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Screening for Antifungal Peptides and Their Modes of Action in *Aspergillus nidulans*▽†

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Many short cationic peptides have been identified as potent antimicrobial agents, but their modes of action are not well understood. Peptide synthesis on cellulose membranes has resulted in the generation of peptide libraries, while high-throughput assays have been developed to test their antibacterial activities. In this paper a microtiter plate-based screening method for fungi has been developed and used to test nine antibacterial peptides against the model fungus *Aspergillus nidulans*. Microscopical studies using sublethal peptide concentrations caused defects in polarized growth, including increased branch formation and depolarized hyphae. We characterized the mode of action for one of our target peptides, Sub5 (12 amino acids), which has already been shown to possess pharmacological potential as an antibacterial agent and is able to interact with ATP and ATP-dependent enzymes. The MIC for *A. nidulans* is 2 μg/ml, which is in the same range as the MICs reported for bacteria. Fluorescein isothiocyanate (FITC)-labeled Sub5 targeted the cytoplasmic membrane, particularly hyphal tips, and entered the cytoplasm after prolonged exposure, independent of endocytosis. Interestingly, Sub5 peptide treatment disturbed sterol-rich membrane domains, important for tip growth, at hyphal tips. A very similar peptide, FITC-P7, also accumulated on the cell membrane but did not have antibacterial or antifungal activity, suggesting that the cytoplasmic membrane is a first target for the Sub5 peptide; however, the antifungal activity seems to be correlated with the ability to enter the cytoplasm, where the peptides might act on other targets.

Fungal infections in plants and animals and fungal contamination of food for humans and livestock result in substantial worldwide economic losses. In addition, certain human fungal infections, such as those caused by *Aspergillus fumigatus*, *Cryptococcus neoformans*, or *Candida albicans*, are gaining importance because of the rising number of immunocompromised patients (1); however, existing treatments for these infections are limited to only a small number of antifungal drugs, such as azoles, echinocandins, and polyenes (10, 12, 43). Given the negative economic and health impacts of these fungi, as well as the tendency of microbes to rapidly develop multidrug resistance, there is a strong argument for continued investigations into antifungal peptides as treatment alternatives.

Cationic host defense peptides, also referred to as antimicrobial peptides, are part of the innate immune system. All forms of life, from bacteria to vertebrates, appear to produce these peptides (11); the skin of amphibians, for example, is an especially rich source (37). It has been shown that cationic host defense peptides kill Gram-positive and Gram-negative bacteria, fungi, parasites, and enveloped viruses. Recent research has shown that cationic host defense peptides can also alter the immune response in mammals, for example, to prevent septic shock (29), in addition to having direct killing capabilities. Despite these findings and their potential importance to human health, there is only a limited understanding of the antimicrobial mode of action, especially for short peptides. It is assumed that the primary site of interaction between cationic peptides and bacteria is the membrane (5). Increasingly, evidence suggests that cationic peptides have internal targets (2); however, interaction with the negatively charged membrane also appears to be an important first step.

Short cationic host defense peptides, 6 to 18 amino acids in length, can be easily synthesized, varied, and/or modified and have the potential to become lead structures in the development of new antimicrobial/antiviral drugs. To further improve the development, a rapid and simple assay has been designed to screen for short peptides with antimicrobial activity against bacteria, for example, *Pseudomonas aeruginosa*, using SPOT synthesis (16, 19). With the assistance of one fully automated robot, approximately 1,000 different peptides per week can be screened for antimicrobial activity against a range of different microbes; many peptides with tremendous therapeutic potential have been discovered using this approach (3, 14, 15, 19). One major drawback for peptide research aimed at medical applications has been the instability of short, unstructured peptides in blood. By using a novel arginine derivate-protected peptide, rapid degradation in mouse serum can be prevented, as was demonstrated with the peptide Sub3 (22). Short antimicrobial peptides, like HHC-53, have been found to exhibit activity against several multidrug-resistant clinical bacterial isolates (3, 8). One peptide that is similar to the peptides used in this study was employed successfully in the treatment of *Staphylococcus aureus*-infected mice (unpublished results). Several short antimicrobial peptides, like Bac2A, indolicidin, Sub3, and Sub5, have been shown to have the ability to interact

