Defensins from Insects and Plants Interact with Fungal Glucosylceramides*

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Growth of the yeast species Candida albicans and Pichia pastoris is inhibited by RsAFP2, a plant defensin isolated from radish seed (Raphanus sativus), at micromolar concentrations. In contrast, gcs-deletion mutants of both yeast species are resistant toward RsAFP2. GCS genes encode UDP-glucosylceramide glucosyltransferases, which catalyze the final step in the biosynthesis of the membrane lipid glucosylceramide. In an enzyme-linked immunosorbent assay-based binding assay, RsAFP2 was found to interact with glucosylceramides isolated from P. pastoris but not with soybean nor human glucosylceramides. Furthermore, the P. pastoris parental strain is sensitive toward RsAFP2-induced membrane permeabilization, whereas the corresponding gcs-deletion mutant is highly resistant to RsAFP2-mediated membrane permeabilization. A model for the mode of action of RsAFP2 is presented in which all of these findings are linked. Similarly to RsAFP2, heliomicin, a defensin-like peptide from the insect Heliotis virescens, is active on C. albicans and P. pastoris parental strains but displays no activity on the gcs-deletion mutants of both yeast species. Furthermore, heliomicin interacts with glucosylceramides isolated from P. pastoris and soybean but not with human glucosylceramides. These data indicate that structurally homologous antifungal peptides present in species from different eukaryotic kingdoms interact with the same target in the fungal plasma membrane, namely glucosylceramides, and as such support the hypothesis that defensins from plants and insects have evolved from a single precursor.

Innate immunity is an ancient defense strategy used by multicellular organisms to control the natural flora and combat pathogens. This strategy involves, among other responses, the production of cationic antimicrobial peptides (AMPs) that generally have a broad activity spectrum. Until now, only one class of AMPs is found to be conserved among plants, invertebrates, and vertebrates, namely defensins. Defensins are small, highly basic cysteine-rich peptides that share a common three-dimensional structure (for review, see Ref. 1). The global fold of plant defensins comprises a cysteine-stabilized αβ motif consisting of an α-helix and a triple-stranded β-sheet, organized in a βαββ architecture and stabilized by four disulfide bridges. Most plant defensins possess antifungal or antibacterial activity in vitro but are noncytotoxic to either mammalian or plant cells. Insect defensins combine an α-helix and a double-stranded β-sheet stabilized by three disulfide bridges, organized in a cysteine-stabilized αβ motif as found in plant defensins (2, 3). An even higher homology to plant defensins is found for the insect defensin-like peptide heliomicin, which carries a triple-stranded β-sheet in a βαββ fold forming a cysteine-stabilized αβ motif (4). In addition to similarities in their global folds, heliomicin and the radish plant defensin RsAFP2 exhibit very similar distributions of hydrophobic residues (4) and display similar biological activities: both peptides are antifungal rather than antibacterial (5, 6).

Most cationic antimicrobial peptides induce membrane permeabilization after initial electrostatic binding to negatively charged phospholipids on the target cell surface. In contrast, plant defensins induce membrane permeabilization through specific interaction with high affinity binding sites on fungal cells (7–9). Via a genetic complementation approach, IPT1 was identified as a gene determining sensitivity toward the dahlia plant defensin Dm-AMP1 in Saccharomyces cerevisiae (10). IPT1 encodes an enzyme involved in the last step of the synthesis of the sphingolipid mannosyldinositolphosphorylceramide (M(IP)₃C) (11). Strains with a nonfunctional IPT1 allele lacked M(IP)₃C in their membranes bound significantly less DmAMP1 compared with the parental S. cerevisiae strain and were highly resistant to DmAMP1-induced membrane permeabilization. Possibly, membrane patches containing sphingolipids act as binding sites for DmAMP1. Interaction between DmAMP1 and these sphingolipids could lead to insertion of the plant defensin into the membrane resulting in membrane destabilization (10, 12).

