Translocation of cell-penetrating peptides into *Candida* fungal pathogens

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Abstract: Cell-penetrating peptides (CPPs) are small peptides capable of crossing cellular membranes while carrying molecular cargo. Although they have been widely studied for their ability to translocate nucleic acids, small molecules, and proteins into mammalian cells, studies of their interaction with fungal cells are limited. In this work, we evaluated the translocation of eleven fluorescently labeled peptides into the important human fungal pathogens *Candida albicans* and *C. glabrata* and explored the mechanisms of translocation. Seven of these peptides (cecropin B, penetratin, pVEC, MAP, SynB, (KFF)$_3$K, and MPG) exhibited substantial translocation (>80% of cells) into both species in a concentration-dependent manner, and an additional peptide (TP-10) exhibiting strong translocation into only *C. glabrata*. Vacuoles were involved in translocation and intracellular trafficking of the peptides in the fungal cells and, for some peptides, escape from the vacuoles and localization in the cytosol were correlated to toxicity toward the fungal cells. Endocytosis was involved in the translocation of cecropin B, MAP, SynB, MPG, (KFF)$_3$K, and TP-10, and cecropin B, penetratin, pVEC, and MAP caused membrane permeabilization during translocation. These results indicate the involvement of multiple translocation mechanisms for some CPPs. Although high levels of translocation were typically associated with toxicity of the peptides toward the fungal cells, SynB was translocated efficiently into *Candida* cells at concentrations that led to minimal toxicity. Our work highlights the potential of CPPs in delivering antifungal molecules and other bioactive cargo to *Candida* pathogens.

Keywords: Cell-penetrating peptides; *Candida*; translocation; drug delivery

Introduction

Candidiasis is a human fungal infection caused by *Candida* species, often *C. albicans* or *C. glabrata*. Infections include relatively minor mucosal infections, such as skin infections and oral thrush, as well as severe systemic infections that can occur in people with compromised immune systems.[1] Available antifungal drugs to treat candidiasis act on a limited number of cellular targets, and the incidence of drug resistance is rising,[2] spurring the demand for novel therapeutic approaches.

One potential approach to improving antifungal therapeutics is to use a drug delivery vehicle to improve cellular recognition and intracellular delivery of new or existing antifungal agents. Cell-penetrating peptides (CPPs) are promising candidates for delivering therapeutic agents and have been widely studied for intracellular delivery of bioactive molecules into mammalian cells. CPPs are typically short, cationic peptides with 30 amino acids or fewer[3] and have the ability to cross cell membranes. A variety of bioactive molecules have been successfully attached to CPPs and delivered intracellularly, including nucleic acids (both DNA and RNA),[4–6] fluorescent proteins,[7,8] and antibodies.[9,10]

Although CPPs show great potential in drug delivery, the structural and functional diversity of CPPs complicates studies of their interactions with cells. Previous mechanistic research on CPP translocation

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Table I. *CPPs tested in this study*

Peptide	Sequence	MW (Da)	Net charge[a]
pVEC	LLIILRRRIRKQAHAHSK	2209.7	+8
(KFF)₃K	KFFKFFKFFK	1413.8	+4
Penetratin	RQIKIWFQNRRMKWKK	2246.8	+7
MAP	KLALKLALKALKAALKLA	1876.0	+5
Pep-1	KETWWETWWTEWSQPKKKRKV	2848.3	+2
hCT	LGTYTQDFNKTFPQTAIGVGAP	2326.6	0
SynB	RGGRLSYSRRRFSTSTGR	2100.3	+6
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	2807.4	+5
PAF26	RKKWFW	950.2	+3
TP-10	AGYLLGKINLKALAALAKKIL	2182.8	+4
Cecropin B (antimicrobial peptide)	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	3835.7	+9

[a] Includes only charges due to amino acid side chains (pH 7) and not N-terminal FAM (-3).

has focused on translocation into mammalian cells and suggests some peptides penetrate cells via an energy-dependent endocytosis process. These CPPs include MAP (synthetic, highly amphiphilic model peptide[11]), TP-10 (fragment of transportan[12]), hCT (derivative of calcitonin[13]), SynB (derivative of the antimicrobial peptide protegrin 1[14]), and PAF26 (hexapeptide with antimicrobial activity[15]). In contrast, other CPPs may enter cells via macropinocytosis,[16] including pVEC (derivative of murine vascular endothelium cadherin[17]) and penetratin (fragment of antennaedia homeodomain[18]). The mechanism of translocation may also include transient pore formation,[19] which is suspected for MPG (derivative of two viruses[20]) and Pep-1 (synthetic peptide[21]). MPG and Pep-1 also contain nuclear localization sequences, which promote the translocation efficacy and solubility of the peptides.[19] For other CPPs, such as (KFF)₃K (synthetic peptide[22]), additional work is required to elucidate the mechanism of translocation. These examples highlight the diversity of CPPs as delivery vehicles and the challenges in understanding their interaction with cells.

