated with LL-37 or Hst 5 for 30 min, cells were spotted onto YPD agar plates (with 10-fold serial dilutions; 10 μl each spot). Cell viability was observed after incubation at 30°C for 24 h.

Transmission electron microscopy (TEM)

Cells treated with 20 μg/ml LL-37 were collected by centrifugation (1,500 × *g* for 5 min) and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C for 30 min. Samples were washed four times with phosphate buffer, and then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer at room temperature for 4 h. Samples were dehydrated through a graded series of acetone solutions and embedded in Spurr's resin (Electron Microscopy Sciences, USA). Ultrathin sections were stained with 4% uranyl acetate and 0.4% lead citrate. The images were obtained using a Philips CM-100 transmission electron microscope (Philips/FEI Corporation, Holland).

Measuring carbohydrate content of the cell wall

Cells (6×10^7 cells/ml) were treated with or without LL-37 (20 μg/ml) for 30 min, harvested by centrifugation and washed with water. To measure cell wall glucan and mannan contents, the acid hydrolysis method was performed as described (Francois, 2006). Briefly, cells were suspended in TE buffer (pH 8.0), vortexed with glass beads and separated by centrifugation (1,000 × *g*) for 1 min. The supernatants were centrifuged again (3,000 × *g*) and pellets were collected. Pellets were suspended in TE buffer, centrifuged (14,000 × *g*), and dried at 60°C for 1 day. Polysaccharides were hydrolyzed to monosaccharides using 72% sulfuric acid at 100°C for 3 h. The acidic solution was neutralized by adding 40 g/L saturated $Ba(OH)_2$ and allowed to precipitate overnight at 4°C. Supernatants were collected by centrifugation. Glucan and mannan were then converted to glucose and mannose respectively using an enzymatic method (Scouten, 1984). Absorbance at 340 nm was then measured using a spectrophotometer (Hitachi Ltd., Japan) for both the samples and standards to determine NADPH levels, which are proportional to the concentration of glucose or mannose in the samples.

Chitin content was measured as described (Bulik *et al.*, 2003) with some modifications. Cells were resuspended in 6% KOH and incubated at 80°C for 90 min. Samples were centrifuged at 13,000 × g for 10 min and washed with PBS. Pellets were resuspended in McIlvaine's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 6.0) and ~0.01 U chitinase, and incubated at 37°C for 24 h. Equal volumes of 0.27 M

sodium borate (pH 9.0) were added and further incubated at 100°C for 10 min. Then, freshly diluted (1:10 with glacial acetic acid) Ehrlich's reagent (10 g *p*-dimethylaminobenzaldehyde in concentrated HCl and glacial acetic acid) was added. The mixture was incubated at 37°C for 20 min, and absorbance at 565 nm was recorded. The content of polysaccharides was calculated as follows: [(mean content for LL-37-treated cells)/(mean content for control cells)] × 100%.

Determining the exposure of β-1,3-glucan on the cell wall

After LL-37 treatment, cells were incubated with 10 μg/ml of a mouse monoclonal antibody against β-1,3-glucan (Biosupplies Inc., Australia), followed by incubation with an FITC-conjugated secondary antibody (goat anti–mouse IgG; Jackson ImmunoResearch, USA) at 4°C. Fluorescence was quantified using a FACSCalibur analyzer (BD Bioscience, USA). Mean fluorescence intensity was measured for 10,000 cells with the FL1 parameter. Relative staining of the cells, as compared with the control is shown.

Total RNA isolation and *C. albicans* DNA microarray analysis

Cells were grown overnight and subsequently diluted in fresh YPD medium to OD_{600} = 0.5. After further incubation for 3 h, cells were treated with either 0 or 5 μg/ml LL-37 for 30 min. Five biological replicates were performed for each condition. Genome-wide analyses using *C. albicans* custom arrays were performed as described (Hsu *et al.*, 2013). Briefly, total RNA was isolated using Trizol Reagent (Ambion, Inc., USA). Samples were shaken at 5,000 rpm for 15 sec with a MagNA Lyser System (Roche Applied Science, USA) and glass beads. After solubilization, phase separation was performed by adding chloroform and centrifugation. Total RNA was then purified using the RNeasy Mini Kit (Qiagen, Germany).

