Internalization of tenecin 3 by a fungal cellular process is essential for its fungicidal effect on Candida albicans

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Tenecin 3 is a glycine-rich, antifungal protein of 78 residues isolated from the insect Tenebrio molitor larva. As an initial step towards understanding the antifungal mechanism of tenecin 3, we examined how this protein interacts with the pathogenic fungus Candida albicans to exert its antifungal action. Tenecin 3 did not induce the release of a fluorescent dye trapped in the artificial membrane vesicles and it did not perturb the membrane potential of C. albicans by the initial interaction. Fluorescence confocal microscopy and flow cytometric analysis revealed that tenecin 3 is rapidly internalized into the cytoplasmic space in energy-dependent and temperature-dependent manners. This internalization is also dependent on the ionic environment and cellular metabolic states. These results suggest that the internalization of tenecin 3 into the cytoplasm of C. albicans is mediated by a fungal cellular process. The internalized tenecin 3 is dispersed in the cytoplasm, and the loss of cell viability occurs after this internalization.

Keywords: antifungal protein; fungus; insect; internalization; tenecin 3.
structure: (a) it has many hydrophilic Gly, His, and Gln residues that constitute ∼ 80 mol % [25]; and (b) the amino-acid sequence does not show any putative α helical or β sheet structural motifs [26]. It also has a highly repeating Gly-X-X-Gly motif, where X can be His, Gln, or Leu. Recently, it was suggested that tenecin 3 has a random structure with very loose turn-like elements [26]. This structural property is not significantly affected by temperature, pH, or by the presence of organic solvents or SDS [26]. These sequence and structural characteristics of tenecin 3 may provide a basis of discrimination mechanisms between bacteria and fungi in its antimicrobial action.

In this study, as an initial step in understanding the antifungal mechanism of tenecin 3, we examined how tenecin 3 interacts with the pathogenic fungus C. albicans and how this interaction is related to its antifungal activity. Our results suggest that tenecin 3 is internalized into the cytoplasm by a fungal cellular process and that it exerts fungicidal activity after internalization.

**MATERIALS AND METHODS**

**Strain and materials**

*C. albicans* TIMM 1768, *Saccharomyces cerevisiae* KCTC 7296, *Escherichia coli* KCTC 1682, *Proteus vulgaris* KCTC 2433, *Bacillus subtilis* KCTC 1918, and *Streptococcus aureus* KCTC 3096 were used in this study for antimicrobial assays. *E. coli* BL21(DE3) was used for bacterial expression of the recombinant tenecin 3 proteins. Melittin was prepared by a solid-phase peptide synthesis procedure. Fluorescein isothiocyanate (FITC), 6-carboxyfluorescein (CF), phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer), amphotericin B, 2,4-dinitrophenol, and propidium iodide (PI) were all purchased from Sigma.

**Purification of recombinant tenecin 3 protein**

The intact tenecin 3 was purified as the recombinant protein from *E. coli* BL21(DE3) cells containing plasmid pAFT-1, as described previously [27]. To construct a plasmid expressing the recombinant tenecin 3 fused to a His6 tag at the N-terminus, the *EcoRI/HindIII* DNA fragment containing the tenecin 3 coding sequence of pMC35 [28] was cloned into the *EcoRI/HindIII* site of pRSET B (Invitrogen). The resulting plasmid pRSAF-1 was used to express the His6-tagged fusion tenecin 3 (His-tenecin 3). *E. coli* BL21(DE3) cells containing pRSAF-1 were grown to a concentration of 1 mg mL⁻¹ in Luria–Bertani medium supplemented with ampicillin (50 μg mL⁻¹). Isopropyl thio-β-D-galactoside (IPTG) was added to the culture at 1 mM and the culture was allowed to grow for an additional 3 h. The cells were harvested by centrifugation. The pelletted cells were resuspended in a column buffer (10 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) and sonicated. After sonication, the supernatant fraction was subjected to Ni²⁺-chelating affinity chromatography as described previously [27]. The recombinant protein containing fraction was further purified by RP-HPLC with a semiprep C₁₈ 218TP column (Vydac, Hesperia, CA, USA). The final purified protein was analyzed by SDS/PAGE. A His₆-tagged protein expressed from the pRSET C vector itself was also purified by the same method and used as a control protein.