Most studies on the translocation mechanisms of CPPs have focused on translocation in mammalian cells, and studies of the interactions of CPPs with fungal cells, including *Candida* cells, are very limited.[7,8,23–26] To expand the application of CPPs to delivering molecules to *Candida* species, an improved understanding of the interaction of CPPs with *Candida* cells is required. One key structural difference between mammalian cells and fungal cells is the presence of a cell wall in fungal cells. The cell wall is composed of chitin, glucans, mannans, and glycoproteins and provides an additional barrier for CPP transport into fungal cells compared to mammalian cells.[27] Another key difference is that fungal cells have vacuoles, which are involved in a number of biological processes in fungal cells, including endocytosis, pH and salt balance maintenance, and phosphate degradation.[28] The effect of these structures on CPP translocation and trafficking has not been described previously, and an understanding of

their role will facilitate the use and design of CPPs for delivering molecular cargo to fungal cells.

To improve the understanding of how CPPs translocate into fungal cells, we studied the translocation of known CPPs into two *Candida* pathogens, *C. albicans* and *C. glabrata*. We evaluated the translocation and toxicity of the CPPs and explored their mechanisms of translocation. Some peptides previously shown to enter mammalian cells were also translocated into *Candida* species, while others exhibited little to no translocation. Our analysis of subcellular localization of CPPs provides insight into intracellular trafficking of the peptides, as well as translocation mechanisms. Further experiments to explore the translocation mechanisms indicate the translocation of some CPPs in fungal cells may differ from the mechanisms proposed for mammalian cells. Our data suggest translocation of CPPs into fungal cells often correlates with toxicity toward the cells, but some peptides are taken up by *Candida* cells with little effect on viability.

Results and Discussion

Translocation of CPPs into Candida *species*

To study the interaction of CPPs with *Candida* species, we selected peptides representing a variety of structures and native origins to study CPP translocation into *Candida* cells (Table I). All peptides were synthesized commercially with an N-terminal 5-carboxyfluorescein label (FAM), which served as the cargo for the CPP and as the reporter to detect translocation. Our set of peptides includes peptides shown previously to translocate into mammalian cells (all CPPs) or microbial cells, including bacteria and fungi (pVEC, (KFF)₃K, penetratin, MAP, PAF26, and TP-10).[7,8,23–26] The CPPs pVEC, (KFF)₃K, penetratin, and TP-10 were shown to enter *C. albicans* previously.[7,23] Some peptides are thought to be transported by energy-dependent endocytic mechanisms (MAP, hCT, TP-10, SynB, and PAF26) or macropinocytosis (pVEC and penetratin), while others undergo pore formation (penetratin, MPG and Pep-1) or
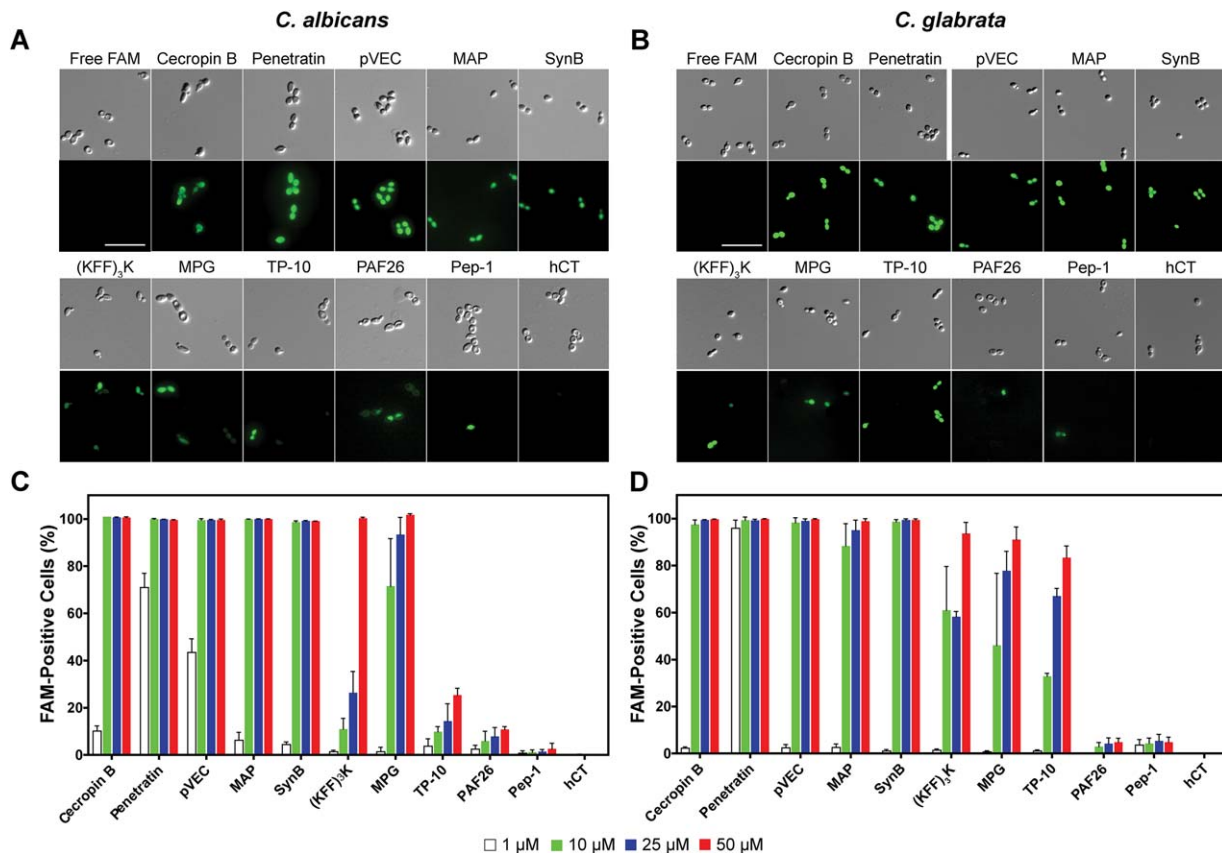
Figure 1. Translocation of FAM-labeled CPPs into *Candida* cells. DIC and FAM fluorescence images for translocation into (A) *C. albicans* and (B) *C. glabrata*. Flow cytometry data for translocation into (C) *C. albicans* and (D) *C. glabrata*. Cells were incubated for 1 h at 30°C with 25 μM peptides for imaging or serial dilutions of peptides (1–50 μM) for flow cytometry. Surface-bound peptides were removed by trypsin prior to imaging or flow cytometry, and controls with free FAM were included. For (A) and (B), scale bar = 10 μm. For (C) and (D), error bars represent the standard error of the mean for three separate experiments (N = 3).