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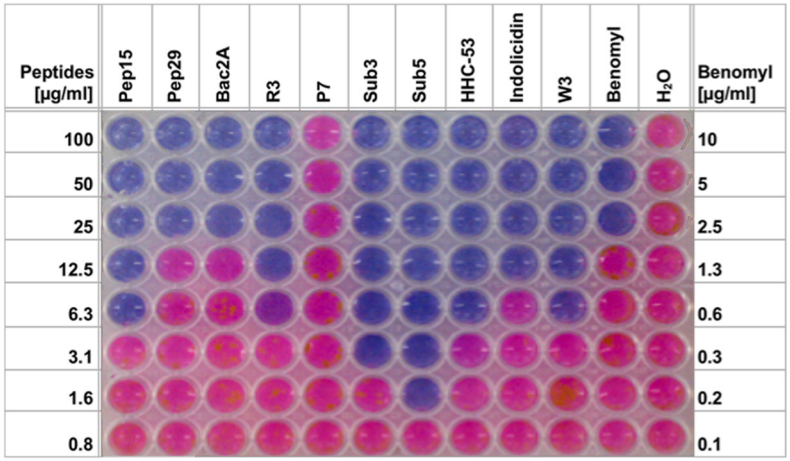

B

Peptide ID	Sequence												hydrophobicity	net charge	MIC (μg/ml)
W3§	V	R	W	R	I	R	V	A	V	I	R	A	66%	+4	5 ± 1
Pep15	V	R	L	R	I	R	V	W	V	I	R	A	66%	+4	5 ± 1
Pep29	K	L	W	W	M	I	R	R	W				66%	+3	21 ± 13
Bac2A#	R	L	A	R	I	V	V	I	R	V	A	R	66%	+4	11 ± 2
R3*	R	L	R	R	I	V	V	I	R	V	A	R	58%	+5	7 ± 2
P7*	R	L	A	R	I	V	P	I	R	V	A	R	58%	+4	>100
Sub3*	R	R	W	R	I	V	V	I	R	V	R	R	50%	+6	3 ± 1
Sub5*	R	R	W	K	I	V	V	I	R	W	R	R	50%	+6	2 ± 1
FITC-Sub5	R	R	W	K	I	V	V	I	R	W	R	R	50%	+6	2 ± 1
FITC-P7	R	L	A	R	I	V	P	I	R	V	A	R	58%	+4	>100
FITC-NC	G	A	T	P	E	D	L	N	Q	K	L	S	25%	-1	>100
HHC-53§	F	R	R	W	W	K	W	F	K				55%	+4	15 ± 5
Indolicidin§	I	L	P	W	K	W	P	W	W	P	W	R	R	53%	+3	9 ± 7

FIG. 1. Standard setup for peptide activity tests in a 96-well microtiter plate. (A) Each well contains 10^4 conidiospores of *A. nidulans* strain RMS011 in 200 μl of MM and 100 μM resazurin. The starting peptide concentration (first row) was 100 μg/ml, and a 10 μg/ml benomyl dilution series was conducted from the top to the bottom of the microtiter plate. Benomyl and H_2O were used as positive and negative controls, respectively. Along the *y* axis, peptide concentrations are shown on the left side, and benomyl concentrations are shown on the right. The peptides tested are labeled along the upper *x* axis. The microtiter plate was incubated statically at 37°C. This picture was taken after a 20-h incubation. (B) Sequence of peptides used in this study. Hydrophobicity and net charge have been calculated with the APD2 antimicrobial peptide predictor program (42). Peptides marked by typographic symbols have been previously published: W3 (14); Bac2A (44); R3, P7, Sub3, and Sub5 (16); HHC-53 (3); and indolicidin (35). MICs (μg/ml) are sample means (± SD) of at least four experiments.

with ATP and ATP-dependent enzymes, suggesting that these peptides could also have internal targets (17).

Here, we develop a screening method for antifungal activity of short cationic host defense peptides using the filamentous fungus *Aspergillus nidulans* as a model organism. *A. nidulans* shares a high degree of similarity with a number of economically and medically important fungi, such as *A. fumigatus*, an opportunistic pathogenic fungus frequently identified in immunocompromised individuals, the aflatoxin producer *Aspergillus flavus*, which causes food and feed storage problems, and the industrially important *Aspergillus oryzae* and *Aspergillus niger* (9, 20, 23). In addition to identification of antifungal peptides, a range of different tools in the cell biology were employed to assess the mode of action.

