Efg1 Controls Caspofungin-Induced Cell Aggregation of *Candida albicans* through the Adhesin Als1

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Received 28 July 2011/accepted 18 October 2011

Echinocandin drugs such as caspofungin (CASP), micafungin, and anidulafungin inhibit fungal cell wall biogenesis by blocking Fks1-mediated β-glucan deposition into the cell surface. Candida have become suitable drugs to treat life-threatening diseases caused by several fungal species, including *Candida albicans*, that are pathogenic for humans. Here, we present the discovery of a novel CASP-induced flocculation phenotype of *C. albicans*, which formed large cell aggregates in the presence of CASP. High concentrations of sugars such as mannose or glucose inhibit CASP-induced flocculation and improve survival of *C. albicans* cells exposed to CASP. Notably, exposure of *C. albicans* cells to CASP triggers Efg1-dependent expression of the adhesin ALS1 and induces invasive growth on agar plates. Indeed, cells lacking either Efg1 or Als1 show strongly diminished CASP-induced flocculation, and the absence of Efg1 leads to marked CASP hypersensitivity. On the other hand, CASP-induced invasive growth is enhanced in cells lacking Efg1. Hence, CASP stress drives an Efg1-dependent response, indicating that this multifunctional transcriptional regulator, which is otherwise involved in filamentation, white-to-opaque switching, and virulence, also modulates cell wall remodeling upon CASP challenge. Taken together, our data suggest that CASP-induced cell wall damage activates Efg1 in parallel with the known cell integrity stress signaling pathway to coordinate cell wall remodeling.

Fungi that are pathogenic for humans have emerged as an important public health problem during the past decades. Systemic or disseminated infections in immunocompromised individuals caused by fungi such as *Candida, Aspergillus*, and *Cryptococcus* spp. are often associated with high mortality rates (39). Clinical therapy for treatment of invasive fungal infections involves a classic repertoire of antifungal drugs, including amphotericin B, fluconazole, and itraconazole. However, a number of adverse side effects, fungistatic rather than fungicidal activity, and increasing drug resistance led to the development of second-generation azoles such as voriconazole, posaconazole, and ravuconazole as well as of compounds with a novel mechanism of action such as the echinocandins (10–11).

Echinocandins such as caspofungin (CASP), micafungin, and anidulafungin are semisynthetic lipopeptides produced via chemical modification of fungal secondary metabolites. Caspofungin, for example, is synthesized from pneumocandin B0 from *Glucaea lozoyensis* (28). In contrast to azoles, which block ergosterol biosynthesis, the fungicidal action of echinocandins is mediated via inhibition of biosynthesis of β-(1,3)-β-glucan, an essential cell wall component unique to the fungal kingdom and absent in the mammalian host (12, 26).

*Candida* spp. represent the major fungal pathogens affecting humans. In addition to causing superficial mucosal conditions such as thrush and vaginitis, they can cause life-threatening systemic infections (6) in immunocompromised individuals that result in mortality rates exceeding 50% (39). Thus, the commensal *Candida* spp. can colonize a variety of host niches. During adherence to host tissues and organs or following invasion, fungal pathogens encounter various host defense mechanisms, some of which impose diverse forms of stress such as variable pH, carbon dioxide, metal ion deprivation, hormones, temperature changes, oxidative stress, and nutrient limitation. Furthermore, *C. albicans* has to cope with host immune surveillance, as well as with treatments that include antifungal drugs, posing additional stresses that compromise survival. Thus, fungal cells must constantly adapt to changing environmental conditions, mainly by activating signaling pathways that coordinate adaptive stress responses (33). Biofilm formation is an efficient way for *C. albicans* to become hyperresistant to azole antifungals. In *Saccharomyces cerevisiae*, flocculation contributes to stress resistance (9, 49).

The molecular target of echinocandins in *C. albicans* is the FKS1 subunit of the β-(1,3)-β-glucan synthase (12). Resistance to CASP is frequently associated with mutations in the FKS1 gene (1, 17). An efflux-based mechanism that operates through the Cdr2 ABC transporter may also contribute to CASP tolerance in laboratory strains as well as in clinical isolates (47). *C. albicans* cells rapidly respond to CASP treatment, since cell wall stress triggers the *C. albicans* cell wall integrity protein kinase C signaling pathway (PKC). Within a few minutes following exposure to CASP, mitogen-activated protein kinase
is activated when cells grow on surfaces. In addition, Mkc1 calcium bursts as well as low temperature, implying cross-talk between signaling pathways such as oxidative stress, osmotic stress, and the Efg1-dependent regulon in parallel with other stress-induced signaling pathways such as the cell wall integrity pathway that operates to provide protection from further damage and to coordinate cell wall remodeling during antifungal stress.