For cRNA synthesis, 1 μg total RNA was amplified using the Quick-Amp Labeling Kit (Agilent Technologies, USA) and labeled with Cy3 (PerkinElmer, Life Sciences, USA) by *in vitro* transcription reactions. Cy3-labeled cRNA (0.6 μg) was fragmented and labeled cRNA was then hybridized to the *C. albicans* oligo microarray (Agilent Technologies) at 65°C for 17 h. The images were obtained by an Agilent microarray scanner, and analyzed using Feature Extraction 10.5.5.1 software (Agilent Technologies).

Analysis of DNA microarray data

Data analysis was conducted using JMP Genomics (SAS Institute Inc., USA). First, an array-to-array correlation heat map was generated for the 10 arrays (five replicate samples from cells with or without LL-37 treatment). Array correlations were >0.98, and no intensity-dependent structures were revealed. For the normalization step, therefore, only a mean adjustment was applied to each array. This decision minimized artificial manipulation of the data while still providing high-quality data. To compare transcript abundance between samples, a paired *t*-test was applied for each of the 6,202 genes on the array. Microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE45177.

Table 1. Primers used for qPCR analysis

Gene	Primer sequence
EFB1	Forward, 5′-TAAGAAGGCTGCTAAAGGTCCAA-3′ Reverse, 5′-ATCCCATGGTTTGACATCCAA-3′
KRE6	Forward, 5′-TCCAACAAGCATTATCAGCA-3′ Reverse, 5′-CATCACCAACAAACCATCGT-3′
NRG1	Forward, 5′-GATCATGCCAAAATACGGTCAAA-3′ Reverse, 5′-TGACGACAAAGCAAGGGAGTT-3′
TPO3	Forward, 5′-TGCTGCTTATGGTTCATCTTGTTT-3′ Reverse, 5′-CAGTCAGCACGTGGTATTTGTTATT-3′
HGT12	Forward, 5′-GCTATTTGTGGTGAATCCTTTGC-3′ Reverse, 5′-GCCAGTTACTTGCGGTACACAAG-3′
RHR2	Forward, 5′-CCACATCCACAAGGTTACCA-3′ Reverse, 5′-AGCACCTTTACCTGCGGTTAT-3′
GAP2	Forward, 5′-TGCCTTTGCTTTCGCTGGTA-3′ Reverse, 5′-TTGGGTTTTCGGTTTCAGCA-3′
TRY4	Forward, 5′-TTGCCGTCAGTAGGATTAGAAAGAG-3′ Reverse, 5′-GAATCGGAACCACTTTTACTGTCA-3′

Quantitative real-time PCR (qPCR)

Total RNA (1 mg) was reverse transcribed into single-stranded cDNA using M-MLV reverse transcriptase (Promega, USA) and oligo$(dT)_{18}$ primers (MDBio, Inc.). qPCR was carried out using the 7500 Real-Time PCR system (Applied Biosystems, USA). Reactions were subjected to 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each sample was analyzed in triplicate. Data analysis was performed using Real-Time PCR System Sequence Detection Software v1.4 (Applied Biosystems). An average C_T (cycle threshold) value was obtained and normalized to the average C_T value for *EFB1*. Primers used for qPCR are listed in Table 1.

Statistical analysis

Statistically significant differences were determined using the two-tailed Student's *t*-test.

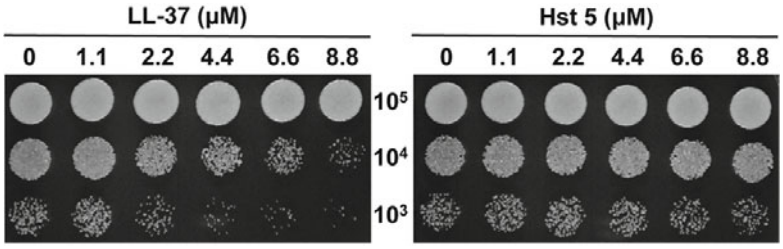

Fig. 1. LL-37 possesses candidacidal activity. Cell susceptibility to LL-37 and Hst 5 was compared by spot assay. SC5314 strain was treated with different concentrations of LL-37 or Hst 5 and ten-fold serial dilutions of the cells were spotted onto YPD agar plates.