MATERIALS AND METHODS

Strains, culture conditions, and microtiter plate assay. We used supplemented minimal medium (MM), prepared as previously described in the literature (13), for culturing our model organism, *A. nidulans* strain RMS011 (*pabaA1 yA2 ΔargB::trpCΔB veA1 trpC801*). Ninety-six-well microtiter plates (polystyrol) were loaded with 200 μl of supplemented MM and 10^4 conidiospores of *A. nidulans* per well. Dilution series for peptides were prepared with an initial concentration of 100 μg/ml. The peptides tested here are listed in Fig. 1B. We used resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide; Aldrich) as the redox indicator at a final concentration of 100 μM from a stock solution of 100 mM in double-distilled H_2O (ddH_2O). Benomyl [*methyl*-1-(butylcarbamoyl)-2-benzimidazole carbamate; Aldrich], a microtubule-destabilizing drug, taken from a stock solution of 1 mg/ml in dimethyl sulfoxide (DMSO), was used at final concentrations of 0.1 to 10.0 μg/ml in the medium. The color change of resazurin in microtiter plates was measured at 570 nm using a microtiter plate reader (Flash Scan 550; Analytik Jena).

Peptide synthesis. All peptides used in this assay were purchased from UM^{pep} (Nordwestuckermark, Germany). The peptides were synthesized according to standard Fmoc (9-fluorenylmethoxy carbonyl) synthesis and purified by reverse-phase high-performance liquid chromatography (HPLC; purity grade of >85%). For fluorescein isothiocyanate (FITC) labeling, three β-alanines were added as spacers at the N terminus during the peptide synthesis, and then FITC was coupled to the N terminus.

Protoplast preparation. A total of 10^8 to 10^9 conidia of *A. nidulans* were inoculated in 200 ml of supplemented MM and then incubated on a shaker for 10 h at 30°C. Germinated conidia were harvested by filtering a stock sample through Miracloth and then resuspended in 10 ml of osmotic medium (1.2 M $MgSO_4$, 10 mM Na_2PO_4, pH 5.8) with Glucanex (15 mg/ml; Novozyme) and

TABLE 1. Comparison of peptide MICs for different microorganisms[c]

Peptide name	MIC (μM) for the indicated organism[a]							
	A. nidulans (filamentous fungus)	Gram-negative bacteria			Gram-positive bacteria			*C. albicans* (yeast)
		P. aeruginosa	*E. coli*	*S.* Typhimurium	*S. epidermidis*	*S. aureus*	*E. faecalis*	
Bac2A	8 ± 1	35	12	24	3	12	12	6
R3	5 ± 1	5	3	5	1	5	5	11
P7[b]	>70	>176	>176	>176	>176	>176	>176	>176
Sub3	1.8 ± 0.6	1.2	0.3	2.4	0.6	1.2	2.4	2.4
Sub5	1.1 ± 0.6	1.1	2.3	4.6	0.6	1.1	1.1	2.3
W3	3 ± 1	4	1	4	1	8	8	8
Pep 15	3 ± 1	8	2	8	2	4	4	16
Pep 29	15 ± 9	6	3	12	1	6	23	6
Indolicidin	5 ± 4	33	8	4	2	16	16	8

[a] MICs are expressed as sample means ± SD (n = 4). *E. coli*, *Escherichia coli*; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; *S. epidermidis*, *Staphylococcus epidermidis*; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*.
[b] The highest-tested concentration of P7 against *A. nidulans* was 100 μg/ml; for all other organisms a concentration of 250 μg/ml was used.
[c] Data from previous studies (15, 18).

bovine serum albumin (BSA; 0.6 mg/ml). Cell wall digestion was performed for 2 h at 30°C, with gentle shaking until sufficient protoplasts were released (assessed microscopically). The resultant protoplast suspension was overlaid with 10 ml of trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7) and centrifuged at 5,000 rpm for 15 min. Protoplasts that floated between the upper and lower phase were collected and transferred into a new tube. These protoplasts were then washed with 10 ml of STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5), centrifuged at 7,000 rpm for 10 min, and resuspended in 100 μl of STC.

Light and fluorescence microscopy. *A. nidulans* cells were grown on coverslips in 450 μl of supplemented MM for live-cell imaging of germlings and young hyphae. Cells were incubated at 27 to 30°C overnight. Calcofluor white was used at a final concentration of 10 μg/ml in the medium. FITC-labeled peptides were used at final concentrations of 2 to 20 μg/ml from stock solutions of 1 mg/ml in ddH2O. FM4-64 was used at a final concentration of 1 μM from stock solutions of 10 mM in DMSO. Filipin (Sigma) was used at a final concentration of 1 μg/ml from stock solutions of 10 mg/ml in methanol. Cellular images were captured at room temperature using an Axio Imager microscope (Zeiss) using filter set 38 (excitation, 470/40; emission, 525/50) for FITC-labeled peptides, filter set 43 (excitation, 545/25; emission, 605/70) for monomeric red fluorescent protein (mRFP1), and filter set 49 (excitation, 365; emission, 445/50) for the calcofluor white and filipin staining stages. Images were collected and analyzed using AxioVision (Zeiss) software. In order to analyze the internalization of FM4-64- and FITC-labeled peptides, images were captured at room temperature using a Leica TCS SP5 confocal microscope and analyzed using LASAF (Leica) software.