In the yeast S. cerevisiae, the main sphingolipids are (mannosylated) inositolphosphorylceramides (namely IPC, MIPC, and M(IP)₃C). There is growing evidence that fungi maintain two separate pools of ceramides to be used for the synthesis of different sphingolipids (13–15). Ceramide backbones with very long chain C₂₄ and C₂₆ fatty acids bound to the sphingobase 4-hydroxyxyphospho-4-hydroxyxyphosphoglycolic acid are directed to the synthesis of the inositolphosphoryl-containing sphingolipids (Fig. 1A), whereas ceramide backbones with C₁₆ or C₁₈ fatty acids linked to the...
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P. pastoris and C. albicans Mutant Strains Lacking GlcCer

Antifungal Activity Assay—Antifungal activity of protein samples against yeast strains was assayed by microscopic analysis of glucosylceramide (GlcCer) (Fig. 1B). GlcCer have never been found in S. cerevisiae under various culture conditions (16). This is in contrast with many fungal and yeast species in which both inositol-phosphoryl-containing sphingolipids and significant amounts of GlcCer have been detected (17, 18).

S. cerevisiae is resistant toward the radish plant defensin RsAFP2 and completely lacks GlcCer, which points toward a possible role of GlcCer in RsAFP2-mediated growth inhibition. To address this hypothesis, RsAFP2 sensitivity of Pichia pastoris and Candida albicans was evaluated. In contrast to S. cerevisiae, membranes of both yeast species contain GlcCer (16, 19). In this paper, we present for the first time evidence that the antifungal plant defensin RsAFP2 and the highly homologous insect defensin-like peptide heliomicin interact with fungal GlcCer in a first step leading to fungal growth arrest.

EXPERIMENTAL PROCEDURES

Materials and Microorganisms—The antifungal peptides DmAmp1, RsAFP2, and RsAFP2-Y38G were isolated as described previously (6, 20, 21). Nonimmune and anti-RsAFP2 sera from rabbit were used as described previously (6). Helicin and anti-helicin rabbit anti-serum were kindly provided by Dr. P. Bulet (Institut de Biologie Moleculaire et Cellulaire, CNRS, Strasbourg, France) and Dr. J. L. Dimarcq (Entomed, Strasbourg, France), respectively. Anti-GlcCer serum from rabbit (22) was kindly provided by Dr. L. Brade (Research Center Borstel, Center for Medicine and Biosciences, Borstel, Germany). Ergosterol, phosphatase-coupled goat anti-rabbit immunoglobulins, and GlcCer isolated from human (Gaucher’s spleen) were purchased from Sigma. GlcCer isolated from soybean was purchased from Avanti Polar Lipids (Alabaster, AL). SYTOX Green was obtained from Molecular Probes (Eugene, OR). Yeast strains used in this study are S. cerevisiae strain BY4741 (Invertrogen), P. pastoris strain OS115 (Invitrogen) and the corresponding P. pastoris gcs-deletion strain (15), C. albicans strain SC5314 CA41 (23) and the corresponding C. albicans gcs-deletion strain (13).

Antifungal Activity Assay—Antifungal activity of protein samples against yeast strains was assayed by microscopic analysis of liquid cultures grown in microtiter plates as described previously (8–10). Growth medium used was either PDB (24 g/liter potato dextrose broth, Sigma) or PDB/YPD (24 g/liter PDB; 2 g/liter yeast extract, Difco; 4 g/liter Peptone, Difco) supplemented with 0.25 M SDS (Sigma) or PDB/YPD (24 g/liter PDB; 2 g/liter yeast extract, Difco; 4 g/liter Peptone, Difco) supplemented with 50 mM Sigma) or PDB/YPD (24 g/liter PDB; 2 g/liter yeast extract, Difco; 4 g/liter Peptone, Difco) supplemented with 50 mM Sigma). Growth medium used was either PDB (24 g/liter potato dextrose broth, Sigma) or PDB/YPD, pH 7.0, in the presence of either the antagonizing compound or distilled water. After 30 min of preincubation, the antifungal activity of RsAFP2 was assayed by incubating 10 μl of an overnight P. pastoris culture in 1 ml of fresh PDB/YPD, pH 7.0, and 50 μl of fresh PDB/YPD, pH 7.0. Hundred-fold dilutions of cell suspension were incubated with RsAFP2 in white 96-well microtiter plates (PE white; PerkinElmer Life Sciences) for 2 h at room temperature, after which the fluorescence was measured as described previously (9). Membrane permeabilization induced by P. pastoris and C. albicans Mutant Strains Lacking GlcCer Are Resistant to RsAFP2—To investigate a possible role of GlcCer in RsAFP2-mediated growth inhibition, mutants of P. pastoris and C. albicans which are completely devoid of GlcCer were used (13). The gcs-deletion mutants and their corresponding parental strains were tested for sensitivity to growth inhibition by RsAFP2. C. albicans and P. pastoris are sensitive to RsAFP2 at concentrations of 1–2 μM and higher, whereas the corresponding gcs-deletion strains are at least 20-fold more resistant to RsAFP2 (Table I). To test whether the GCS gene is also involved in conferring sensitivity toward other plant defensins, the gcs-deletion strains and the corresponding parental strains were tested for sensitivity to growth