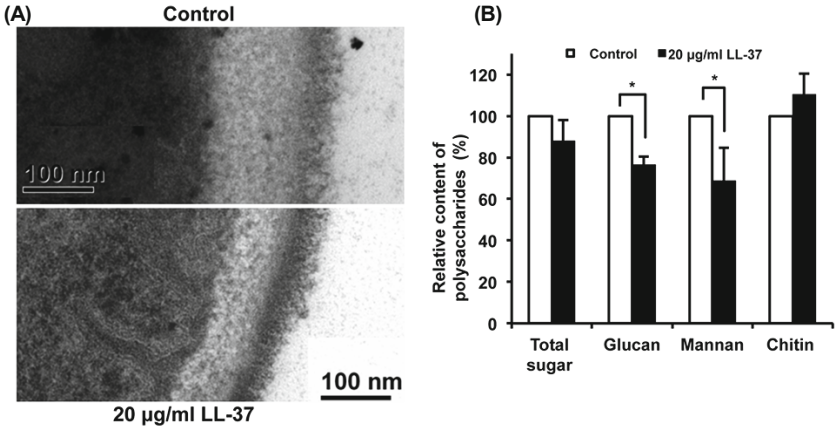

Fig. 2. LL-37 changes the cell wall and alters cell wall composition in *C. albicans*. (A) Cell wall thickness was compared between wild type and LL-37-treated (20 μg/ml) cells. Representative TEM images are shown. For each cell, 10 parts were analyzed. Results are the mean ± SD from 25 cells. $^{**}P < 0.01$, as compared with controls. (B) Cell wall polysaccharides were quantified and compared between LL-37-treated (20 μg/ml) and control cells. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared with controls. Results are the mean ± SD for three independent assays.

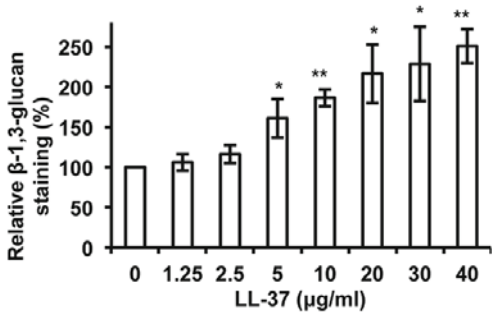

Fig. 3. LL-37 affects cell wall construction and remodeling in *C. albicans*. After LL-37 treatment, staining of β-1,3-glucan on the cell surface was quantified by flow cytometric analysis. Data was obtained from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared with controls.

Results

Antimicrobial activity of LL-37 against *C. albicans*

In our previous study (Tsai *et al.*, 2011b), the ability of LL-37 to kill *C. albicans* was quantitatively measured by a Fun-1 assay. Under our experimental condition, LL-37 had an obviously candidacidal effect beginning at doses 20 μg/ml (~4.4 μM) and the LC50 of LL-37 was about 30 μg/ml (~6.6 μM). In this work, we further compared the effect of LL-37 and Hst 5 on the killing of *C. albicans*. The anti-*Candida* activity of LL-37 was dose-dependent; 40 μg/ml (~8.8 μM) LL-37 strongly inhibited the fungal growth, but 5 μg/ml (~1.1 μM) LL-37 had a slight inhibition effect on *Candida* growth. Interesting, the viability of *C. albicans* cells was less affected by Hst 5 compared to that with LL-37 treatment (Fig. 1).