RESULTS AND DISCUSSION

Microtiter plate-based screening assay for antifungal peptides. Filamentous fungi grow by tip extension and branching to form hyphae and mycelia. Since the mycelia are normally heterogeneously distributed in liquid culture, simple turbidity measurements are unsuitable for following fungal growth; this tends to hamper the application of microtiter plate-based screening methods (6). In this study, we used resazurin as a color indicator for metabolic activity. Resazurin is a blue, nonfluorescent dye that is converted to pink and fluorescent resurfin in the presence of a respiring organism (31, 39). The color change can be measured by light absorbance at 570 to 600 nm. Resazurin at 100 μM changed color from blue to pink after overnight incubation with 10^4 fresh conidiospores of *A. nidulans* in supplemented minimal medium (Fig. 1A, far right column); however, resazurin remained blue at benomyl concentrations greater than 1.3 μg/ml, indicating fungal growth inhibition (Fig. 1A, second column from right). This color assay was used to determine the antifungal activities of given peptides.

In order to investigate the importance of spore concentration on peptide inhibition potential, peptide activity was measured with different spore concentrations as inocula (10^5 to 10^2 per well) (see Fig. S1A in the supplemental material). Spore concentrations less of than 2.5×10^4 per well resulted in the same degree of sensitivity to the peptide Sub5; therefore, a spore concentration of 10^4 per well was used in subsequent experimentation.

All peptides listed in Fig. 1B are synthetic derivatives of bactenecin and the naturally occurring peptide indolicidin, with the exception of peptide HHC-53. Bactenecin and indolicidin were discovered in bovine neutrophils (34, 36). All derivatives are linear peptides and were created using amino acid substitutions or sequence scrambling of their original versions. HHC-53 was discovered using a quantitative structure-activity relationship (QSAR) approach and was based on semi-random peptide libraries (3, 26). All peptides used in this study consist of 9 to 13 amino acids and are composed of mainly basic and hydrophobic amino acids.

The antifungal activity of each peptide was tested at various concentrations, ranging from 0.8 to 100 μg/ml (Fig. 1A). Following stationary incubation (37°C for 18 h), a clear color change from blue to pink was expected if the fungus continued to grow in the presence of a given peptide. According to the color changes observed, the peptides Sub5 and Sub3 showed high antifungal activity while most other peptides displayed only moderate activity, and P7 showed no antifungal activity.

Most peptides used in this study have already been tested on a range of different microorganisms, including Gram-positive and -negative bacteria and *C. albicans* (15, 18). The MICs arising out of previous work were quite variable (Table 1) (15, 18). In order to calculate a MIC for *A. nidulans*, we quantified the color change using a plate reader. The color change was measured at 570 nm every 2 h (see Fig. S1B and C in the supplemental material). Our data sets for HHC-53 and Sub5 are shown as examples. The color change was visible to the naked eye after 8 to 10 h and was quantified after 8 h using a plate reader. Higher peptide concentrations slowed down the reduction of resazurin, and concentrations above the threshold

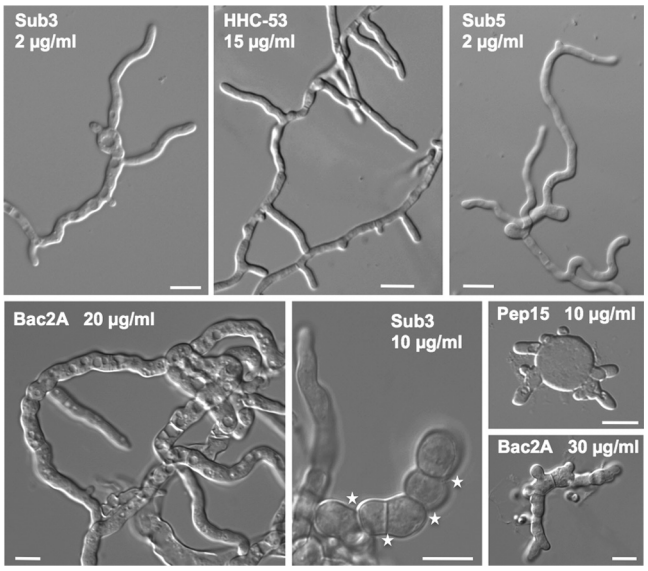

FIG. 2. Morphological phenotypes of hyphae incubated with peptides at various concentrations. Differential interference contrast (DIC) micrographs of *A. nidulans* hyphae grown at 30°C overnight in 450 μl of MM. At concentrations around the MIC almost all spores germinated, but more branching and meandering hyphae were observed (upper panel). At concentrations above the MIC, where most spores did not germinate, thicker hyphae and depolarized cells were observed (lower panels). Septa are marked by asterisks in the culture with Sub3. Bar, 10 μm.