unknown mechanisms ((KFF)$_3$K).[11–19] We also included cecropin B, a well-known antimicrobial peptide with antifungal activity, to compare its translocation with peptides previously identified as CPPs. *C. albicans* and *C. glabrata* were selected as target cells, because they are frequently isolated from patients with candidiasis.[29]

To screen the CPPs for translocation into *Candida* species, we incubated *C. albicans* and *C. glabrata* cells with each of the CPPs. We identified the CPPs that cross the barriers of these fungal cells using fluorescence microscopy to visualize the location of the fluorescein-labeled peptides [Fig. 1(A,B)]. Cells were treated with trypsin prior to imaging to remove peptide associated with the cell surface.[7,30] A high level of translocation efficacy was observed in both types of fungal cells for several peptides with a relatively high net charge ($\geq$ +4), including penetratin, pVEC, MAP, SynB, (KFF)$_3$K, and MPG. PAF26 and Pep-1, which have a lower net charge ($<$ +4), showed limited levels of translocation, while hCT (no net charge) could not be detected entering the *Candida* cells. The antimicrobial peptide cecropin B (+ 9) exhibited a high level of translocation, suggesting

cecropin B can function as a CPP in addition to an antimicrobial peptide.

We quantified translocation and the effect of peptide concentration on translocation in *C. albicans* and *C. glabrata* using flow cytometry. Single cells were identified, and the percentage of these cells positive for FAM fluorescence was determined for each species [Fig. 1(C,D)]. We detected a dose-dependent fluorescence signal for each of the peptides that showed substantial translocation in the fluorescence microcopy assay. The flow cytometry data confirmed the limited translocation for PAF26 and Pep-1 and the lack of translocation for hCT that we observed by fluorescence microscopy.

For most of the peptides, the behavior in *C. albicans* and *C. glabrata* was very similar. The exception to this was TP-10, which showed significantly enhanced translocation in *C. glabrata* compared to *C. albicans*. Although these two species are closely related, they do have unique characteristics that could affect the interaction of the cells with CPPs. For example, they express different membrane-anchored proteases and other proteins,[31,32] which may alter degradation of CPPs and their interaction with the cell membrane.

Role of net charge in translocation

For *Candida* cells, our data indicate that net charge of the peptides plays a role in translocation, with higher levels of positive charge generally leading to higher levels of translocation. As the phosphate heads of the membrane lipids are negatively charged, positively charged residues in peptides will lead to electrostatic interactions that bring peptide in close contact with the cell membrane. CPPs with higher net charges ($\geq +4$) including pVEC, penetratin, MAP, SynB, (KFF)$_3$K, MPG, TP-10 and Cecropin B would have stronger electrostatic interactions, leading to a stronger interaction with the cell membrane and explaining their higher translocation efficacy. In our experiments, we used a FAM label as the cargo for our peptides. This FAM label has a net negative charge of -3 at the pH of our experiments, which would reduce the overall net charge of the peptide-cargo constructs. Additional studies evaluating other cargo molecules will be important in understanding the impact of the peptide charge alone and in determining whether properties of cargo molecules strongly influence the translocation effects of the peptides.