### Table I

| Yeast species       | MIC (μM) |
|---------------------|----------|
|                     | RsAFP2   | DmAmp1 | Helicin |
| S. cerevisiae       | >400     | 0.32    | >20     |
| S. pombe            | >400     | ND      | ND      |
| P. pastoris         | 2.0      | 1.25    | 2.5     |
| P. pastoris gcs-deletion mutant | >400 | 1.25 | >400 |
| C. albicans         | 2.5      | 5.0     | 2.5     |
| C. albicans gcs-deletion mutant | >400 | 5.0 | >20     |

* MIC values are the minimal concentrations (μM) of the peptides required to inhibit the growth of a yeast strain by 10%. Data are the means of duplicate measurements. Standard errors were typically below 6.5%.

# Microplate Binding Assay (ELISA)—Interaction of antifungal peptides with GlcCer was evaluated by using an ELISA-based assay as described previously (27–30). Stock solutions of all glycolipids were prepared in methanol:chloroform:water (16:16:5, v/v/v) at a concentration of 500 μg/ml. Lipids were applied in 75-μl aliquots to the wells of microtiter plates and allowed to dry overnight at room temperature. All subsequent handling steps were performed at 37 °C. Blocking buffer was 1% (w/v) gelatin (cold fish skin, Sigma) in phosphate-buffered saline, and washing buffer was 1% blocking buffer. Anti-RsAFP2 and anti-helicin rabbit antisera and phosphatase-coupled goat anti-rabbit immunoglobulin were 1,000-fold diluted in washing buffer. Pooled values are means of triplicates adjusted for the plate background. Plate background values are the absorbance readings of methanol:chloroform:water (16:16:5, v/v/v)-coated wells incubated with peptides and antisera.

### RESULTS

P. pastoris and C. albicans Mutant Strains Lacking GlcCer Are Resistant to RsAFP2—To investigate a possible role of GlcCer in RsAFP2-mediated growth inhibition, mutants of P. pastoris and C. albicans which are completely devoid of GlcCer were used (13). The gcs-deletion mutants and their corresponding parental strains were tested for sensitivity to growth inhibition by RsAFP2. C. albicans and P. pastoris are sensitive to RsAFP2 at concentrations of 1–2 μM and higher, whereas the corresponding gcs-deletion strains are at least 20-fold more resistant to RsAFP2 (Table I). To test whether the GCS gene is also involved in conferring sensitivity toward other plant defensins, the gcs-deletion strains and the corresponding parental strains were tested for sensitivity to growth
and the fatty acid moieties of fungal and plant GlcCer were analyzed by reversed phase HPLC after hydrolysis of the purified GlcCer and conversion of the sphingobases into their 2,4-dinitrophenol derivatives. Whereas soybean GlcCer contain mainly the diunsaturated sphingobases (4E,8E)-sphinga-4,8-diene (d18:2\textsubscript{trans},trans) and (4E,SE)-sphinga-4,8-diene (d18:2\textsubscript{trans},cis), the dominating sphingobase of GlcCer from \textit{P. pastoris} is diunsaturated but carries a methyl group at C-9 (4E,SE)-9-methylsphinga-4,8-diene (9-methyl-d18:2\textsubscript{trans},trans) (Fig. 2 and Table II). Two additional glycolipids were used as controls in the assay. GlcCer from human (Gaucher's) spleen containing mainly (4E)-sphing-4-ename (d18:1\textsubscript{trans}) (Sigma; Matreya Inc., State College, PA) and monogalactosyldiacylglycerols from soybean, which contain a \(\beta\)-galactosyl headgroup and a diacylglycerol backbone instead of ceramide. The fatty acids of \textit{P. pastoris} and soybean GlcCer differ only in the carbon chain length. Although soybean GlcCer contain saturated C\textsubscript{16}, C\textsubscript{22}, and C\textsubscript{24} \(\alpha\)-hydroxy fatty acids, a C\textsubscript{16} \(\alpha\)-hydroxy fatty acid is dominating in GlcCer from \textit{P. pastoris} (Table II). In contrast, GlcCer from human (Gaucher's) spleen contain mainly nonhydroxy C\textsubscript{24} and C\textsubscript{22} very long chain fatty acids (Sigma; Matreya).