(for HHC-53, 25 μg of peptide/ml; Sub5, 3.1 μg of peptide/ml) inhibited the reduction of resazurin (see Fig. S1C). Since resazurin reduction began no later than 16 h into the incubation period at peptide concentrations showing the reduction of resazurin, our MICs were calculated at the 18-h incubation time point.

The MICs are reported as sample means (± standard deviations [SD]) of four microtiter plates. The MICs of all peptides against *A. nidulans* are within a range similar to the MICs of other microorganisms. No peptides presented in Table 1 show exclusively antifungal or antibacterial activity.

Morphological phenotype. In order to better understand the mode of antifungal activity of our peptides, we investigated effects on hyphal morphology. After spores were exposed to peptide concentrations around their MICs, more than 95% of the spores were still capable of germinating successfully; however, hyphae showed increased branching, tip splitting, and multiple branches from single compartments. In addition, hyphae did not grow straight but curved (Fig. 2, upper panel); these phenotypes were not observed in all samples, however, and some hyphae appeared largely unaffected. With peptide concentrations above the MICs, less than 1% of the spores formed hyphae, and all of them displayed strong morphological phenotypes: hyphae were much thicker and appeared irregularly swollen (Fig. 2, lower panel).

Localization of fluorescently labeled peptides. The Sub5 peptide (RRWKIVVIRWRR; 1,724 Da) was selected for further analysis and synthesized with an N-terminal FITC label. In addition, nonactive peptides P7 and NC were also labeled with FITC and used as negative controls. The MIC of the FITC-labeled Sub5 was the same as that of the unlabeled derivative (Fig. 1B), and FITC-labeled P7 and NC showed no antifungal activity. When peptides were added, just before observation, to hyphae of *A. nidulans* grown on coverslips, both FITC-Sub5 and FITC-P7 localized to the cell surface and septa (Fig. 3A and data not shown). *A. nidulans* hyphal tips showed a stronger

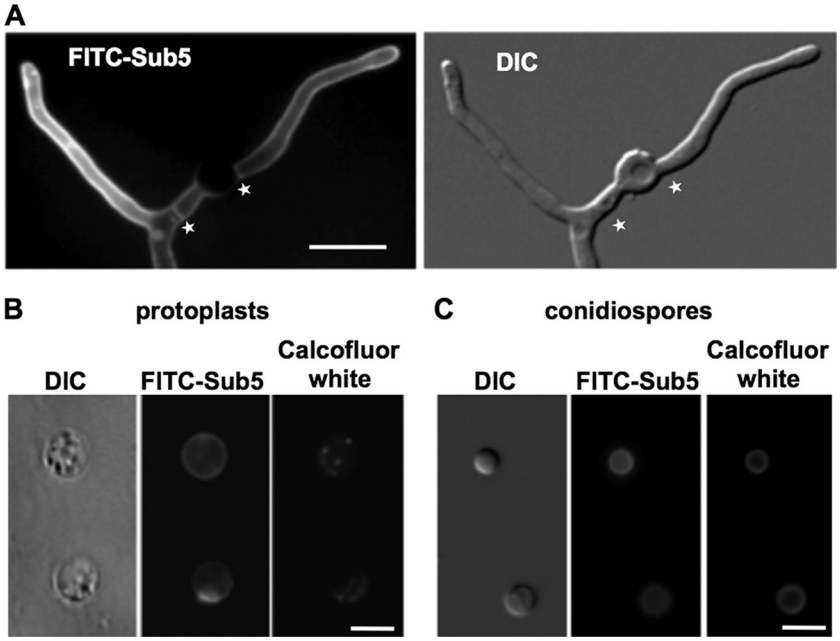

FIG. 3. Localization of FITC-Sub5. (A) *A. nidulans* hyphae after a 5-min exposure to FITC-Sub5 (10 μg/ml). FITC-Sub5 was identified at the cortex of hyphae and septa (asterisks). Bar, 10 μm. (B) Localization of FITC-Sub5 in protoplasts. Protoplasts were prepared using cell wall digestion, then stained with calcofluor white, and incubated with FITC-Sub5. Bar, 5 μm. (C) Localization of FITC-Sub5 in conidiospores. Conidiospores were stained with calcofluor white and incubated with FITC-Sub5. Bar, 5 μm.