Comparison to translocation into mammalian cells

Although each of the CPPs was previously shown to translocate into mammalian cells,[30,33] only pVEC, (KFF)$_3$K, penetratin, and TP-10 were previously shown to translocate into *Candida* cells.[7,23] Our results indicate that many of the CPPs that function in mammalian cells do function in *C. albicans* and *C. glabrata*, but translocation into mammalian cells does not guarantee translocation in fungal cells. Three of the peptides tested showed little to no translocation in the two *Candida* species. In the case of Pep-1, association of the peptide with the cell wall contributed to the low level of translocation: when cells incubated with Pep-1 were imaged prior to trypsin treatment, the peptide was observed on the cell surface (Supporting Information Fig. S1). However, for hCT and PAF26, the origin of the difference is less clear, since these peptides did not localize at the cell surface.

Subcellular localization of peptides

To gain insight into the intracellular trafficking of the CPPs, we examined our microscopy images (Fig. 1). In some images, peptides appeared to localize in the vacuole [e.g. see FAM image for PAF26 in Fig. 1(A)]. In other images, the vacuoles that typically are easily visible were no longer present [e.g. see differential interference contrast (DIC) images for MAP and penetratin in Fig. 1(B)]. Yeast vacuoles are very important for maintaining homeostasis, and they are highly involved in transmembrane transport.[28]

To better understand the relationship between CPP trafficking, vacuoles, and vacuole loss, we used Cell-Tracker Blue, a yeast vacuole stain, to track the vacuoles during incubation of *C. albicans* cells with the peptide pVEC [Fig. 2(A)]. When cells were incubated with a low concentration of pVEC, they retained their vacuoles and exhibited colocalization of a low level of FAM fluorescence with vacuole stain fluorescence. At higher concentrations of peptide, we observed total loss of vacuole stain fluorescence, an enhancement of FAM fluorescence intensity, and a shift to cytosolic fluorescence. For cells treated with 10 μM of pVEC, the differential FAM fluorescence was most apparent. Cells retaining their vacuoles showed a brighter vacuole stain signal but a weaker FAM fluorescence, whereas cells that lost vacuoles had a stronger cytosolic FAM signal and no vacuolar fluorescence. The differential fluorescence intensity arises from the sensitivity of the 5-FAM fluorescein derivative we used. 5-FAM is very sensitive to pH and exhibits higher fluorescence intensity at pH 7.5 and lower intensity at pH 6.6.[34] Because the cytosolic pH ($\sim$7.4) is relatively higher than the one inside vacuoles ($\sim$6.2),[35] the fluorescence intensity of the peptides is lower in vacuoles than in the cytosol.[36] Vacuole loss can also be identified by using flow cytometry to observe the FAM signal shift as the peptides move from the vacuoles to the cytosol at higher concentrations [Fig. 2(B)]. We quantified the cellular localization by gating two distinct populations in the flow cytometry data: one with a stronger FAM intensity but no vacuole stain fluorescence and the other with both vacuole stain fluorescence and lower intensity FAM fluorescence. These data show that vacuole loss is associated with high levels of translocation of pVEC.

Based on the results for pVEC, we used flow cytometry to evaluate the subcellular localization in *C. albicans* and *C. glabrata* for the peptides with significant translocation (Fig. 3). Subcellular vacuole localization was observed for each of the CPPs, suggesting vacuoles are generally involved in the translocation mechanism of CPPs for fungal cells. However, the proportion of FAM-positive cells that exhibited vacuole localization was lower for some peptides (e.g. pVEC, penetratin) compared with other peptides (e.g. SynB, MPG, (KFF)$_3$K) (Supporting Information Fig. S2), indicating differences in translocation mechanisms and/or trafficking amongst the peptides.