**MATERIALS AND METHODS**

*C. albicans* strains, growth conditions, and growth inhibition assays. The *C. albicans* strains used in this study are listed in Table 1. Gene deletions were created by using the C.m.LEU2 and C.A.HIS1 marker cassettes as described in reference 36. Rich (yeast extract-peptone-dextrose [YPD]) medium and synthetic complete (SC) medium, supplemented with appropriate auxotrophic components, were prepared as described elsewhere (23a). Unless otherwise indicated, strains were grown routinely at 30°C. RPMI 1640 was obtained from PAA Laboratories (Pasching, Austria). Caspofungin (Merck & Co., Whitehouse Station, NJ) and Calcofluor White M2R (Sigma-Aldrich, Vienna, Austria) were dissolved in dimethyl sulfoxide and added to RPMI 1640, followed by NaOH to a pH of 7.4. Plates were scanned using an HPScanjet G3010 Scanjet and Adobe Photoshop CS3 software.

**Flocculation and survival assays.** Flocculation was measured as described previously (23), using minor modifications. Strains were routinely grown to the early logarithmic growth phase in YPD medium before CASP was added. Alternatively, cells grown to the late exponential growth phase in YPD were diluted in YPD supplemented with additional glucose or mannose (2.5% to 17% [wt/vol]) and grown to the early-logarithmic-growth phase before the addition of CASP. Unless otherwise indicated, cells were treated for 3 h before the OD_{600} was determined directly after swirling of the cuvette (OD1) and some 15 min later without swirling (OD2). The calculated ratio R = (OD1 × 100)/OD2 represents the percentage of cells still in suspension, shown as the percentage of surviving cells by assuming a lag phase of 30 min and a generation time of 60 min. Colony growth was inspected and recorded after 48 to 72 h of incubation at 30°C. Plates were scanned using an HPScanjet G3010 Scanjet and Adobe Photoshop CS3 software.

**TABLE 1. Fungal strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Short name</th>
<th>Genotype(s)</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA-IF100</td>
<td></td>
<td>arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/ his1Δ, URA3/ura3Δ</td>
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</tr>
<tr>
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<td>ura3Δ::imm434/ura3Δ::imm434</td>
<td></td>
</tr>
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Here, we isolated and investigated a novel flocculation phenotype evoked in CASP-exposed *C. albicans* cells. Interestingly, high concentrations of sugars in the growth medium inhibited CASP-induced flocculation and considerably reduced the fungicidal activity of CASP toward *C. albicans* cells. Exposure of *C. albicans* to subinhibitory CASP concentrations resulted in Efg1-dependent expression of ALS1 encoding a major fungal adhesin. Interestingly, we identified Als1 as a critical factor required for CASP-induced flocculation, suggesting that flocculation is a fungal protection mechanism whose purpose is to counteract the effects of extensive cell surface damage. These data show an Efg1-dependent stress response to CASP challenge of *C. albicans*. Our data suggest that CASP-induced cell wall damage triggers activation of a subset of genes from the Efg1-dependent regulon in parallel with other stress-induced signaling pathways such as the cell wall integrity pathway that operates to provide protection from further damage and to coordinate cell wall remodeling during antifungal stress.
(Amersham Biosciences, Little Chalfont, Buckinghamshire, England). Northern blots were hybridized with PCR-amplified probes and radiolabeled by incorporation of [\( ^{32}P \)]dCTP and the use of a MegaPrime labeling kit (Amersham). Methylene blue staining was used as a control to ensure equal RNA loading. The radiolabeled probes were added to the prehybridization solution after purification on a Nick column (Amersham). Membranes were washed three times at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% sodium dodecyl sulfate (SDS) and three times in 1× SSC-1% SDS and then exposed to X-ray films at −70°C for appropriate time periods.

Preparation of yeast extracts and immunoblotting. For immunoblotting, cell extracts were prepared by the TCA (trichloroacetic acid) method exactly as previously described (31). Overnight cultures were diluted to an OD\(_{600}\) of 0.2 and grown until they reached an OD\(_{600}\) of 1, before addition of CASP to the cultures. Aliquots were harvested after the indicated time intervals. Cell lysates equivalent to an OD\(_{600}\) of 0.5 were separated in 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany). Phosphorylated Mkc1 was detected using an anti-phospho-p44/42 MAPK (Thr\(^{202}\)/Tyr\(^{204}\)) antibody (Cell Signaling Technologies, Beverly, MA). Immunoblots were developed using an ECL chemiluminescence detection system under the conditions recommended by the manufacturer (Amersham).