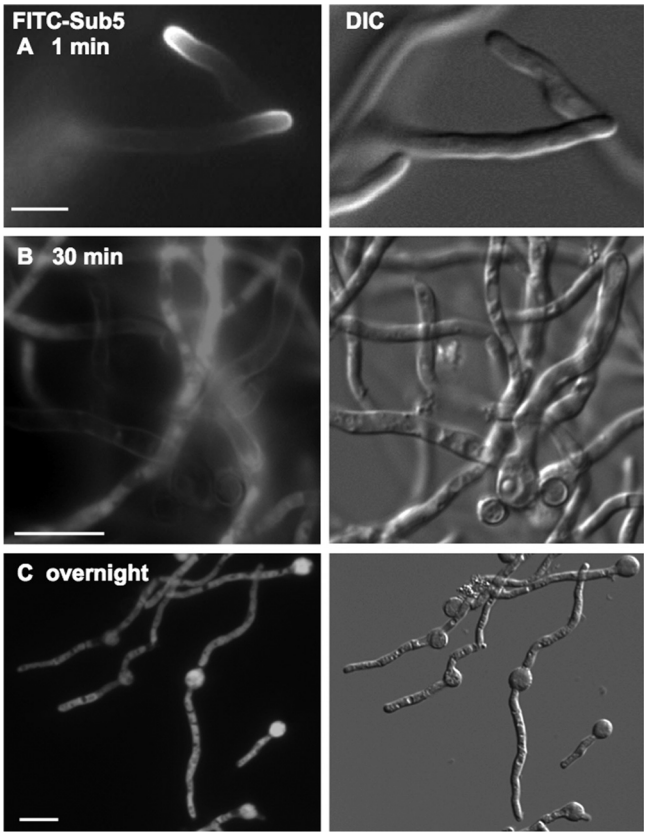

FIG. 4. Time series of FITC-Sub5 internalization. *A. nidulans* hyphae exposed to 10 μg/ml FITC-Sub5 for 1 min (A), 30 min (B), and overnight (C). (A) FITC-Sub5 localized at hyphal cortices, particularly at hyphal tips. (B) FITC-Sub5 localized to the cytoplasm in many hyphae. (C) FITC-Sub5 migrated as far as the cytoplasm in almost all hyphae. After overnight incubation, germlings did not develop into mycelia but remained as germlings. Bar, 10 μm.

signal than the hyphal body (Fig. 4A). In contrast, the FITC-NC peptide did not stain septa or cell surfaces (data not shown). FITC-P7, like FITC-Sub5, localized to the cell surface but showed no antifungal activity, indicating that the localization to cell surface of the peptides does not imply antifungal activity. The localization pattern at the cell surface was not dependent on the concentration of the peptide (1 to 10 μg/ml).

In order to address the question of whether the peptides localized on the cytoplasmic membrane or on the cell wall, we analyzed peptide localization in protoplasts. Microscopic investigation confirmed that protoplasts were produced by the partial digestion of hyphal cell walls and concentrated in high-osmotic buffer. Protoplasts stained using calcofluor white showed that the cell wall had largely disintegrated and that only some dots remained (Fig. 3B). Both peptides (FITC-Sub5 and FITC-P7) remained localized on the surface of protoplasts the same as on the surface of conidiospores surrounded with the cell wall, indicating that they localized to the cytoplasmic membrane (Fig. 3B and C).

Internalization of peptide and fungal cell viability. While FITC-Sub5 (10 μg/ml) localized to the plasma membrane of *A. nidulans*, especially at hyphal tips (Fig. 4A), continuous exposure led to a strong cytoplasmic signal in many hyphae after 30 min (Fig. 4B) and in most hyphae after 60 min. Germling growth ceased, and no further elongation of hyphae was observed when cells were incubated overnight (Fig. 4C). In comparison, FITC-P7 and FITC-NC did not penetrate fungal cells, even after overnight incubation, which suggests a connection between antifungal activity of the peptide and internalization of the peptide.