**Assay of antimicrobial activity**

Bacteria were grown in Luria–Bertani medium (1% NaCl, 1% bacto-trypton, 0.5% yeast extract) and maintained on a Luria–Bertani agar plate. The fungal cells were grown in yeast malt medium (0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, 1% dextrose) and maintained on a yeast peptone dextrose (0.5% yeast extract, 1% bacto peptone, 2% dextrose) agar plate. The antibacterial and antifungal activities were assayed by the broth-microdilution methods as described previously [27,29]. The minimal inhibitory concentration (MIC) of His-tenecin 3 or melittin was examined by incubating 10³–10⁴ CFU per mL of cells with serial dilutions of each peptide in a 96-well microtiter plate. The bacterial or fungal growth was assessed by measuring the D₆₅₀ after a 12 h incubation at 37°C. The minimal protein concentration required to fully suppress the growth was determined as the MIC value.

**Carboxyfluorescein leakage measurement**

CF-encapsulated large unilamellar vesicles (LUV) composed of PtdCho and PtdSer (3:1, w/w) were prepared by the reverse-phase ether evaporation method [30] with 100 mM CF in NaCl/P, (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₃HPO₄, 1.4 mM KH₂PO₄), pH 7.4, as a high salt buffer or 10 mM sodium phosphate buffer (SPB), pH 7.4, as a low salt buffer. The initially formed vesicles were extruded through a 0.1 μm Nucleopore filter (Avestin Inc., Ottawa, Canada). To remove the free CF dye, the vesicles were passed through a Bio-Gel A 0.5 m gel (BioRad) column (1.5×30 cm) and eluted with each buffer. The separated LUV fraction was diluted with each buffer to the final concentration of 6.36 μM phosphate and mixed with His-tenecin 3 or melittin at 25°C. After 5 min incubation, 10 μL of Triton-X 100 was added (0.05%, w/v) in order to disrupt the vesicles thoroughly. The leakage of CF from LUV was monitored by measuring the fluorescence intensity at 520 nm, when excited at 490 nm, on a Shimadzu RF-5000 spectrofluorometer.

**Preparation of FITC-labeled His-tenecin 3**

FITC was freshly dissolved in dimethylsulfoxide to 10 mg mL⁻¹, and added to 1 mg mL⁻¹ of His-tenecin 3 in 100 mM sodium bicarbonate, pH 9.3, at 1 mg mL⁻¹ FITC. After incubation for 4 h in the dark at room temperature, 1 M ethanolamine was added to inactivate the residual FITC. The solution was left in the dark for an additional 2 h and subjected to gel-filtration column chromatography using a 120-cm Sephadex G-50 column to remove the unconjugated dye. The FITC-labeled His-tenecin 3 (FITC-tenecin 3) was further purified by RP-HPLC with the C₁₈ column. The conjugation between FITC and tenecin 3 was verified by an intense fluorescent band under an UV light on an SDS polyacrylamide gel. A similar labeling procedure was applied for the preparation of the His₆-tagged control protein.

**Confocal laser scanning microscopy**

Intracellular localization of the FITC-labeled His-tenecin 3 (FITC-tenecin 3) in *C. albicans* was analyzed by confocal laser scanning microscopy. *C. albicans* cells were inoculated
into 3 mL of yeast malt medium and incubated at 30°C for 12 h. The cells were then diluted 1:50 in Saubouraud dextrose broth medium (SB medium; 1% bacto peptone, 4% glucose) and incubated at 30°C for 3 h to enrich the population of exponentially growing cells. The number of cells was adjusted to 10⁶ cells per mL by diluting in the SB medium. In some cases, the stationary phase cells were used instead of the exponential phase cells. FITC-tenecin 3 was added to 100 μL of the cell suspension at 12.5 μM, and the cells were incubated at 37°C for 15 min. The cells were pelleted by centrifugation at ≈4000 g for 2 min and washed three times with the ice-cold NaCl/Pi buffer supplemented with 0.01% NaN₃. The cells were washed three times with ice-cold NaCl/Pi supplemented with 0.01% NaN₃. Intracellular localization of FITC-tenecin 3 was examined by the Leica TCS-4D system connected to a Leica DAS upright confocal microscope.