Mechanisms for translocation into fungal cells

Our localization experiments revealed the important relationship between vacuoles and translocation. Previous work with yeast vacuoles suggested vacuoles are involved in endocytosis,[28,37] so we next studied endocytosis of the CPPs. To explore whether the mechanisms of translocation in fungal cells
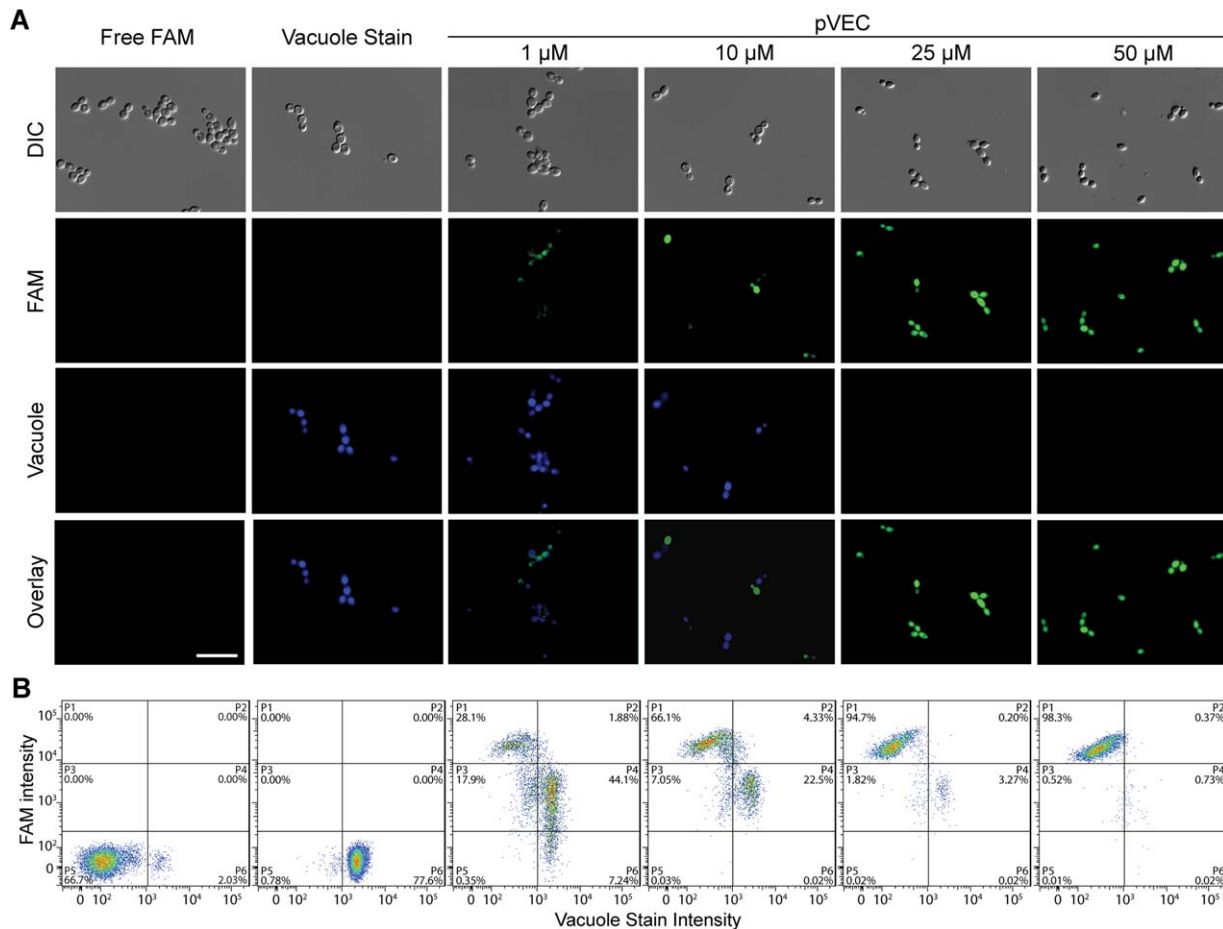
Figure 2. Intracellular distribution of pVEC in *C. albicans* at different peptide concentrations. (A) DIC and fluorescence micros-copy images showing location of FAM-labeled pVEC and location of vacuoles in cells. (B) Flow cytometry data illustrating shift of FAM and vacuolar fluorescence. Cells were incubated with serial dilutions of pVEC (1–50 μM) at 30°C for 1 h and treated with trypsin to remove surface-bound peptide. CellTracker Blue vacuolar stain was added, and the samples were incubated at room temperature for 10 min prior to analysis. For (A), scale bar = 10 μm.

involve endotytosis, we first studied the transloca-tion process in *C. albicans* under conditions that inhibit ATP synthesis. Most ATP-dependent pro-cesses, including endocytosis, are limited at 4°C.[38] Sodium azide (NaN$_3$) also inhibits energy-dependent endocytosis by inhibiting the function of cytochrome-c-oxidase for ATP synthesis.[39,40] We observed a sig-nificant reduction in translocation for six of the eight peptides (cecropin B, MAP, SynB, MPG, (KFF)$_3$K, and TP-10) in the presence of 25 mM NaN$_3$ and at low temperature (Fig. 4), suggesting these peptides utilize energy-dependent endocytosis as their translocation mechanisms. pVEC and pene-tratin were not highly affected by the addition of NaN$_3$ or the lower temperature, suggesting an energy-independent translocation process.

Membrane destabilization is also involved in the translocation mechanism for some CPPs.[14,15,41] To evaluate whether membrane destabilization plays a role in translocation of the peptides in our study, we used propidium iodide (PI) to identify pore formation in the membranes during CPP translocation into

C. albicans and *C. glabrata*. Cells are normally impermeable to PI, but PI fluorescence can be detected in cells with destabilized membranes, which typically represents cells losing viability.[42] Using fluorescence microscopy, we observed that pVEC, penetratin, MAP, and cecropin B contributed to membrane permeabilization of *C. albicans* at a moderate concentration (10 μM) [Fig. 5(A)]. We con-firmed these results by flow cytometry [Fig. 5(B,C)] and found almost all cells incubated with these pep-tides were both PI- and FAM-positive, indicating translocation of these peptides is correlated to cell permeabilization. In contrast, (KFF)$_3$K, SynB, and MPG showed FAM fluorescence with little to no PI fluorescence, suggesting these CPPs did not lead to general defects in membrane integrity at this concentration.