Using the ELISA-based binding assay, RsAFP2 was found to interact in a dose-dependent manner with purified GlcCer from \textit{P. pastoris} (Fig. 3A). No significant interaction of RsAFP2 with different concentrations of neither human or soybean GlcCer or with soybean monogalactosyldiacylglycerols could be detected, indicating that RsAFP2 interacts selectively with \textit{P. pastoris} GlcCer.

Because sphingolipids, including GlcCer, are associated with sterols in the plasma membrane to form rafts (31, 32), we further investigated the effect of ergosterol, the main fungal sterol, on the interaction of RsAFP2 with fungal GlcCer. In a similar approach, we demonstrated recently the positive effect of ergosterol on the interaction of DmAMP1 with sphingolipids from \textit{S. cerevisiae} (12). Interaction of 50 nM RsAFP2 with different concentrations of ergosterol (ranging from 0.1 nmol to 5 nmol coated/well) was assessed. Under these conditions, no interaction of RsAFP2 with ergosterol could be observed (data not shown). Furthermore, interaction of 50 nM RsAFP2 with lipid mixtures consisting of 1 nmol of GlcCer and different amounts of ergosterol was assessed. However, no increase in RsAFP2 interaction with fungal GlcCer in the presence of various concentrations of ergosterol could be observed (data not shown).

\textbf{Link between the Antifungal Activity of RsAFP2 and Its Interaction with Fungal GlcCer}—To address the link between the antifungal activity of RsAFP2 and its interaction with fungal GlcCer, the interaction of an RsAFP2 variant that is devoid of antifungal activity (RsAFP2(Y38G)) (21) with fungal GlcCer was assessed. Similarly to RsAFP2, RsAFP2(Y38G) interacts with GlcCer from \textit{P. pastoris}, but not with GlcCer from human origin (Fig. 4). These results indicate that RsAFP2(Y38G) is competent for GlcCer binding but is impaired in the induction of fungal growth inhibition. Hence, interaction of RsAFP2 with fungal GlcCer is not sufficient to induce fungal growth arrest. Based on this observation, it could be anticipated that \textit{P. pastoris} cells pretreated with RsAFP2(Y38G) should be more resistant to subsequent treatment with RsAFP2. Therefore, we investigated a putative antagonism between RsAFP2(Y38G) and RsAFP2 by preincubating \textit{P. pastoris} cells with either 40 \(\mu\)M RsAFP2(Y38G) or water and assessing the sensitivity of the preincubated \textit{P. pastoris} cells to RsAFP2. The antifungal activity of RsAFP2 was not affected by the presence of RsAFP2(Y38G). The MIC value of RsAFP2 was the same in the absence or presence of RsAFP2(Y38G) (MIC = 2 \(\mu\)M). Hence, no antagonism between RsAFP2(Y38G) and RsAFP2 was ob-