Analysis of membrane depolarization

*C. albicans* cells were harvested at the mid-log phase, and the number of cells was adjusted to 10⁶ per mL with SB medium. The cell suspension of 100 μL was mixed with His-tenecin 3, amphotericin B, or 2,4-dinitrophenol, and incubated at 37°C for 20 min. Then, 3,3'-dipentylxoxycarbocyanine (DiOC₅) was added at 0.5 μM and the total volume was adjusted to 400 μL with SB buffer, as described previously [31]. After the 5 min incubation, flow cytometric analysis with the FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) was carried out. The fluorescence of DiOC₅ was monitored in the FL1-H channel and analyzed with the CELLQUEST software package provided by the manufacturer.

Flow cytometric analysis of internalization of tenecin 3 and cell-viability

Internalization of tenecin 3 into *C. albicans* was investigated by flow cytometric analysis. *C. albicans* cells harvested at the mid-log growth phase were diluted to 10⁶ cells per mL in SB medium and preincubated for 10 min on ice. The cell suspension of 100 μL was incubated with 12.5 μM of the FITC-labeled His-tenecin 3 for 15 min at 0°C, 15°C, and 37°C in the absence or presence of 0.05% NaN₃. The cells were washed three times with ice-cold NaCl/P, and subjected to flow cytometric analysis using the FACSCalibur flow cytometer. To ensure that fluorescence signals would not result from the fluorescence by the FITC-tenecin 3 bound to the extracellular cell surface, a parallel experiment was carried out: cells were treated with 4% Trypan blue before the flow cytometric analysis, as described previously [32]. The fluorescence of FITC was monitored in the FL1-H channel. As a control experiment, the cell suspension was treated with 7.3 μM melittin. The melittin-treated cells were further incubated with PI on ice for 30 min, and PI uptake was monitored in the FL2-H channel.

For the time-course analysis of tenecin 3 internalization and PI uptake by *C. albicans*, the cell suspension was incubated with 12.5 μM of FITC-labeled His-tenecin 3 or unlabeled His-tenecin 3 at 37°C. Each 100 μL aliquot of cells was removed at specified time intervals and washed three times with ice-cold NaCl/P, supplemented with 0.01% NaN₃. For cells treated with the unlabeled His-tenecin 3, 50 μg·mL⁻¹ of PI was added, and the cells were further incubated on ice for 30 min. In both cases, the final cell suspensions were subjected to flow cytometric analysis using the FACSCalibur flow cytometer. The FITC-tenecin 3 internalization and PI uptake were monitored in the FL1-H channel and FL2-H channel, respectively.

**RESULTS**

Preparation of the recombinant tenecin 3 proteins

We prepared the intact tenecin 3 as a recombinant protein according to the method that was previously developed for efficient bacterial expression and purification of the recombinant tenecin 3 protein having the same sequence of the native protein [27]. In an initial experiment for the FITC-labeling of protein, intact tenecin 3 was used. Unfortunately, the intact tenecin 3 was not efficiently labeled with FITC even though tenecin 3 has one lysine residue. Instead, we used a recombinant tenecin 3 protein fused to a His₆ tag because it has one more lysine residue in the linker peptide between the His₆ tag and the tenecin 3 sequences. For the preparation of recombinant His-tenecin 3, the coding sequence of tenecin 3 was cloned into plasmid pRSET B to generate pRSAF-1. E. coli BL21(DE3) cells containing pRSAF-1 were constructed to express the recombinant tenecin 3 protein fused to a His₆ tag (His-tenecin 3). Following a 3 h induction with IPTG, His-tenecin 3 accumulated in the cells (Fig. 1, lane 2). Ni²⁺-chelating affinity chromatography and subsequent RP-HPLC produced highly purified His-tenecin 3 as judged by a single band on an SDS polyacrylamide gel (Fig. 1, lane 3). His-tenecin 3 migrated as a ≈16.5-kDa protein on the gel, which is larger than the calculated molecular mass of 12.3 kDa. A similar gel mobility was previously observed with natural tenecin 3 on SDS/PAGE [23]. Indeed, His-tenecin 3 was much more efficiently coupled to FITC than the intact tenecin 3, probably due to the presence of one more lysine in the linker peptide. The antimicrobial activity of His-tenecin 3 was tested against Gram positive and Gram negative bacteria, and fungi, using broth microdilution methods [27,29]. Similar to the intact tenecin 3, His-tenecin 3 repressed the growth of *C. albicans* and *S. cerevisiae* and did not show antibacterial activity against both Gram positive and Gram negative bacteria (Table 1). The MIC of His-tenecin 3 for *C. albicans* was comparable to that of the intact tenecin 3 (Table 1), [23,27]. Both the intact tenecin 3 and His-tenecin 3 also showed a salt-dependency for antifungal activity; in the SB media supplemented with 150 mM NaCl, they showed no apparent growth inhibition of *C. albicans* in the range of 2–15 μM protein concentration (Table 1). In contrast, the His-tagged control protein expressed from the pRSET C vector itself, which has the same His₆ tag and linker sequence as His-tenecin 3, did not show antifungal activity (Table 1).