Relationship between subcelluar localization and translocation

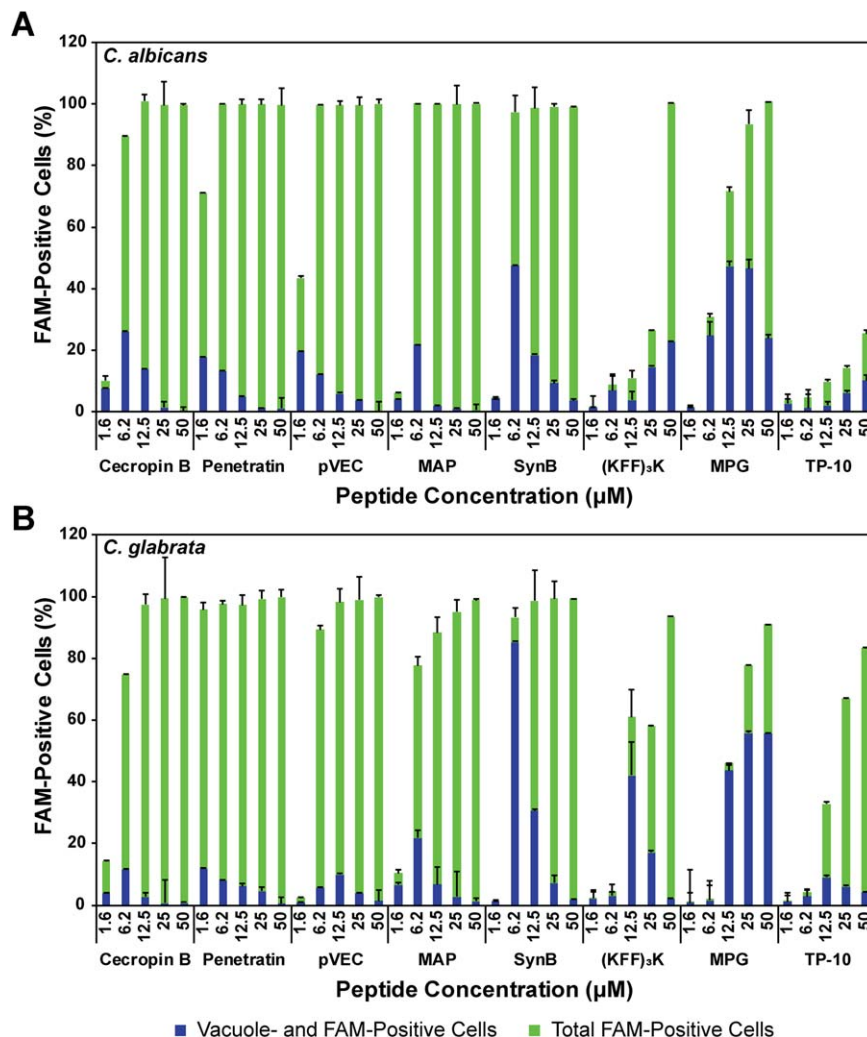Our results for subcellular localization, membrane stability, and endocytic inhibition together provide

Figure 3. Quantification of cellular location of CPPs in (A) *C. albicans* and (B) *C. glabrata*. Cells were incubated with serial dilutions of peptide (1–50 μM) at 30°C for 1 h, washed with trypsin, and incubated with CellTracker Blue vacuolar stain at room temperature for 10 min. Flow cytometry data were collected for FAM (peptide) fluorescence and vacuolar stain fluorescence. The percentage of cells with FAM fluorescence and with both FAM and vacuolar fluorescence were quantified. Error bars represent the standard error of the mean for three separate experiments ($N = 3$).

insight into the translocation mechanisms for the CPPs in fungal cells (Table II). The combination of significant vacuolar localization, lack of strong membane destabilization, and inhibition of translocation by NaN_3 and low temperature suggests endocytosis is involved in the translocation of a peptide; we observed these characteristics for the translocation of SynB and $(KFF)_3K$. Stronger cytosolic localization along with high PI permeability, as we observed at high concentrations for cecropin B, pVEC, penetratin, and MAP, indicates the possibility of direct translocation into the cytosol, though endocytosis might still be possible at concentrations resulting in vacular localization.

Interestingly, the data for several of the peptides indicate multiple mechanisms may be involved in their translocation into fungal cells. For example, the NaN_3 and low temperature data for pVEC suggest an energy-independent mechanism, such as

macropinocytosis or direct pore formation, which is consistent with the PI data. However, the microscopy images and flow cytometry data (Fig. 2) also indicate vacuolar trafficking, which is more consistent with energy-dependent endocytosis. The possibility of multiple mechanisms is consistent with prior work with pVEC in mammalian cells. Elmquist *et al.*[45] found that a clathrin-dependent endocytic pathway is involved in the translocation of pVEC, yet they still observed modest translocation at low temperatures to also implicate non-endocytic pathways. Likewise, MAP and cecropin B have high PI permeability consistent with direct translocation, yet they exhibit the energy-dependent translocation and vacuolar trafficking consistent with endocytosis, which could indicate multiple mechanisms are also involved in translocation of these peptides.