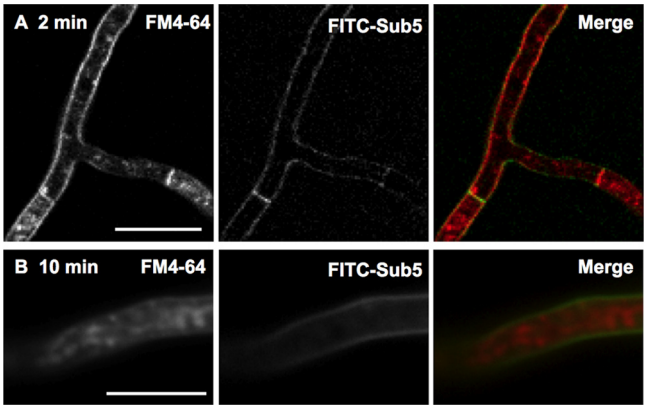

FIG. 5. Double staining of FITC-Sub5 using the membrane dye FM4-64. (A) Double staining of FM4-64 and FITC-Sub5 for 2 min resulted in an overlapping signal at the cell membrane and at septa. (B) After 10 min, FM4-64 infiltrated endosomal organelles while FITC-Sub5 remained localized at the cell surface. The images were taken with a confocal microscope. Bar, 10 μm.

In order to investigate how FITC-Sub5 was internalized from the cell surface into the cytoplasm, we used the membrane-selective fluorescent dye FM4-64 to visualize endocytosis (32). Hyphae were stained with FM4-64 and FITC-labeled peptides (10 μg/ml) and immediately observed under a fluorescence microscope. The hyphae showed an overlapping signal of FITC-labeled peptides with FM4-64 at the level of the cell membrane (Fig. 5A). After 10 min, FM4-64 was internalized into endosomal organelles; in contrast, FITC-Sub5 remained at the cell surface (Fig. 5B). The cytoplasmic signal of peptides appeared slower in the cytoplasm than the endosomal signal of FM4-64. The different kinetics suggests that the internalization of peptides occurs independently of endocytosis.

The localization of FITC-Sub5 that accumulated at hyphal tips was reminiscent of sterol-rich membrane domains, which are located at the hyphal tips of filamentous fungi, are important for cell signaling and polarity, and are essential for tip growth (7, 33, 38). These membrane domains were identified in several fungi using the sterol-binding fluorescent dye filipin (Fig. 6A) (24, 30, 38). After a 5-min incubation with FITC-Sub5 (10 μg/ml), hyphae were stained with filipin for 5 min, and both the filipin and FITC-Sub5 signals were observed at hyphal tips; however, when hyphae were incubated with FITC-Sub5 for 25 min prior to staining with filipin for 5 min, filipin staining was observed not at hyphal tips but, rather, within the hyphae. These results indicate that the peptide Sub5 disturbs these sterol-rich membrane domains although it was unclear whether the effect was direct or indirect. Disturbance of sterol-rich membrane domains in the fungal hyphae of *A. nidulans* was not observed after treatment with FITC-P7 or only filipin (1 μg/ml for 30 min).

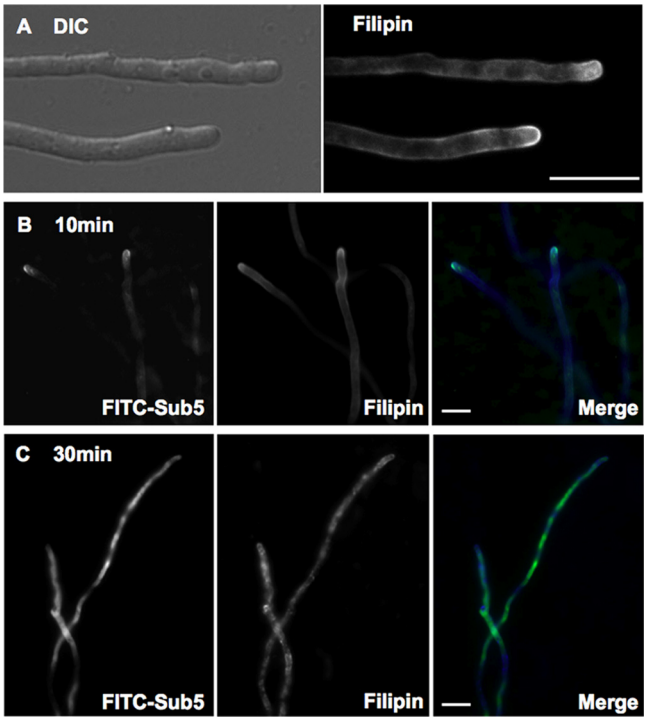

FIG. 6. Effect of FITC-Sub5 on sterol-rich membrane domains. (A) Hyphae stained with filipin (1 μg/ml) for 5 min. Sterol-rich membrane domains were visible at hyphal tips. (B) After a 5-min incubation with FITC-Sub5 (10 μg/ml) and then staining with filipin (1 μg/ml) for 5 min, FITC-Sub5 and the sterol-rich membrane domains were observed at hyphal tips. (C) After a 25-min incubation with FITC-Sub5 (10 μg/ml) and 5 min with filipin (1 μg/ml), FITC-Sub5 was internalized into the cytoplasm. Filipin staining was not observed at hyphal tips, suggesting disruption of the sterol-rich domains. Bar, 10 μm.