![Fig. 2. Sphingobase composition of GlcCer from soybean meal and \textit{P. pastoris}](image-url)
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*TABLE II*

| Sphingobase | Fatty acid | Ref. |
|-------------|------------|------|
| *P. pastoris* |            |      |
| 9-Methyl-d18:2<sup>trans</sup> | 69 | 18:0-(2-OH) | 85 | This work and 16 |
| d18:2<sup>trans</sup> | 19 | 16:0-(2-OH) | 6 |
| d18:1<sup>trans</sup> | 12 | 17:0-(2-OH) | 6 |
| Soybean |            |      |
| d18:2<sup>trans</sup> | 62 | 16:0-(2-OH) | 64 | This work and 48 |
| 18:2<sup>trans</sup> | 33 | 24:0-(2-OH) | 18 |
| t18:1<sup>cis</sup> | 3 | 22:0-(2-OH) | 16 |
| Human |            |      |
| d18:1<sup>trans</sup> | ND<sup>a</sup> | 24:0 | 33 | Sigma; Matreya, Inc. |

<sup>a</sup> Relative amounts in mol % are given. Components of less than 2 mol % are omitted.

<sup>b</sup> ND, not determined.

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**FIG. 3. Interaction of RsAFP2 and heliomicin with GlcCer.** Dose-response curves are presented for the interaction of RsAFP2 (A) and heliomicin (B) with GlcCer from *P. pastoris* (squares), human spleen (triangles), soybean (diamonds), and monogalactosyldiacylglycerols from soybean (circles). RsAFP2 and heliomicin were used at a concentration of 50 nM. Data are the means ± S.E. of triplicates.

**FIG. 4. Interaction of an inactive variant of RsAFP2 with fungal GlcCer.** The interaction of 50 nM RsAFP2 (white bars) or the biologically inactive variant RsAFP2(Y38G) (gray bars) with GlcCer from *P. pastoris* and human spleen is presented. Each microtiter plate well was coated with 0.5 nmol of each glycolipid. Data are the means ± S.E. of triplicates.

It was observed that some plant defensins bind reversibly to fungal and yeast cells (7). In this respect, it is possible that RsAFP2(Y38G), which does not display antifungal activity and therefore probably cannot reach and/or affect the fungal target, can be displaced from its primary binding site by RsAFP2. Hence, this approach is not appropriate to demonstrate the link between the antifungal activity of RsAFP2 and its interaction with fungal GlcCer.

Therefore, we investigated whether anti-GlcCer antibodies (22) can inhibit the antifungal action of RsAFP2 by binding to GlcCer and hence, blocking the RsAFP2 target on the fungal plasma membrane. To this end, we have preincubated a *P. pastoris* cell suspension with either a rabbit antiserum against GlcCer (1/20 diluted), a nonimmune rabbit serum (1/20 diluted) as a control, or water. The antifungal activity of RsAFP2 was found to be reduced 4-fold in the presence of the rabbit antiserum against GlcCer (MIC of RsAFP2 = 8 μM) compared with the appropriate controls (MIC of RsAFP2 = 2 μM). From these data, it can be concluded that the antifungal action of RsAFP2 can be reduced by blocking its target, namely fungal GlcCer, and that the observed interaction between RsAFP2 and fungal GlcCer in the ELISA-based binding assay is relevant for its antifungal activity.

RsAFP2 Induces Permeabilization of *P. pastoris* Cells but Not of the Corresponding gcs-Deletion Mutant—To determine the role of GlcCer in RsAFP2-mediated membrane permeabilization of yeast cells, an assay based on the uptake of SYTOX Green was used (9). SYTOX Green is an organic compound that only penetrates cells with compromised plasma membranes and fluoresces upon interaction with nucleic acids (33). RsAFP2-induced SYTOX Green uptake was assessed in *P. pastoris* parental strain and the corresponding gcs-deletion mutant. SYTOX Green uptake in the *P. pastoris* parental strain increases significantly upon treatment with RsAFP2 at concentrations higher than 2 μM (Fig. 5). No such RsAFP2-induced membrane permeabilization can be detected in the gcs-deletion mutant up to 10 μM. However, a minor increase of permeabilization in the gcs-deletion mutant was observed for RsAFP2 concentrations higher than 10 μM, consistent with previously observed DmAMP1-induced permeabilization of the *S. cerevisiae* parental strain and a DmAMP1-resistant *ipt1* mutant (9). Plant defensin-induced permeabilization occurring at high plant defensin concentrations (10–40 μM) is proposed to result from an interaction of the peptides with the membrane in a binding site-independent manner (9).