This indicates that the His₆ tag and linker sequence had little effect on the tenecin 3 activity. Therefore, we used His-tenecin 3 in the protein-localization experiments as an alternative to the intact tenecin 3. However, both the intact tenecin 3 and His-tenecin 3 were used in the other experiments to rule out any possible effects of the His₆ tag and linker sequence of His-tenecin 3.

Interaction between tenecin 3 and artificial membrane vesicles

We examined whether or not the interaction of tenecin 3 with *C. albicans* would occur through the pore-formation on
the plasma membrane because the pore formation is assumed to be a major antimicrobial action mechanism. The leakage of CF dye trapped in an artificial membrane liposome (PtdCho/PtdSer, 3 : 1) of LUVs, which could mimic the biological membrane, was examined during the incubation with tenecin 3. Melittin, an antifungal peptide, whose antimicrobial activity is based on the formation of transmembrane channels [14], was used as a positive control peptide. As antifungal activity of tenecin 3 is dependent on the salt concentration, the leakage experiment was performed in both high salt buffer (NaCl/Pi) and low salt buffer (SPB). In SPB buffer, tenecin 3 was fully active against C. albicans. His-tenecin 3, melittin, and the intact tenecin 3 were treated in the range of 80–500 nM, 2–200 nM, and 100–400 nM, respectively. The protein concentration ranges of the tenecin 3 proteins were supposed to be effective relative to that of melittin (Table 1). Fig. 2 shows that the CF dye was not released by the treatment of the intact tenecin 3 or His-tenecin 3 in either NaCl/Pi or SPB buffer, while CF was released from LUV after the treatment of melittin in a dose-dependent manner. The salt-independent stability of the liposome during the incubation with tenecin 3 indicates that tenecin 3 does not disrupt the lipid membrane through direct interaction such as pore formation.
Table 1. Antimicrobial activity of the recombinant tenecin 3 protein. At 15 μM protein concentration, or greater, suppression of bacterial or fungal growth was not observed. Tenecin 3, intact tenecin 3. His-control, His6-tagged control protein. ND, not determined.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Tenecin 3</th>
<th>His-tenecin 3</th>
<th>Tenecin 3* (150 mM NaCl)</th>
<th>His-tenecin 3* (150 mM NaCl)</th>
<th>His-control</th>
<th>Melittin</th>
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</thead>
<tbody>
<tr>
<td>Gram positive bacteria</td>
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<td><em>B. subtilis</em> KCTC 1918</td>
<td>≥15</td>
<td>≥15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.19 ± 0.05</td>
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<tr>
<td><em>S. aureus</em> KCTC 3096</td>
<td>≥15</td>
<td>≥15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>Gram negative bacteria</td>
<td></td>
<td></td>
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<tr>
<td><em>E. coli</em> KCTC 1682</td>
<td>≥15</td>
<td>≥15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.56 ± 0.14</td>
</tr>
<tr>
<td><em>P. vulgaris</em> KCTC 2433</td>
<td>≥15</td>
<td>≥15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.55 ± 0.18</td>
</tr>
<tr>
<td>Fungi</td>
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<tr>
<td><em>C. albicans</em> TIMM1768</td>
<td>10.5 ± 2.3</td>
<td>12.3 ± 1.1</td>
<td>≥15</td>
<td>≥15</td>
<td>≥15</td>
<td>3.5 ± 0.6</td>
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<tr>
<td><em>S. cerevisiae</em> KCTC 1678</td>
<td>3.8 ± 0.8</td>
<td>4.1 ± 0.9</td>
<td>≥15</td>
<td>≥15</td>
<td>≥15</td>
<td>0.35 ± 0.5</td>
</tr>
</tbody>
</table>

*Antifungal activity was assayed in the presence of 150 mM NaCl.