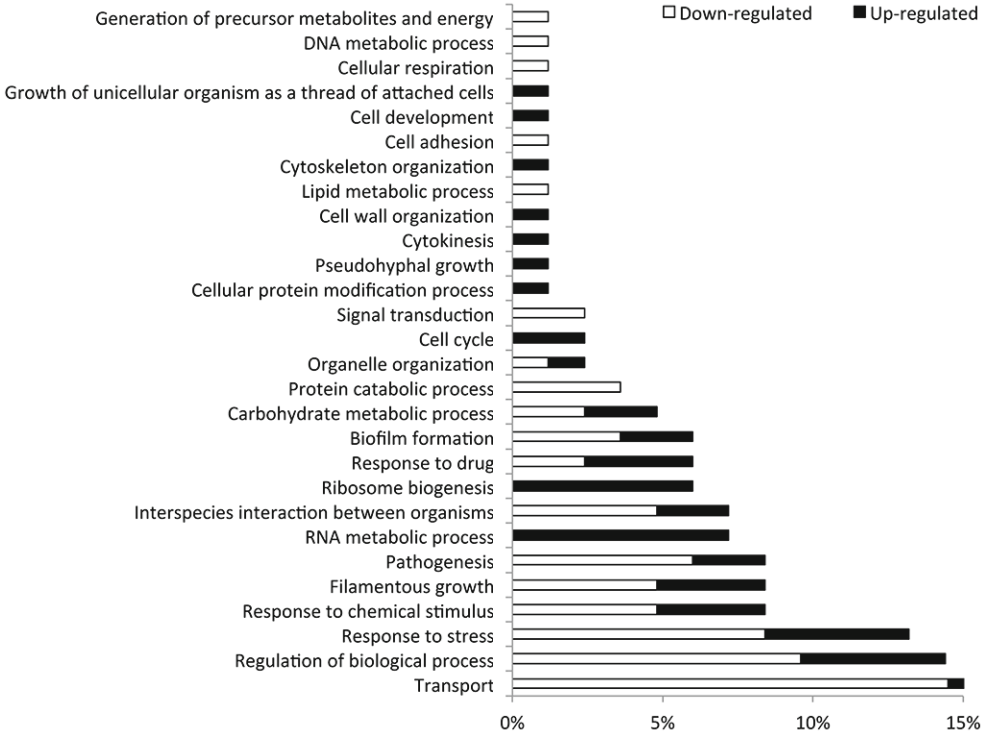

Fig. 4. Identification of genes affected by LL-37 using DNA microarray analysis. Genes with a fold change > 1.5 and $P < 0.05$ were considered significant by LL-37 treatment. Five biological replicates were analyzed. Differentially expressed genes were then classified using gene ontology terms. Among these genes, 51.8% of the genes were annotated as "biological process unknown".

Table 2. DNA microarray analysis reveals the transcriptional regulation of *C. albicans* genes in response to LL-37