Microscopy and viability assay. Microscopic analysis was performed using an IX81 microscope (Olympus). Images of living unfixed cells were captured using a Hamamatsu Orca-ER camera (Olympus) and CellR software (Olympus). For viability assays, untreated and CASP-treated cells were stained using FUN1 and Calcofluor White and a Live/Dead yeast viability kit (Molecular Probes, Eugene, Oregon) as recommended by the manufacturer. The plasma membrane integrity and metabolic function of yeasts determined the conversion of yellow-green fluorescent intracellular staining of FUN1 into images showing red-orange fluorescent intravacuolar structures. Images were manipulated by adjusting brightness and contrast to the same levels in all pictures taken with the same fluorescence filters (excitation settings of 579 to 596 nm and emission settings of 618 to 664 nm for red fluorescent proteins; excitation settings of 457 to 487 nm and emission settings of 503 to 538 nm for green fluorescent proteins [GFP]) before merging images were created using CellR software. For measuring levels of GFP expressed by \( P_{\text{AAL}}-\text{GFP} \) reporter strains, whole cells were tagged for quantification. Intensity values were recorded using CellR software. Background values were subtracted, and mean values corresponding to a minimum of 100 cells for each set of conditions were calculated.

Agar invasion assay. Cells were grown on YPD plates for 4 days at 30°C before tap water was used to wash colonies from the agar surface. Plates were scanned from the bottom to visualize degrees of agar invasion. Cross-sections of colonies were analyzed using a Discovery V12 stereoscope equipped with an Axiocam MR5 camera (Zeiss) to determine levels of invasion.

RESULTS

CASP treatment causes flocculation of \( C. \) albicans. During our experiments performed with CASP-treated cells, we observed pronounced flocculation of \( C. \) albicans cultures in the presence of CASP. Cells grown in liquid YPD or RPMI medium started to flocculate approximately 60 min after CASP addition. In glass tubes, this effect was clearly visible macroscopically after 3 h of treatment with CASP at 10 and 100 ng/ml or left untreated. Cultures were photographed immediately after vortex mixing. Some 15 min later, the same tubes were measured again without vortex mixing. Values shown represent the results of the second measurement relative to those of the first measurement. All treatments and measurements were done in triplicate; standard deviations are shown as error bars. *; \( P < 0.05; \) **; \( P < 0.01 \) (Student’s \( t \) test) relative to the untreated control results. (C) High sugar concentrations inhibit CASP-induced flocculation. Wild-type strain SC5314 was grown to the exponential growth phase in YPD medium or RPMI medium supplemented with the indicated concentrations of mannose (Man). CASP was added at a final concentration of 100 ng/ml. After 3 h of CASP treatment, the optical density of the cultures was measured as described for panel B. *; **; ***; \( P < 0.001 \) (Student’s \( t \) test).

FIG. 1. \( C. \) albicans flocculation in response to CASP. (A) \( C. \) albicans wild-type strain (SC5314) growing in the exponential growth phase at 30°C in YPD or RPMI 1640 medium was treated for 3 h with CASP at 10 or 100 ng/ml or left untreated. Cultures were photographed 2 min after vortex mixing of the culture tubes. ctrl, control. (B) The cultures described for panel A were used for measuring the OD\(_{600}\) after 1 and 3 h of treatment. The optical density of the cultures was measured directly after vortex mixing. Some 15 min later, the same tubes were measured again without vortex mixing. Values shown represent the results of the second measurement relative to those of the first measurement. All treatments and measurements were done in triplicate; standard deviations are shown as error bars. *; \( P < 0.05; \) **; \( P < 0.01 \) (Student’s \( t \) test) relative to the untreated control results. (C) High sugar concentrations inhibit CASP-induced flocculation. Wild-type strain SC5314 was grown to the exponential growth phase in YPD medium or RPMI medium supplemented with the indicated concentrations of mannose (Man). CASP was added at a final concentration of 100 ng/ml. After 3 h of CASP treatment, the optical density of the cultures was measured as described for panel B. *; **; ***; \( P < 0.001 \) (Student’s \( t \) test).
B and i [46]). Moreover, CASP-induced flocculation occurred in clinical isolates of *C. tropicalis* and *C. lusitaniae* also (data not shown).

Cell aggregation might represent an active response by *C. albicans* for coping with CASP-induced cell wall alterations or might be, for example, a consequence of cell death. Even a subinhibitory concentration of CASP (10 ng/ml) induced flocculation (Fig. 1B). Notably, another cell wall-damaging compound, Calcofluor White (CFW), triggered similar cell aggregations (WT strain; see Fig. S1A and B in the supplemental material), indicating an active response to cell wall stress.