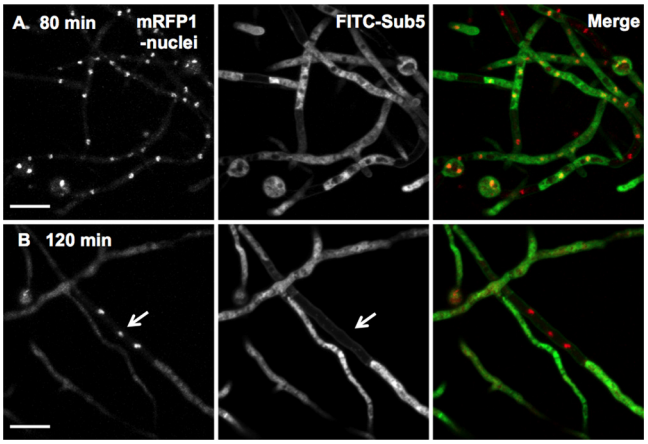

FIG. 7. Correlation between FITC-Sub5 internalization and cell death. Hyphae with mRFP1-labeled nuclei were incubated with FITC-Sub5 (10 μg/ml) for 80 min (A) and 120 min (B). (A) FITC-Sub5 localized in the cytoplasm of most hyphae, and mRFP1-labeled nuclei were visible. (B) mRFP1-tagged nuclei disappeared in most hyphae. mRFP1-tagged nuclei were observed in a small number of compartments where FITC-Sub5 was not internalized (arrow). Bar, 10 μm.

Preferential peptide binding with membrane domains has also been observed in bacteria. Increasingly, the evidence points to a lateral distribution of lipids that are heterogeneously distributed in bacterial membranes (25). The predominant anionic lipids of bacterial membranes, phosphatidylglycerols, are segregated into membrane domains (41) while cardiolipin is enriched at the polar and septal regions of the cytoplasmic membrane (21, 28). Phosphatidylglycerol domains are important for division site selection (27), and cardiolipin domains regulate the localization of osmosensory transporters (29). In the case of the interaction between antimicrobial cationic host defense peptides and bacteria, the peptide is assumed to interact with the anionic lipids of bacterial membranes, thereby impairing lipid domain formation, which inhibits membrane domain function (4, 5). Moreover, several mechanisms, such as toroidal pore, barrel-stave, and carpet models, have been proposed by which the peptides insert into membrane bilayers to form pores and induce membrane lysis (2). A mechanism similar to these may account for the internalization of Sub5 and could be applicable to other peptides as well.

The connection between cytoplasmic localization of FITC-Sub5 and fungal cell death was investigated using histone-H1 tagged with mRFP1 as a marker for viability (40). After treatment with FITC-Sub5 (10 μg/ml) for 80 min, FITC-Sub5 was observed in the cytoplasm of almost all hyphae, and mRFP1-labeled nuclei were clearly visible (Fig. 7A). After a 120-min exposure, mRFP1-labeled nuclei were no longer visible in most hyphae (Fig. 7B); the mRFP1-tagged nuclei were observed only in a small number of compartments (<1%) where FITC-Sub5 had not yet infiltrated the cytoplasm but remained at the cytoplasmic membrane (Fig. 7B, arrow). The effect on nuclei could still be explained if there were action only on the cytoplasmic membrane which caused cell death and subsequent nuclear degradation. However, the fact that we observed hyphal compartments where the FITC-Sub5 signal remained at the cytoplasmic membrane and nuclei appeared normal favors the notion that internalization of the peptide is important for the deleterious effect of the peptide. It will be one of the important tasks for future research to identify such intracellular targets of Sub5 and understand the mechanism of action. The results showing that short antimicrobial peptides, like Bac2A, indolicidin, Sub3, and Sub5, have the ability to interact with ATP and ATP-dependent enzymes suggest that these peptides could also have internal targets (17).

All peptides investigated in this study have also been found to be antibacterial, indicating a lack of specificity. While the findings of our study provide important information about the antifungal potential of certain peptides, as well as the mode of action for FITC-Sub5, an important aim is also the identification of microbe-specific peptides; to this end, the high-throughput assay developed in this paper will be very important toward screening large peptide libraries for antifungal activity. Once such peptides have been identified, the assay can be used to further optimize peptide design; experiments using amino acid substitutions or random sequence scrambling have proven to be effective in that regard (15, 19).

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