GO term[a]	No. of genes (percentage)[b]	Gene names
Biological process unknown	43 (51.8%)	ATO7, HRQ2, LDG3, PGA34, PGA45, PHO113, orf19.1062, orf19.1679, orf19.1691, orf19.1748, orf19.2212, orf19.2457, orf19.258, orf19.2701, orf19.3107, orf19.3337, orf19.3905, orf19.3906, orf19.3908, orf19.3924, orf19.3954.1, orf19.4189, orf19.4819, orf19.5863, orf19.6084, orf19.6200, orf19.633, orf19.6575, orf19.7305, orf19.873, ADH3, MET18, orf19.2468, orf19.4567; **orf19.1287**, **orf19.204**, **orf19.3888.2**, **orf19.3988**, **orf19.5020**, **orf19.5262**, **orf19.5267**, **orf19.6556**, **orf19.851**
Transport	13 (15.7%)	AGP2, GAP2, GIT1, HGT10, HGT12, HGT17, JEN2, NAG4, OPT4, RAS2, TNA1, orf19.3232, **TPO3**
Regulation of biological process	12 (14.5%)	GAP2, HMS1, PHO112, RAS2, SAP4, SAP6, TRY4, orf19.230, **ASH1**, **KRR1**, **NRG1**, **SPC19**
Response to stress	11 (13.3%)	AGP2, HGT12, HMS1, PHO112, RAS2, RHR2, SAP4, **ASH1**, **NRG1**, **RTA3**, **orf19.2330**
Response to chemical stimulus	7 (8.4%)	HGT12, HMS1, NAG4, orf19.3232, **NRG1**, **TPO3**, **orf19.6586**
Filamentous growth	7 (8.4%)	CTN3, HGT12, HMS1, RAS2, **ASH1**, **NRG1**, **orf19.2330**
Pathogenesis	7 (8.4%)	ICL1, NAG4, SAP4, SAP5, SAP6, **ASH1**, **NRG1**
RNA metabolic process	6 (7.2%)	**KRR1**, **KTI11**, **MAK16**, **NSA2**, **UTP21**, **orf19.2330**
Interspecies interaction between organisms	6 (7.2%)	HYR1, SAP4, SAP5, SAP6, **NRG1**, **YWP1**
Ribosome biogenesis	5 (6%)	**KRR1**, **MAK16**, **NSA2**, **UTP21**, **orf19.2330**
Response to drugs	5 (6%)	NAG4, orf19.3232, **NRG1**, **TPO3**, **orf19.6586**
Biofilm formation	5 (6%)	HYR1, RHR2, TRY4, **NRG1**, **YWP1**
Carbohydrate metabolic process	4 (4.8%)	ICL1, RHR2, **KRE6**, **RHD1**
Protein catabolic process	3 (3.6%)	SAP4, SAP5, SAP6
Signal transduction	2 (2.4%)	HMS1, RAS2
Organelle organization	2 (2.4%)	orf19.5042, **SPC19**
Cell cycle	2 (2.4%)	**KRE6**, **SPC19**
Cellular protein modification process	1 (1.2%)	**KTI11**
Pseudohyphal growth	1 (1.2%)	**NRG1**
Cytokinesis	1 (1.2%)	**KRE6**
Cell wall organization	1 (1.2%)	**KRE6**
Lipid metabolic process	1 (1.2%)	AGP2
Cytoskeleton organization	1 (1.2%)	**SPC19**
Cell adhesion	1 (1.2%)	RHR2
Cell development	1 (1.2%)	**NRG1**
Growth of unicellular organism as a thread of attached cells	1 (1.2%)	**NRG1**
Cellular respiration	1 (1.2%)	CTN1
Generation of precursor metabolites and energy	1 (1.2%)	CTN1
DNA metabolic process	1 (1.2%)	orf19.5042

[a]Gene ontology terms are adapted from the *Candida* genome database (http://www.candidagenome.org).

LL-37 alters cell wall architecture

The cell wall is the outermost layer of *C. albicans*. As such, it directly encounters environmental changes and stresses, and maintaining cell wall integrity is crucial for cell survival. In addition, our previous studies indicated that LL-37 inhibits *C. albicans* attachment to polystyrene and epithelial cell by interacting with carbohydrates and/ or cell wall proteins (Tsai *et al.*, 2011a, 2011b). To determine if LL-37 can affect cell wall architecture, cells were visualized by TEM (Fig. 2A) and cell wall polysaccharides were quantified (Fig. 2B). To better demonstrate the strong effect of LL-37, we used a lethal dose of LL-37 (20 μg/ml) in these studies. Although this concentration of LL-37 was candidacidal, we did show the obvious effect of LL-37 on the alteration of *C. albicans* cell wall. The images revealed that LL-37 treatment reduced *C. albicans* cell wall thickness by ~30% (Fig. 2A).

Polysaccharides are major components of the *C. albicans* cell wall. To further characterize the effect of LL-37, cell wall polysaccharides were quantified. Total sugar content within the *C. albicans* cell wall was slightly decreased (~10%) in cells treated with LL-37 as compared with controls (Fig. 2B). Amounts of glucan and mannan were, however, reduced by ~25% and ~30% in LL-37-treated cells, respectively. The levels of chitin were no significantly different between LL-37-treated and control cells (Fig. 2B). These results suggest that LL-37 affects the *C. albicans* cell wall by disrupting its integrity and altering its composition.