Induced cell-cell adhesion in fungi usually requires cell surface proteins (so-called “adhesins” or “flocculins”) that specifically bind amino acid or sugar residues on the surface of neighboring cells. One type of adhesion requires the lectin-like binding of adhesins to surface-exposed carbohydrate moieties. This can be competitively inhibited by certain sugars (56). To characterize the *C. albicans* flocculation response to CASP, we therefore used different concentrations of mannose to increase the sugar content of the YPD medium. After subjecting the cultures to 3 h of exposure to CASP, we quantified the degree of flocculation by measuring the sedimentation. In drug-treated, flocculating cultures, we again observed a 40% decrease in the OD₆₀₀. In untreated control cultures, the OD₆₀₀ decreased by only about 3% within the same period (Fig. 1C). Notably, supplementation with 2.5% and 5% mannose influenced flocculation only slightly. However, cellular aggregation was strongly reduced in cultures supplemented with 10% and 17% mannose (Fig. 1C). A similar effect on flocculation was observed in the presence of high glucose levels (see Fig. S1C in the supplemental material). Hence, high sugar concentrations significantly inhibited CASP-induced cell aggregation. To test the dependency of adhesion on sugar levels, we added a variety of different sugars and sugar alcohols to YPD liquid medium and measured flocculation in the presence of CASP. In addition to 10% mannose and 10% glucose, supplementation with fructose, sorbose, saccharose, galactose, and maltose inhibited CASP-induced flocculation. Likewise, pentose sugars such as xylose and arabinose as well as the sugar alcohols mannitol and sorbitol reduced flocculation in liquid cultures (see Fig. S1D in the supplemental material).

Furthermore, we asked whether high sugar concentrations also influence the antifungal efficacy of CASP. Hence, we used FUN1 staining to quantify survival of fungal cells after CASP treatment in the presence and absence of high sugar concentrations. The metabolic activities of yeast cells convert intracellular green fluorescent FUN1 staining into red fluorescent, intravacuolar signals (32). Fluorescence microscopy pictures were taken after 3 h of drug treatment, and viability was determined according to the results of red vacuolar staining. In YPD medium supplemented with CASP at 100 ng/ml, the vast majority of cells showed green cytoplasmic staining, indicating dead cells. In contrast, most cells grown in YPD medium containing 10% glucose retained their metabolic activity at the same CASP concentrations, indicating better survival and, thus, reduced CASP potency (Fig. 2A). With mannose, a similar increase in the rate of survival was observed (data not shown).

Interestingly, we observed that addition of 0.3 M NaCl also improved the survival of *C. albicans* cells exposed to CASP, even though flocculation remained unchanged (Fig. 2A). Thus, high external osmolarity might be advantageous for cells with damaged cell walls. To quantify cell survival, we split cultures of wild-type *C. albicans*, growing exponentially in YPD medium or YPD supplemented with additional glucose, NaCl, or KCl at the indicated concentrations, into two halves; one half was treated with CASP for 3 h, whereas the second half remained untreated. All cultures were then diluted and recovered in YPD medium for 6 h, followed by measurement of the OD₆₀₀ of the now nonflocculating cultures and calculation of the number of live cells present immediately after CASP treatment. This additional recovery step was required because of difficulties in obtaining reliable cell counts of flocculating cultures. The rates of cell survival are shown relative to untreated control culture results. Remarkably, the presence of glucose increased cell survival after CASP treatment severalfold compared to regular YPD growth results (Fig. 2B). Notably, even though microscopy indicated survival of most cells in the presence of high levels of sugar, only about 23% of cells were recovered compared to the untreated culture results. Hence, the CASP effect or the response to that effect might still impair cell proliferation, rendering a fraction of cells unable to recover from cell wall stress. Interestingly, we also observed an increase of survival in the presence of 0.2 and 0.3 M NaCl or 0.3 M KCl (Fig. 2B).

On agar plates supplemented with high glucose concentrations, *C. albicans* cells grew when exposed to CASP at up to 350 ng/ml. A gradient of glucose introduced into the agar plate clearly demonstrated improved survival with increasing concentrations of sugar (Fig. 2C). Similar positive effects on growth were observed with other types of sugars, including mannose, fructose, galactose, saccharose, and arabinose, albeit the degrees of improvement were slightly different (see Fig. S2A in the supplemental material).

At low CASP concentrations, addition of 10% glucose to YPD agar plates also improved growth of the CASP-hypersensitive *mkc1* mutant strain (Fig. 2D). However, only partial rescue was observed, since at CASP concentrations higher than 50 ng/ml, glucose failed to rescue growth of the *mkc1* mutant. These data indicated that Mk1 is not required for the protective effect of high levels of sugar in the presence of CASP. Moreover, high levels of external glucose are not sufficient to protect cells from severe defects in the cell wall organization of an *mkc1* mutant strain.