In contrast to native RsAFP2, RsAFP2(Y38G) did not induce membrane permeabilization on parental *P. pastoris* nor on the
Gs-d-eletion mutant (results not shown). These results indicate that interaction of the antifungal plant defensins with specific fungal membrane components on one hand, and subsequent membrane permeabilization on the other hand, are uncoupled events in the process leading to fungal growth inhibition.

The Insect Defensin-like Peptide Helio micin Interacts with the Same Fungal Target as RsAFP2—The three-dimensional structure of helio micin, an insect defensin-like peptide from the lepidopteran *Heliothis virescens*, is highly homologous to radish plant defensin RsAFP2 (1, 4). In addition, both peptides display similar biological activities. Therefore, a possible involvement of fungal GlcCer in helio micin-induced antifungal activity was assessed. As observed for RsAFP2, growth of *C. albicans* and *P. pastoris* is inhibited by helio micin starting from 2 μM, whereas gcs-deletion mutants of both yeast strains are at least 20-fold more resistant (Table 1). In addition, helio micin was found to interact with fungal GlcCer in a dose-dependent manner, but not with human GlcCer or soybean monogalactosyldiacylglycerols (Fig. 3B). In contrast to RsAFP2, however, helio micin also interacts with soybean GlcCer.

Furthermore, the ability of RsAFP2 to compete with helio micin for *P. pastoris* GlcCer interaction was tested. Interaction of helio micin with *P. pastoris* GlcCer cannot be competed for by a 50-fold excess of RsAFP2 and vice versa (results not shown), indicating that helio micin and RsAFP2 are probably interacting with different motifs of the fungal GlcCer.

**DISCUSSION**

In this study, we present clear evidence that the radish antifungal defensin RsAFP2 and the highly homologous insect defensin-like peptide helio micin both interact directly with fungal GlcCer. We have discussed previously the evolutionary conservation of defensins among different kingdoms of eukaryotes (1). Based on a comparison of their overall three-dimensional structure, there is a close relationship among plant defensins, insect defensins, and mammalian β-defensins. In some cases this “interkingdom” structural homology is even stronger than the structural homology among different types of defensins from the same species, such as mammalian α- and β-defensins (1). This suggests that defensins are ancient peptides conserved across the eukaryotic kingdom, originating before the evolutionary divergence of plants and animals. Possibly, defensins have evolved from a single precursor, being a molecule with an overall structure resembling that of plant defensins. Evidence presented in this paper substantiates this hypothesis further, as a plant defensin and an insect defensin-like peptide both interact with conserved binding sites on susceptible fungi, namely GlcCer. To our knowledge, this is the first demonstration that related defense components from organisms belonging to different kingdoms target the same microbial structure.

The interaction of RsAFP2 with fungal GlcCer was found to be selective because RsAFP2 did not interact with human or plant GlcCer nor with plant monogalactosyldiacylglycerols. Differences in ceramide structure among fungal, plant, and human GlcCer probably account for the observed binding selectivity. GlcCer from various fungal and yeast species, such as *P. pastoris* and *C. albicans*, are identical (Glc-9-methyl-d18:2/h18:0 = N-2'-hydroxyoctadecanoyl-1-O-β-D-glucopyranosyl-(4E,8E)-9-methylsphinga-4,8-diene) (17). Fungal GlcCer show a number of structural features that distinguish them from those found in mammals and plants, such as the 9-methyl group branching of the sphingoid base, variable levels of unsaturation and length of the fatty acid chain. The exact structural features of fungal GlcCer which are important for the interaction with the antifungal defensins and/or subsequent cell growth inhibition are currently being assessed via a genetic deletion approach in *P. pastoris* and ELISA-based binding studies of the corresponding GlcCer. The finding that helio micin, in contrast to RsAFP2, interacts with fungal and plant GlcCer points toward different GlcCer-interacting characteristics for RsAFP2 and helio micin. This is also reflected by the observation that interaction of helio micin with fungal GlcCer could not be competed for by RsAFP2 and vice versa, indicating that helio micin and RsAFP2 probably interact with different structural motifs of the fungal GlcCer.