LL-37 affects cell wall remodeling

Because cell wall carbohydrates were modulated by LL-37, this suggests that construction of the *C. albicans* cell wall may be altered by LL-37 treatment. Moreover, LL-37 inhibits *C. albicans* adhesion by interacting with components of the cell wall, including the major exoglucanase, Xog1 (Tsai *et al.*, 2011a; Chang *et al.*, 2012). Therefore, LL-37 alters *C. albicans* cell wall construction, either by disrupting cell wall biogenesis

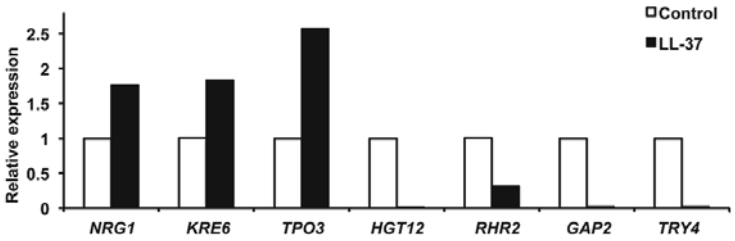

Fig. 5. Quantitative real-time RT-PCR to verify selected gene expression patterns identified from DNA microarray analysis. Total RNA was reverse transcribed, and the resulting cDNA was subjected to qPCR. Expression levels for controls were set to 1.0. Relative expression levels for LL-37-treated cells (as compared with controls) are shown. Results are the mean from triplicates for one assay.

or by inhibiting cell wall reconstruction.

β-1,3-Glucan is generally masked by mannan, and very little β-1,3-glucan is exposed on the cell surface (Martinez-Esparza *et al.*, 2006; Wheeler and Fink, 2006). We hypothesized that LL-37 affects cell wall reorganization, which would result in β-1,3-glucan exposure. Cell surface levels of β-1,3-glucan were thus detected using an antibody against this carbohydrate. Indeed, cells treated with LL-37 (≥5 μg/ml) had significantly more β-1,3-glucan exposed on the cell surface as compared with controls (Fig. 3).

Taken together, these results suggest that LL-37 disrupts the process of cell wall reconstruction, leading to the exposure of cell wall β-1,3-glucan.

Genome-wide analysis of LL-37-treated cells

Although we have shown that LL-37 affects cell wall architecture and cell wall remodeling in *C. albicans*, LL-37 may have many other critical functions, except for its candidacidal activity, which need to be identified. To explore these functions at the genomic level, DNA microarray analysis was used to detect *C. albicans* genes whose expression was affected by LL-37 treatment. In this experiment, the cells were treated with sub-lethal dose of LL-37 (5 μg/ml), which almost cannot cause cell death (Fig. 1) and is possibly to induce different cell responses. Transcripts from LL-37–treated cells were compared with the controls. Among the 6,202 *C. albicans* genes that were evaluated, 83 genes exhibited a significant change in expression ($P<0.05$) that was >1.5-fold ($\log_2 = 0.585$). Twenty four of these genes were up-regulated in response to LL-37, whereas 59 were down-regulated. Base on *C. albicans* genome annotation (http://www.candidagenome.org), these genes are involved in a wide variety of biological processes, including RNA metabolism; ribosome biogenesis; transport; cellular responses to chemicals, drugs, and stresses; carbohydrate metabolism; cell wall organization; filamentous growth; and biofilm formation (Fig. 4 and Table 2).

Here we highlight some of the differentially expressed genes. For example, *TPO3*, *NRG1* and *KRE6* were up-regulated in LL-37-treated cells, whereas *HGT12*, *RHR2*, *GAP2* and *TRY4* were down-regulated (Table 2). The *NRG1* gene product is a transcriptional regulator that controls filamentous growth, stress responses, and biofilm formation (Braun *et al.*, 2001; Murad *et al.*, 2001; Vyas *et al.*, 2005). The *KRE6* gene product is involved in β-1,6-glucan synthesis (Mio *et al.*, 1997). *TPO3* encodes a putative polyamine transporter, its gene expression decreases in hyphae cells and regulated by the transcriptional regulator Nrg1 (Murad *et al.*, 2001). *HGT12* encodes a glucose, fructose, mannose transporter, which plays a role in macrophage-induced hyphal growth (Luo *et al.*, 2007). *RHR2* encodes glycerol 3-phosphatase, which plays a role in biofilm and regulated by Nrg1 (Murad *et al.*, 2001; Desai *et al.*, 2013). *GAP2* encodes general amino acid permease, which regulated by Nrg1 and the transcriptional co-regulator Tup1, induced in biofilm formation (Nobile *et al.*, 2012). *TRY4* is a C2H2 transcription factor, required for yeast cell adherence to silicone substrate and induced in biofilm formation (Finkel *et al.*, 2012; Nobile *et al.*, 2012). Real-time PCR verified the expression patterns of *NRG1*, *KRE6*, *TPO3* and *HGT12*, *RHR2*, *GAP2*, *TRY4* (Fig. 5).