**Als1 mediates CASP-induced flocculation.** To further elucidate the mechanisms underlying CASP-dependent flocculation, we first focused on the *C. albicans* PKC pathway, since PKC signaling is required for the cell wall damage response and CASP tolerance in *S. cerevisiae* (43), as well as in *C. albicans* (59). Indeed, immunoblotting demonstrated rapid and strong Mk1 activation in the presence of CASP. However, Mk1 phosphorylation occurred both in normal YPD medium and in medium supplemented with high levels of sugar (see Fig. S2B in the supplemental material). This indicates that Mk1 mediates the response to cell wall stress even under conditions of high sugar levels. Furthermore, cell-cell aggregation was not decreased in a strain lacking Mk1 (data not shown). Hence, even though Mk1 mediates CASP tolerance and is activated in response to the drug, the flocculation response bypassed Mk1 signaling.
The aggregation of CASP-treated cells suggested overexpression of adhesin-like proteins on the cell surface. The ALS genes encode cell surface glycoproteins that mediate adherence of *C. albicans* to various surfaces, as well as cell-cell adhesion (reviewed in reference 22). Indeed, microarray data showed a pronounced induction of *ALS1* by CASP (data not shown). We confirmed the induction of *ALS1* expression by the use of GFP reporter strains (19). A strain expressing GFP...
under the control of the \textit{ALS1} promoter showed strongly increased fluorescent signals in the presence of CASP (Fig. 3A). Notably, this induction was specific for \textit{ALS1}, since we did not observe CASP-dependent promoter induction for \textit{ALS3}, \textit{ALS5}, \textit{ALS6}, \textit{ALS7}, or \textit{ALS9} (Fig. 3B). Als1 is a major cell surface adhesion molecule, mediating cell-cell adhesion. Moreover, Als1 is a downstream effector of the \textit{EFG1} regulatory pathway (16). Notably, Als1 is required for rapamycin-induced aggregation of \textit{C. albicans} (2). Thus, we hypothesized that Als1 also plays a role in the CASP-dependent flocculation phenotype. Indeed, the \textit{als1ΔΔ} mutant strain showed strongly reduced flocculation in liquid medium (Fig. 3C), confirming that the observed cellular aggregation in the presence of CASP requires \textit{ALS1}. Taken together, these data strongly support the idea that Als1 is the major cell surface protein necessary and sufficient for CASP-induced flocculation.

Interestingly, Als1-mediated flocculation was diminished by addition of various sugars but not by high external osmolarity in general, since adding NaCl and KCl failed to inhibit cellular aggregation (see Fig. S1D in the supplemental material). However, the Als-dependent adhesion was insensitive to the presence of sugar, since the proteins lacked the conserved PA14 domain required for lectin-like binding (8, 45). Hence, we asked whether \textit{ALS1} is still upregulated by CASP in the presence of high glucose concentrations. We repeated the GFP reporter assays with a strain harboring \textit{P}_{\text{ALS1}}-\text{GFP} in the presence of additional 10% glucose. Indeed, the quantification of fluorescence signals demonstrated a complete lack of CASP-
induced GFP expression in high-sugar medium (Fig. 3D). This suggests that the reduced flocculation was the result of a dampened stress response. Furthermore, the reduced flocculation was due to the lack of Als1 upregulation rather than a consequence of sugar binding to the adhesin.

**Efg1 is required for CASP tolerance and CASP-induced flocculation.** Northern analysis further showed that ALS1 induction in response to CASP also strictly required functional Efg1. CRH11, a glycosylphosphatidylinositol (GPI)-anchored cell wall transglycosylase whose expression is induced by CASP (7, 29, 38), is upregulated in both wild-type and efg1 mutant cells, whereas ALS1 expression is not induced in the mutant strain (Fig. 4A). Moreover, CASP-dependent flocculation was completely absent in cells lacking Efg1 (Fig. 4B). Notably, Efg1

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**FIG. 4.** Efg1 is required for CASP tolerance and flocculation. (A) Northern analysis of ALS1 expression. Wild-type SC5314 and efg1Δ/Δ strains were grown to the exponential growth phase followed by treatment with CASP at 10 ng/ml. Samples were taken at indicated time points. Total RNA was isolated, and mRNA levels of ALS1 were detected by Northern blotting. CRH11 served as a control for an Efg1-independent CASP-responsive gene and ACT1 as a loading control. (B) Wild-type SC5314 and efg1Δ/Δ cells in the exponential growth phase at 30°C in glass tubes were treated with CASP at 80 ng/ml for 3 h. Cultures were photographed 2 min after vortex-mixing (upper panel). To quantify flocculation, the OD600 of cultures was measured directly after vortex-mixing. The same tubes were measured again without prior mixing at 5 min, 10 min, 15 min, and 20 min after the first measurement. Treatments and measurements were done in triplicate; standard deviations are indicated as error bars (lower panel). *, P < 0.01; **, P < 0.001 (Student’s t test). (C) Identical volumes of 10-fold serial dilutions of two independent efg1 mutant strains were spotted onto YPD plates containing CASP at 250 ng/ml along with their parental strains (SC5314 and CAI-4). Colony growth was inspected after 48 h of incubation at 30°C. (D) Strains CAYF5 (PADH1-ALS1) and CADS1 (PADH1-ALS1-efg1Δ/Δ) and the corresponding wild type were grown to the exponential growth phase at 30°C in synthetic medium before treatment with CASP at 10 or 50 ng/ml for 3 h. The optical density of the cultures was measured directly after vortex-mixing. Some 15 min later, the same tubes were measured again without vortex-mixing. Values shown represent the results of the second measurement relative to the results of the first measurement. All treatments and measurements were done in triplicate; standard deviations are shown as error bars. *, P < 0.05; **, P < 0.01 (Student’s t test) relative to the untreated control results.
is a master transcriptional regulator of many biological and pathophysiological processes, including morphogenesis and filamentation (54), white-to-opaque switching (21, 51), biofilm formation (44), and even virulence (20, 52). Efg1 also plays a major role in the regulation of cell wall genes and, as a further consequence, in the cell wall organization of \textit{C. albicans} (50).