For some peptides, our results implicate a translocation mechanism in *Candida* cells that differs
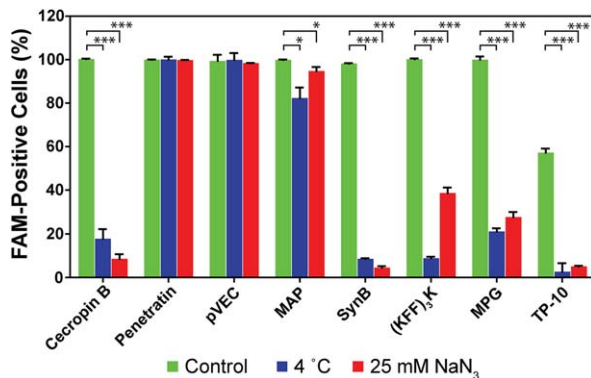
Figure 4. CPP translocation into *C. albicans* under conditions that inhibit energy-dependent endocytosis. Cells were incubated with peptides (10 μM) for 1 h. Control samples were incubated at 30°C. Endocytosis was inhibited by adding 25 mM NaN_3 or by changing the incubation temperature to 4°C. The percentage of cells exhibiting FAM fluorescence was quantified by flow cytometry. Error bars represent the standard error of the mean for three separate experiments ($N = 3$). Statistical significance was analyzed with a one-way ANOVA test ($\alpha = 0.1$), and the number of asterisks indicates the level of significance (* for $P \leq 0.01$ and *** for $P \leq 0.0001$).

from previous studies with mammalian cells. MPG and TP-10 were previously suggested to destabilize and make pores in the membrane of HeLa and melanoma cells, respectively.[41,44] However, our PI data,

NaN_3 assay, and localization images indicate an endocytic process is involved for *Candida* cells. The difference in structure of the cell types may help explain this discrepancy. Hallbrink *et al.*[44] and Simeoni *et al.*[41] showed the translocation and pore formation of MPG, Pep-1, and TP-10 were associated with the close interaction between peptides and cell membranes. However, the additional barrier of the cell wall in fungal cells could interfere with this close interaction. This idea is consistent with our observation that Pep-1 associated with the surface and did not translocate into fungal cells (Supporting Information Fig. S1). Additionally, differences in cell-surface properties and proteins could change the overall structure and behavior of CPPs. For example, *Candida* cells could have a receptor that recognizes MPG and TP-10, causing a shift in the translocation mechanism in fungal cells to endocytosis, even though membrane destabilization occurs in mammalian cells.

Toxicity of CPPs toward *Candida* cells
Yeast vacuoles are necessary for cells to maintain homeostasis, so the loss of vacuoles due to CPPs may lead to toxicity toward fungal cells. To examine the toxicity of CPPs toward *C. albicans* and *C.*
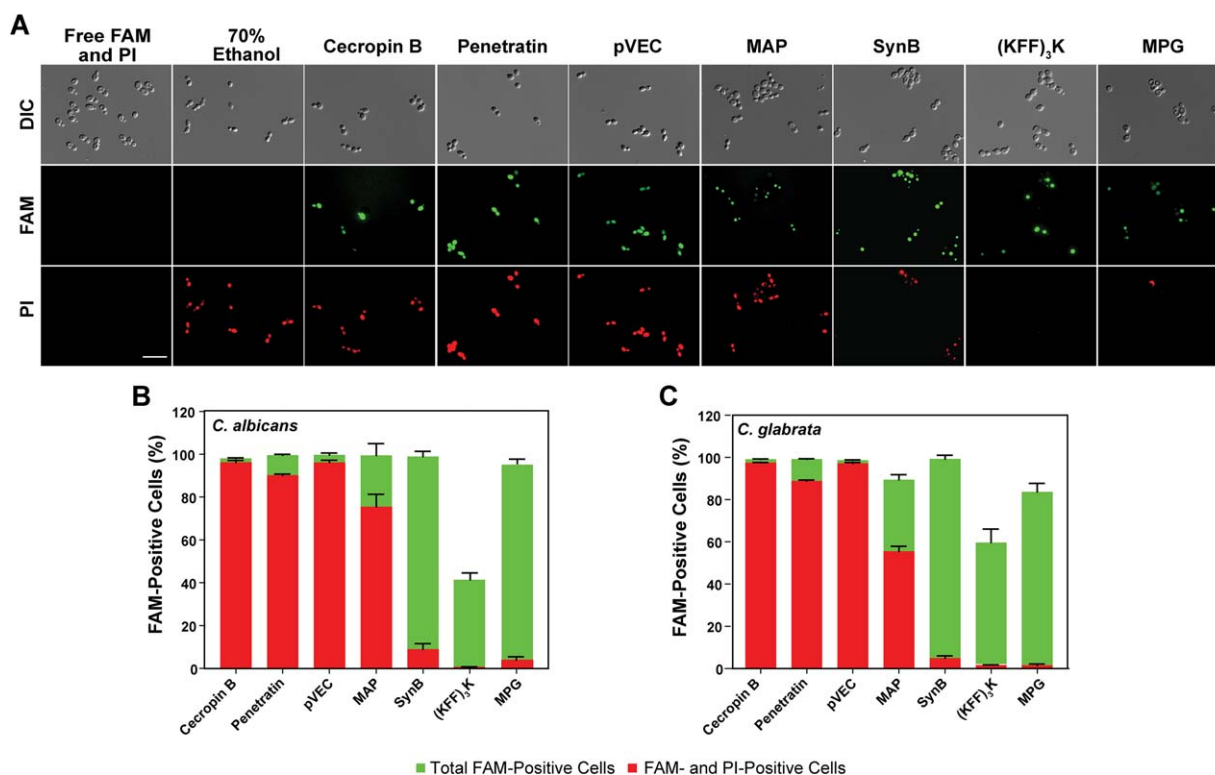