Intracellular localization of tenecin 3

Intracellular localization of tenecin 3 during interaction with *C. albicans* was investigated by confocal fluorescence microscopy using His-tenecin 3 coupled to FITC. The FITC-tenecin 3 showed the same MIC value for *C. albicans* as that of the unlabeled His-tenecin 3, indicating that the FITC-labeling did not affect the antifungal activity of His-tenecin 3. A His6-tagged control protein expressed from the expression vector (pRSET C) itself (Fig. 1, lane 4) was also labeled with FITC and used as a control. Fig. 3 shows the results of confocal fluorescence microscopy of *C. albicans* cells treated with FITC-tenecin 3. When *C. albicans* cells harvested at the mid-log phase were incubated with FITC-tenecin 3 for 15 min, uniformly dispersed staining patterns were observed in the region corresponding to the cytoplasmic space (Fig. 3C), while FITC itself or the FITC-labeled control protein were not internalized under our experimental conditions (Fig. 3A, B). Tenecin 3 was not localized in a specific organelle because the staining covered the whole cytoplasm. However, there was a central fluorescence-void region, which seemed to be a fungal vacuole or nucleus. In contrast to the cells harvested at the mid-log phase, the cells harvested at the stationary phase were not stained well with FITC-tenecin 3 (Fig. 3D). This finding suggests that internalization of tenecin 3 is highly dependent on the cell growth state.

Effects of tenecin 3 on the *C. albicans* membrane potential

We examined whether or not internalization of tenecin 3 into *C. albicans* would accompany the dissipation of the electrochemical gradient across the cell membrane, as the disruption of membrane function may be an action mechanism of tenecin 3. The membrane potential change after the intact tenecin 3 or His-tenecin 3 treatment was measured by flow cytometric analysis using a membrane-potential-sensitive fluorescent dye, DiOC5 [31]. DiOC5 is a cationic, membrane-permeable dye that accumulates inside the cell on polarized membranes. Therefore, the fluorescence decreases upon membrane-depolarization [33]. Amphotericin B and 2,4-dinitrophenol were used as reference drugs, which are well known as a membrane-perturbing antifungal drug and an ionophore dissipating the membrane potential, respectively [34,35]. The treatment of amphotericin B caused the decrease of fluorescence inside the cells in a dose-dependent manner, which is indicative of the membrane-depolarization (Fig. 4A). 2,4-Dinitrophenol also caused membrane depolarization, although the depolarization was relatively small compared to amphotericin B (Fig. 4B). However, neither His-tenecin 3 nor the intact tenecin 3 could depolarize the membrane potential even when a high tenecin 3 concentration of 12.5 μM and an incubation time enough for internalization of tenecin 3 were applied (Fig. 4C, D). No effect of tenecin 3 on the cell membrane potential suggests that the membrane integrity of *C. albicans* remains unchanged after internalization of tenecin 3.

Factors affecting internalization of tenecin 3 into *C. albicans*

We examined whether or not the internalization of tenecin 3 was influenced by incubation temperatures and by the metabolic states of *C. albicans* because these parameters are indicative of the dependency of the tenecin 3 internalization on some cellular functions of *C. albicans*. The cells harvested at the mid-log growth phase were incubated with FITC-tenecin 3 for 15 min at different temperatures in the absence or presence of NaN3 as a respiration inhibitor. The population of *C. albicans* cells showing the fluorescence of the internalized tenecin 3 was analyzed by a flow cytometer. In this experiment, we used a 1:6 dilution mixture of FITC-tenecin 3 and unlabeled His-tenecin 3, as the cells treated with the undiluted FITC-tenecin 3 protein fluoresced too intensely in the FL-1H channel. As a control experiment, the cells were treated with 4% Trypan blue, a quenching reagent of the extracellular fluorescence [32], after incubation with FITC-tenecin 3. The Trypan blue treatment did not alter the fluorescence signal in cell populations, demonstrating that the fluorescence signals did not result from the fluorescence by the FITC-tenecin 3 bound to the extracellular cell surface. As shown in Fig. 5, internalization of FITC-tenecin 3 was highly dependent on incubation temperatures and the presence of the respiration...
inhibitor NaN₃. Tenecin 3 was internalized into the cell at 37°C, but not in the presence of NaN₃, suggesting that internalization of tenecin 3 is mediated by a cellular function requiring cellular energy consumption. However, this internalization process did not occur at 0°C, while slower internalization was observed at the intermediate temperature of 15°C. The lack of internalization at 0°C and the slower internalization at 15°C could be due to slower metabolic processes at the lower temperatures. These internalization properties of tenecin 3 are similar to those of cellular endocytosis [36]. As the antifungal activity of tenecin 3 was repressed by the high salt concentration, internalization of FITC-tenecin 3 was examined in the same SB medium supplemented with 150 mM NaCl at 37°C. The presence of 150 mM NaCl inhibited internalization of tenecin 3 (Fig. 5). In contrast, the interaction of melittin with C. albicans cells, as observed with PI uptake by the nonviable cells [37], was...
not temperature, energy, or salt dependent (Fig. 5), indicating that the interaction does not require a cellular-metabolic function. This result is consistent with the antifungal action of melittin based on the formation of membrane-perturbing pores [14].