Discussion

The emergence of *C. albicans* drug-resistance represents a major challenge in healthcare. AMPs are important components of the innate immunity that *C. albicans* must overcome to establish an infection. As such, AMPs are considered as prime candidates for antifungal therapeutics (Reddy *et al.*, 2004). To ensure successful application of AMPs in a clinical setting, however, it is important to determine the susceptibility of *C. albicans* to AMPs, and to understand the effects that AMPs exert on *C. albicans*. Studies concerning the candidacidal effects of AMPs have primarily focused on AMPs, such as Hst 5 (Wunder *et al.*, 2004; Vylkova *et al.*, 2007; Kumar *et al.*, 2011; Li *et al.*, 2013). There are relatively few studies, describing how the α-helical peptide, such as LL-37, kills *C. albicans*.

Previous studies showed that LL-37 associates with cell wall components of *C. albicans*, disrupting the cell membrane (Burton and Steel, 2009) and inhibiting cell adhesion (Tsai *et al.*, 2011a). A strong correlation has been indicated between the permeability of LL-37 across the *C. albicans* membrane and the ability of LL-37 to inhibit *C. albicans* growth (Lopez-Garcia *et al.*, 2005). In addition, LL-37-mediated membrane disruption leads to the efflux of small nucleotides such as ATP (den Hertog *et al.*, 2006). However, several evidences indicate that membrane disruption may not be the primary mechanism by which LL-37 kills microorganisms (Yeaman and Yount, 2003; Brogden, 2005). A study utilizing a model lipid systems and *E. coli* demonstrated that LL-37 disrupts the lipid bilayer via a toroidal pore mechanism (Henzler Wildman *et al.*, 2003). In *Bacillus subtilis*, LL-37 elicits a complex stress response involving the YxdJK two-component signaling systems, inducing the expression of genes involved in a wide variety of functions (Pietiainen *et al.*, 2005). In *Staphylococcus aureus*, LL-37 both permeabilizes the membrane and inhibits synthesis of bac-

terial macromolecules, especially RNA and proteins (Senyurek *et al.*, 2009).

In this study, we expanded the current understanding on the mechanisms of LL-37 and showed that LL-37 induces cell wall alterations and complex cellular responses in *C. albicans*. For example, LL-37–treated cells had lower levels of total sugars in the cell wall as compared with controls, although this difference was not statistically significant (Fig. 2B). One possible explanation for this result is that the phenol/sulfuric method used to measure the total carbohydrates. This method is used to generally detect simple sugars, oligosaccharides, polysaccharides, and their derivatives, but not for the detection of any specific saccharides (Dubois *et al.*, 1956). Indeed, using different enzymatic methods, glucan and mannan levels decreased 25–30% in response to LL-37, whereas chitin levels did not significantly change (Fig. 2B). Therefore, these results support that LL-37 causes alterations of cell wall composition in *C. albicans*.