We therefore tested two independent homozygous \textit{efg1Δ/Δ} deletion strains for CASP susceptibility. Indeed, the lack of Efg1 led to pronounced CASP hypersensitivities in SC5314 as well as CAI-4 (Fig. 4C). Moreover, growth experiments performed with CASP-treated \textit{efg1Δ/Δ} cells demonstrated a protective effect of high external osmolarity on cell survival, since the addition of sugar or sorbitol clearly improved survival of \textit{efg1Δ/Δ} cells in the presence of CASP (see Fig. S2C in the supplemental material). Whereas CASP hypersensitivity can be linked to Efg1 function in cell wall gene regulation and might be caused by an altered cell wall composition in the mutant, the lack of flocculation in the absence of Efg1 suggests an active role for this master regulator in the cellular response to CASP that is mediated by regulating expression of the cell surface adhesin Als1.

Overexpression of Als1 leads to extensive flocculation, as shown earlier by Fu et al. (16). With our sedimentation assay, we confirmed the enhanced aggregation of strains overexpressing \textit{ALS1} under the control of the \textit{ADH1} promoter, even in the absence of Efg1 (Fig. 4D). Addition of CASP slightly increased sedimentation in the \textit{efg1Δ/Δ} background. This effect might have been caused by differences in the cell wall composition of the mutant strain. Furthermore, we cannot exclude the possibility that additional Efg1-independent factors contribute to Als1-mediated cellular aggregation in the presence of CASP.

**CASP induces agar invasion.** \textit{C. albicans} cells respond to contact with solid agar media by activating Mkcl, leading to agar invasion (25). Hence, we tested whether CASP, which otherwise also activates Mkcl, affects agar invasion. \textit{C. albicans} wild-type cells were spotted onto YPD agar plates containing CASP at 10 ng/ml, 50 ng/ml, and 100 ng/ml. After 3 days of incubation at 30°C, cells were washed off the plates. At a CASP concentration of 50 ng/ml or higher, colonies adhered much better to the agar plate compared to the results seen in the absence of the drug (Fig. 5A), indicating enhanced agar invasion. Invasion of \textit{C. albicans} cells into semisolid medium is thought to require Mkcl but to be repressed by Efg1 (25). Therefore, we tested an \textit{efg1Δ/Δ} deletion strain for invasive growth on CASP agar plates. In keeping with its role as a repressor of invasive growth, we found that the lack of Mkcl increased adherence in the presence of low CASP concentrations compared to the results seen with its parental strain (Fig. 5B). Cross-sections of the agar beneath the spotted wild-type and \textit{efg1Δ} mutant colonies showed that growth on YPD agar plates supplemented with CASP at 50 ng/ml induced formation of very dense but short filaments. The \textit{efg1Δ} mutant showed a dense layer of short filaments at a much lower concentration of 10 ng/ml compared to the wild-type control (Fig. 5C). Due to the CASP hypersensitivity of the \textit{efg1Δ} mutant strain, we were unable to compare the invasive growth phenotypes at higher drug concentrations. Hence, based on the available data, it seems reasonable to conclude that induction of invasive growth triggered by CASP is repressed by Efg1.

Taken together, our results identify Efg1 as a central regul-
findings provide novel insights into the mechanisms that control signaling cascades and downstream effector molecules. Our results show that (i) Efg1 is required for basic CASP tolerance, (ii) the upregulation of an Efg1-targeted adhesin in the presence of CASP, whereas Efg1 normally acts as an inhibitor of invasive growth.