**DISCUSSION**

In this study, we have investigated the antifungal mechanism of tenecin 3 isolated from the insect *T. molitor* against *C. albicans*. Both the fluorescence-dye leakage assay using LUV (Fig. 2) and the membrane-potential perturbation assay of *C. albicans* cells (Fig. 4) suggest that the interaction of tenecin 3 with the plasma membrane of *C. albicans* through the formation of transmembrane pores is not a reasonable mechanism for the antifungal action of tenecin 3. This was further supported by the indirect immunostaining of the tenecin 3-treated *C. albicans* cells using anti-(tenecin 3) Ig and FITC-conjugated secondary Ig, showing that no significant fluorescence signal was observed on the outer membrane of *C. albicans* (D. H. Kim & Y. Lee, unpublished data). The results also agree well with the prediction from the characteristics of the amino-acid sequence of tenecin 3 [23]; tenecin 3 is composed mostly of hydrophilic amino acids without amphiphilicity, and appears to be incompatible with the mechanism of channel formation.

The intracellular localization of tenecin 3 was observed by confocal fluorescence microscopy (Fig. 3) and flow cytometric analysis (Figs 5 and 6) with FITC-conjugated tenecin 3. In these experiments, we used His-tenecin 3 instead of intact tenecin 3 because intact tenecin 3 could not be efficiently labeled with FITC. This might result from a difficulty of FITC to have access to the single lysine residue present in the intact tenecin 3. Furthermore, His-tenecin 3 was much more efficiently coupled to FITC, possibly due to the presence of one more lysine in the linker peptide between the His6 tag and the tenecin 3 sequence. As we used His-tenecin 3 as an alternative to the intact tenecin 3 in the localization experiments, we carried out a variety of control experiments to rule out the effects of the His6 tag and linker sequence. First, His-tenecin 3 and the intact tenecin 3 showed the almost same antifungal activity, but the His6-tagged control protein expressed from the pRSET vector, which contains the same His6 tag and linker sequence, did not show any antifungal activity. Secondly, the His6-tagged...
control protein vector was used as a negative control protein. Finally, we used the intact tenecin 3 in addition to His-tenecin 3 in the experiments, such as carboxyfluorescein leakage and membrane depolarization, where the FITC-labeled protein was not necessary. In these experiments, both the intact tenecin 3 and His-tenecin 3 gave rise to the same results. Therefore, we conclude that His-tenecin 3 can represent the intact tenecin 3 in the localization experiments. Confocal fluorescence microscopy showed that \textit{C. albicans} cells take up tenecin 3 into the cell matrix space. As the internalized tenecin 3 is uniformly dispersed in the cytoplasm, the target of tenecin 3 does not seem to be a specific organelle in \textit{C. albicans}. Previously, AFP, an insect antifungal-protein related to tenecin 3, was reported to bind the cell membrane of \textit{C. albicans} [21]. If the action mechanism of AFP is similar to that of tenecin 3, the binding signal of AFP to the fungal membrane would originate from the internalized AFP rather than membrane-bound AFP. It is likely that internalization of tenecin 3 is essential for its antifungal activity because \textit{C. albicans} cells lose their viability after the intracellular localization of tenecin 3. The coincidence of the inhibition of internalization with the loss of the antifungal activity by the high ionic environment may support this possibility.