Previously, we demonstrated that RsAFP2 induces membrane permeabilization in susceptible fungi (9). In this study, we show that RsAFP2 induces membrane permeabilization in a susceptible *P. pastoris* yeast strain at concentrations higher than 2 μM. In contrast, no such RsAFP2-induced membrane permeabilization was detected in the RsAFP2-resistant *P. pastoris* gcs-deletion mutant. Hence, it can be concluded that the GCS gene determines RsAFP2-induced permeabilization. Our findings that fungal GlcCer constitute the RsAFP2 binding site and that the GCS gene determines RsAFP2-induced permeabilization as well as fungal growth inhibition provide strong support for a model in which all of these phenomena are causally linked. We propose a two-step model for the mode of action of RsAFP2, i.e. 1) interaction of RsAFP2 with GlcCer in the fungal plasma membrane (as evidenced by this study) and 2) subsequent permeabilization of the fungal plasma membrane (as demonstrated previously (9)). The finding that RsAFP2(Y38G), an RsAFP2 variant devoid of antifungal activity (21), still interacts to the same extent with fungal GlcCer as native RsAFP2, confirms the hypothesized two-step model for the mode of action of RsAFP2. Indeed, it seems that the mutant RsAFP2(Y38G) is competent for GlcCer binding but is impaired in induction of membrane permeabilization and fungal growth inhibition. In this study, we furthermore demonstrated that antibodies against GlcCer could antagonize the antifungal activity of RsAFP2 by blocking its target on the fungal plasma membrane, namely GlcCer, thereby providing a link between the observed interaction of RsAFP2 with fungal GlcCer on one hand and inhibition of fungal growth on the other hand. Based on these observations, we can conclude that interaction of RsAFP2 with fungal GlcCer is not sufficient but necessary to induce fungal growth arrest. This conclusion is also supported by the finding that helio micin does not seem to be phytotoxic (41), although it interacts with GlcCer from plants.
The composition of the plasma membrane of fungal cells is asymmetric, which is typical of eukaryotic cells, with phosphatidylycerine mainly in the inner leaflet and sterols and sphingolipids in the outer leaflet (38). It has been shown that sphingolipids and sterols are enriched in specific domains in the plasma membranes, the so-called membrane rafts (31, 32). We demonstrated recently that the interaction between the dhahila plant defensin DmAMP1 and inositolphosphophoryl-con-taining sphingolipids from S. cerevisiae is enhanced by 40% in the presence of equimolar concentrations of ergosterol (12), pointing toward a putative involvement of rafts in the DmAMP1 interaction with the fungal plasma membrane. This is in contrast to the interaction between RsAFP2 and fungal GlcCer, which is not altered by different concentrations of ergosterol.

Most antimicrobial peptides studied thus far are known to interact with ubiquitous phospholipids via a charge-based mechanism, explaining their relatively broad antimicrobial spectrum. Fungal GlcCer have not been identified so far as possible targets for antimicrobial peptides. However, verotoxin receptor-mediated endocytosis, verotoxin inhibits protein synthesis resulting in necrosis and/or apoptosis (45–47). Whether RsAFP2-induced fungal growth arrest is a result of the induced membrane permeabilization or results from its interaction with a putative intracellular target is currently not known. Several plant defensins isolated from barley have been shown to inhibit protein synthesis in eukaryotic as well as prokaryotic cell-free systems (39, 40), supporting the theory of intracellular targets for plant defensins. Studies are currently initiated to address the subcellular localization of fluorescently labeled RsAFP2 in susceptible yeast cells and as such to provide crucial information regarding such putative intracellular RsAFP2 targets. In this respect, the RsAFP2-resistant gcs-deletion mutants characterized in this study are considered invaluable tools.

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