Caspofungin is one of the echinocandins that can inhibit β-1,3-glucan synthesis, reducing levels of cellular β-1,3-glucan. Subinhibitory doses of caspofungin exposes β-1,3-glucan and triggers an immune response (Wheeler and Fink, 2006). In Figs. 2B and 3, we showed that LL-37 treatment reduced glucan levels and exposed β-1,3-glucan on the cell surface. Interestingly, PI staining revealed no significant cell membrane loss at sub-lethal concentrations (< 20 μg/ml) of LL-37. Cells treated with lethal concentrations of LL-37 (≥20 μg/ml) exhibited significantly change in their membrane integrity as compared with controls (data not shown). To determine the exposure of β-1,3-glucan detected by the specific antibody was not due to the effect of LL-37 on cell membrane damage, we used two sub-lethal concentrations of LL-37 (lower than 5 μg/ml) in this study. As shown in Fig. 4, LL-37 caused β-1,3-glucan exposure beginning at the dose of 1.25 μg/ml. Moreover, a significant difference in the staining of β-1,3-glucan was observed in the cells treated with LL-37 beginning at 5 μg/ml (Fig. 4), excluding the possibility that β-1,3-glucan exposure induced by LL-37 is due to cell death. A recent study indicates that *C. albicans* β-1,3-glucan exposure upon caspofungin treatment is controlled by the Cek1-mediated MAPK signaling pathway (Galan-Diez *et al.*, 2010). Interestingly, reduced levels of Cek1 phosphorylation were associated with β-1,3-glucan exposure following LL-37 treatment (unpublished data).

Cell wall remodeling depends on cell wall-associated glycoside hydrolases, such as glucanases and chitinase (Adams, 2004; Hurtado-Guerrero *et al.*, 2009). *C. albicans XOG1* is the homolog of *Saccharomyces cerevisiae EXG1* (Adams, 2004; Lesage and Bussey, 2006). *C. albicans* Xog1 protein shows exo-β-1,3-glucanase activity, and participates in cell wall remodeling (Gonzalez *et al.*, 1997). Our previous study indicated that LL-37 can modulate the glucanase activity of Xog1 (Tsai *et al.*, 2011a). Therefore, Xog1 may be responsible for masking β-1,3-glucan during LL-37 treatment. In addition, *PHR2* and *KRE5* are two other genes that function to mask β-1,3-glucan (Wheeler and Fink, 2006). *PHR2* encodes a transglycosylase that is required to cross-link between β-1,3 and β-1,6-glucan (Fonzi, 1999). *KRE5* involves in the synthesis of β-1,6-glucan, which is a minor component of the cell wall (Kollar *et al.*, 1997). It will be interesting to determine the function of *PHR2* and *KRE5* in response to LL-37, particularly their roles in β-1,3-glucan exposure. Together, this work suggests that the candidacidal activity of LL-37 is partially mediated by cell wall remodeling, as LL-37 may affect the enzyme activities, and lead to β-1,3-glucan exposure. This hypothesis, however, needs to have further studies.

Although we try to mimic as physiological conditions as possible in our experiments, it is worth to note that the environments are much more complicated *in vivo* than that *in vitro*. For instance, several components including ions, polysaccharides, proteases, and serum may affect the functions of LL-37 *in vivo*. Lopez-Garcia and colleagues showed that the effect of LL-37 against *C. albicans* was inactivated in high ionic solutions that mimicked human sweat (Lopez-Garcia *et al.*, 2005). Bergsson and colleagues demonstrated that interaction of LL-37 and glycosaminoglycans as well as cleavage of exogenous LL-37 by protease cathepsin D and neutrophil elastase inhibited the ability of LL-37 to kill *Pseudomonas aeruginosa* that caused cystic fibrosis in lungs (Bergsson *et al.*, 2009). In addition, the antibacterial activity of LL-37 was inhibited by human serum (Johansson *et al.*, 1998). LL-37 is commonly produced at a concentration of ~2–5 μg/ml (~0.44–1.1 μM) at mucosal surfaces (Bowdish *et al.*, 2005), and can be reached to 30–1,500 μg/ml (~6.6–330 μM) in infection or inflammation (Nijnik and Hancock, 2009). The impacts of various environmental factors on our findings will be investigated in future studies.

Conclusion

We have demonstrated a connection between LL-37 and the cell wall of *C. albicans*. LL-37 treatment affected the cell wall remodeling process. In addition, the differentially expressed genes in response to LL-37 treatment were identified. This study provides important insights for the future use of AMPs in a clinical setting, and also suggests that LL-37 can be a promising candidate for antifungal therapy.

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