DISCUSSION

CASP treatment strongly affects fungal cell wall homeostasis by blocking proper cell wall biosynthesis, thereby also activating several mechanisms of adaptive stress responses. This is consistent with the fact that cell wall biogenesis and maintenance in *C. albicans* are regulated by a complex network of signaling cascades and downstream effector molecules. Our findings provide novel insights into the mechanisms that contribute to drug-induced stress signaling in *C. albicans*, which is the most prevalent fungus that is pathogenic for humans. Here, we show that (i) Efg1 is required for basic CASP tolerance, (ii) CASP triggers an Efg1-dependent response that is responsible for increased cell-cell adhesion, (iii) CASP induces invasive growth that is negatively regulated by Efg1, and (iv) high sugar or salt concentrations reduce the antifungal potency of CASP.

**Efg1 triggers flocculation in response to cell wall stress in C. albicans.** Treatment of *C. albicans* with CASP results in rapid cell aggregation. In liquid medium supplemented with CASP, cells flocculate and large cell aggregates are visible under the microscope (Fig. 2A). Similar cell aggregation occurs in the presence of CFW. Notably, others have also noticed clumping of *C. albicans* cells in the presence of micafungin (48). This indicates that cell wall damage severely changes cell surface hydrophobicity and, thus, adhesion properties, favoring cell-cell adhesion. However, at concentrations that trigger activation of the cell integrity pathway, caffeine and Congo red fail to induce cellular aggregation (data not shown), indicating that this response does not require the PKC pathway. Our data demonstrate that CASP-triggered cell-cell adhesion also occurs at sublethal concentrations (Fig. 1A and B), implying that it is likely to represent an active response rather than a consequence of cell death.

Interestingly, we show that the major adhesin mediating CASP-induced aggregation is Als1, since *ALS1* mRNA levels are strongly upregulated following addition of CASP. As predicted, deletion of Als1 results in strongly diminished flocculation (Fig. 3 and 4A). A number of functionally and/or structurally related adhesin molecules are known to occur in fungi. Many of them, such as members of the ALS family in *C. albicans*, are implicated in adhesion and cell aggregation (13, 22). Notably, although a total of 8 ALS proteins have been shown to exist in *C. albicans*, CASP-induced flocculation as shown here requires only Als1. Microarray experiments confirmed the dedicated Als1 function in flocculation, since none of the other ALS genes were regulated under these conditions (data not shown). Additionally, constitutive cellular aggregation of cells overexpressing *ALS1* was observed (16) (Fig. 4D). *C. albicans* ALS proteins contain amyloid-forming domains. Amyloid-binding dyes attenuate aggregation of *S. cerevisiae* cells expressing *C. albicans* Als1 or Als5, suggesting that amyloid formation plays a role in *C. albicans* cell-cell adhesion (37, 40, 42). We cannot exclude the possibility that this mechanism is also involved in CASP-induced cellular aggregation. However, we are unable to provide experimental evidence, because the concentrations of amyloid-binding dyes required for inhibition of cell aggregation severely reduced cell viability under our experimental conditions with the strains we used. Furthermore, the results of our studies imply a novel role for Efg1 as a critical downstream regulator of the CASP response, since induction of *ALS1* strictly requires Efg1 (Fig. 4A). This is consistent with previous data that identified Efg1 as a regulator of *ALS1* expression under filamentation-inducing conditions (16) as well as in the presence of rapamycin, which blocks TOR signaling (2). The lack of Efg1 caused marked CASP hypersensitivity (Fig. 4C). However, deletion of *ALS1* failed to cause reduced CASP tolerance (data not shown). Another study addressing the role of Efg1 in biofilm formation and filamentation failed to show CASP hypersensitivity of *efg1Δ/s* cells (60). However, flocculating and nonflocculating cells cannot be easily compared in liquid culture, and MIC determinations have been shown to give results different from those of drug sensitivity tests on agar plates.

**EFG1** is known to play pivotal roles in regulating expression of cell wall genes during morphogenesis as well in its activity as a downstream effector of the PKA signaling pathway (4). Even in the *C. albicans* yeast form, transcriptional levels of numerous cell wall genes are strongly reduced in *efg1A/d* cells (50). Hence, the prominent role of Efg1 in regulating cell wall genes, and the upregulation of an Efg1-targeted adhesin in the presence of CASP, hints that Efg1 is a central regulator of signaling networks that mediate CASP response, as well as of cell-cell adhesion.

Invasive growth on semisolid surfaces was previously found to be connected with activation of the Mkc1 MAPK (25), which is also rapidly activated by CASP. Hence, it is likely that the increased agar invasion of cells lacking Efg1 is also regulated by Mkc1. Since Efg1 normally acts as a repressor of agar invasion (25), we hypothesize an additional and dual role for
this pleiotropic transcriptional regulator in the CASP response. On the one hand, Efγ1 drives ALS1 induction following CASP-induced cell surface damage, while on the other hand, it represses agar invasion.