We have also shown that internalization of tenecin 3 into \textit{C. albicans} is dependent on temperature, cellular-energy consumption, and cell-growth state (Figs 3 and 5). Simple diffusion, facilitated diffusion, active transport of ions, direct penetration across the membrane [38,39], receptor-mediated endocytosis [40], and fluid-phase endocytosis (pinocytosis) [41] are generally well known transport processes for uptake of extracellular molecules by fungi. Simple diffusion and facilitated diffusion are regarded as transport mechanisms for very small molecules, such as ethanol [42], glycerol [43], and several organic acids [44], and these transport mechanisms have been shown to operate without the cellular energy consumption. The active transport of ions across the membrane requires the consumption of chemical energy to pump certain ions against their electrochemical gradient [45]. The transport of a few peptides, such as penetratin and the Tat fragment, also occurs without cellular-energy consumption by the direct membrane penetration mechanism in a temperature-independent manner [46]. In the case of tenecin 3, its dependency on the temperature, cellular energy consumption, and cell growth state for internalization into \textit{C. albicans} indicates that the internalization requires a cellular metabolic process. Considering that the internalization of tenecin 3 also occurs without disruption of the cell membrane integrity (Fig. 4), the uptake of tenecin 3 into \textit{C. albicans} seems to be similar to the internalization characteristics with receptor-mediated endocytosis or fluid-phase pinocytosis [36].

Recent studies on the candidacidal mechanism of human saliva histatin 5 revealed that this histidine rich peptide was also internalized in temperature- and energy-dependent manners [47]. The histatin-binding receptor on the cellular membrane was also identified [48] although the function of this receptor was not known. As both histatin 5 and tenecin 3 are histidine rich (histatin 5, 29%; tenecin 3, 19%), it could be argued that they share a general mechanism as histidine rich, antifungal proteins for internalization into fungal cells. However, they have very different charge characteristics as determined by their theoretical isoelectric points (pI); histatin 5, pI = 10.5 and tenecin 3, pI = 7.0. Furthermore, histatin 5 is localized in the fungal mitochondria [37] rather than the cytoplasmic space where tenecin 3 is localized. In this respect, the internalization mechanism may be different from that of histatin 5.

The antimicrobial spectrum and specificity of antimicrobial proteins have been explained by their mode of action. For the amphipathic antimicrobial peptides that were known to be channel forming peptides, their antimicrobial spectrum and specificity depend on the phospholipid composition of host cell membranes and the presence of specific phospholipids, such as cardiolipin [49]. For example, the discrimination in the antimicrobial activity of cecropin between bacteria and mammalian cells is known to originate from the presence of cholesterol on the mammalian cell membrane [49]. The plant antifungal proteins, such as chitinase, glucanase, and echinocandins, show their specificity to fungal cells by disrupting the fungal cell wall. However, the antifungal specificity of tenecin 3 could be
explained by the fungal-specific internalization, even though the inhibition of fungal-specific macromolecular synthesis or other intracellular damage by tenecin 3 in the fungal cell may be also responsible for the specificity.

The time course uptake of PI after the tenecin 3 treatment (Fig.6) indicates that internalization of tenecin 3 into the cytoplasmic space precedes the fungicidal action of tenecin 3 leading to the loss of cell viability. Although we do not yet know whether internalization of tenecin 3 over the threshold is required for its fungicidal action, it is evident that the fungicidal action requires some intracellular processes after internalization because the internalization was not a direct cause of the loss of cell viability. The nonlytic internalization and nucleic acid binding properties have been reported for some antimicrobial peptides, such as bufolin II isolated from Bufo bufo garagriozans [50], PR-39 from pig intestine [51], and tachyplesin I from Tachypleus tridentatus [52]. Their antimicrobial mechanisms are thought to be related to the inhibition of macromolecular synthesis within the cells. However, the properties related to possible intracellular processes for the fungicidal action of tenecin 3 are not yet known. The identification of the intracellular target molecule of tenecin 3 and the exact mechanism of synthesis within the cells remains to be demonstrated.

In summary, we have found that tenecin 3 is internalized through a fungal cellular process without forming transmembrane pores on the plasma membrane. The internalized tenecin 3 is dispersed in the cytoplasmic space and seems to exert the fungicidal action there.

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