A tantalizing and still open question remains concerning whether flocculation of *C. albicans* cells provides an advantage for stressed cells. If one considers flocculation a social trait in fungi (49), one might speculate that such cooperative behavior promotes survival of cells under adverse conditions or in clinical settings in patients undergoing antifungal therapy. This may contribute to the *in vivo* “survival” or selection of persisting cells, which could thus escape the effects of antifungal treatment. Interestingly, *in vitro* CASP treatment at a high dosage causes a trailing growth effect or paradoxical growth effect such that a fraction of *C. albicans* cells survive otherwise fungicidal drug activity and escape by resuming growth. Our data could provide an additional explanation for these clinically relevant observations in various fungal pathogen studies (3, 53).

On the basis of our data, we propose the following model for the mechanisms governing the *C. albicans* response to CASP-induced cell surface damage (Fig. 6). Mkc1 activates expression of cell wall remodeling and homeostasis genes in response to CASP (our unpublished data). The PKC pathway might promote agar invasion by relieving Efγ1-dependent repression, most likely via Czf1, a regulator of morphogenesis and filamentous growth opposing Efγ1 functions (57–58). It has been suggested that induction of invasive hyphae on laboratory growth medium may resemble mechanisms involved in tissue penetration. Our *in vitro* observations might relate to the response of *C. albicans* to CASP treatment *in vivo*. However, to the best of our knowledge, clinical studies have not addressed whether clinical isolates from patients undergoing CASP therapy display pronounced inversion phenotypes.

Sugar molecules increase *C. albicans* survival in the presence of CASP. Flocculation mediated by the *FLO1* adhesin may be a major mechanism protecting *S. cerevisiae* cells from environmental stress conditions (49). Whether cell wall stress-induced flocculation has a protective role for *C. albicans in vivo* remains an open question. We hypothesize that flocculation is a consequence of dynamic changes in the cell wall composition to compensate for defects caused by echinocandins.

Even though *C. albicans* ALS gene products were previously considered sugar-independent adhesins (24), we observed that addition of sugars strongly inhibited flocculation (Fig. 1C and 2A). This was most likely owing to a lack of Als1 upregulation in the presence of high sugar concentrations. Initially, we thought that if aggregation protected cells located in the center of large cell aggregates, the presence of high sugar concentrations would reduce survival of CASP-treated cells. Interestingly, we observed that high concentrations of sugars such as mannose and glucose make *C. albicans* cells refractory to CASP killing (Fig. 2), thereby diminishing CASP efficacy. Hence, our analysis uncovered a protective function of high sugar concentrations in response to CASP. High sugar concentrations increase external osmolarity, with a protective effect on cellular survival when the cell wall is damaged. Consistently, supplementing the growth medium with NaCl or KCl also increased *C. albicans* survival in the presence of CASP. Notably, the Hog1 kinase that senses stress associated with osmolarity is also involved in the response to CASP (34, 59). Thus, high external osmolarity is likely to stabilize damaged cell walls, thereby counteracting CASP activity or the transcriptional response to CASP. In cells pretreated with high levels of osmolarity, the Hog1-mediated response is transiently activated, which might lead to cross-protection against cell wall stress. Interestingly, only high sugar concentrations and not salt concentrations can dampen the stress response mediated by Efγ1 by preventing Als1 upregulation. However, phosphorylation of Mkc1 demonstrated the activation of the PKC pathway under the same conditions. This confirms that cell surface stress signaling can activate Efγ1 independently of PKC signaling.

In summary, our results link Efγ1, which is otherwise involved in filamentation, white-to-opaque switching, biofilm formation, and environmental signaling as well as other cellular processes, to signaling pathways responding to CASP-induced damage (34, 41, 61). Therefore, our results are consistent with a model that involves the activation of parallel and/or (at least in part) functionally redundant signaling pathways that appropriately and efficiently counteract cell surface damage or impaired cell wall homeostasis. Our unpublished observations seem to exclude the involvement of the cyclic AMP (cAMP)-PKA signaling pathway in CASP-induced Efγ1 activation, since a cdc35 deletion mutant strain, which is defective in cAMP production and therefore most likely lacks PKA activity, was able to flocculate in response to CASP (data not shown). Thus, it would be interesting to identify the as-yet-unknown upstream signaling molecules activating Efγ1 in the presence of CASP, as well as the regulatory mechanism by which Efγ1 activates expression of ALS1.

**ACKNOWLEDGMENTS**

We are indebted to Lois Hoyer for sharing ALS reporter strains and to Yue Fu for als1 deletion and overexpression strains. Michael Tscherner, Denes Hnisz, and Tobias Schwarzmuller are thankfully acknowledged for helpful discussions, strains, and technical help. This work was supported by the EU-FP6 project EURESFUN (STREP-CT-518199; K.K. and S.R.) and in part by the Christian Doppler Society and the UNICELLSYS FP7-Integrated Project (K.K.).

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