Figure 5. Effect of CPPs on integrity of cell membrane. (A) DIC and fluorescence microscopy images showing CPP translocation and PI uptake in *C. albicans*. (B) Flow cytometry data indicating the percentage of cells with CPP translocation (FAM fluorescence) and with PI uptake in *C. albicans*. (C) Flow cytometry data indicating the percentage of cells with CPP translocation and with PI uptake in *C. glabrata*. Following incubation with FAM-labeled CPPs (10 μM), cells were treated with trypsin and then incubated with PI for 1 min immediately prior to imaging or flow cytometry. For (A), scale bar = 10 μm. For (B) and (C), error bars represent the standard error of the mean for three separate experiments ($N = 3$).

Table II. *Summary of subcellular localization and potential translocation mechanism of CPPs*

	Cecropin B	Penetratin	pVEC	MAP	SynB	(KFF)$_3$K	MPG	TP-10
Vacuolar localization	Low	Low	Low	Low	High	High	High	High
PI permeability[a]	++	++	++	++	+	−	−	−
NaN$_3$/4°C endocytosis inhibition	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Potential mechanism[b]	E/D	M/D	E/M	E/D	E	E	E	E
Previously reported mechanism[b]	E/D[43]	E/D[14]	M[17]	D[44]	E[14]	n/a	D[41]	D[44]

[a] Strong PI permeability is indicated by ++, moderate permeability by +, and no significant permeability by −.
[b] Translocation mechanisms are as follows: endocytosis (E), macropinocytosis (M), direct translocation/pore formation (D), or no data available (n/a).

glabrata, we incubated the cells with serial dilutions of the peptides. At high concentrations of peptide, we observed significant toxicity toward both types of *Candida* cells for each peptide that exhibited substantial translocation (Fig. 6), with minimum inhibitory concentrations (MIC50s) ranging from 1 μM to 50 μM (Table III). Little to no antifungal activity could be detected for CPPs that exhibited very low levels of translocation (i.e., Pep-1, PAF26, and hCT in both species and TP-10 in *C. albicans*) (Fig. 6), and MIC50s for these peptides were above 50 μM (Table III). Incubation with free FAM did not lead to any viability loss of *Candida* cells compared to the cells incubated with only Na$_2$HPO$_4$ buffer (data not shown). Interestingly, the CPPs pVEC, penetratin, and MAP exhibited even stronger antifungal activity than the well-known antimicrobial peptide cecropin B. For many of the peptides, the loss of viability could be due to the vacuole loss we observed in our microscopy and flow cytometry data, since vacuoles are essential for fungal cells in controlling the cellular osmotic pressure, salt balance, and pH.[28,46] For the peptides that exhibited substantial PI permeability (pVEC, penetratin, and MAP), the loss of viability could also be due to membrane permeabilization, though it is not clear from our data whether the

permeabilization is a cause or an effect of cell death. Between the two *Candida* species, *C. glabrata* tended to be less sensitive to toxicity of the peptides compared to *C. albicans*, which is consistent with previous research of antimicrobial peptides and other antifungal agents.[47–49] This difference in toxicity of CPPs and antimicrobial peptides between *Candida* species is not yet fully understood, though work has suggested that it may be due to the different compositions of the cell membranes and the presence of different membrane proteins.[47,49]

The toxicity of CPPs toward *Candida* pathogens could prove to be a positive feature of peptides targeted to fungal pathogens, as one motivation for studying CPPs in fungal cells is to use them to deliver antifungal molecules, and the cytotoxicity of the CPP vehicles would potentially increase the therapeutic effects of the cargo being delivered. To function as a delivery vehicle for antifungal therapeutics, toxicity of the CPPs to host cells would need to be minimal. Previous studies suggest TP-10, penetratin, MAP, and cecropin B negatively affect mammalian cell viability,[33,50–53] which would complicate their use in delivery of antifungal therapeutics. In contrast, pVEC and MPG did not significantly reduce viability of mammalian cells,[33,54] and we observed both strong translocation
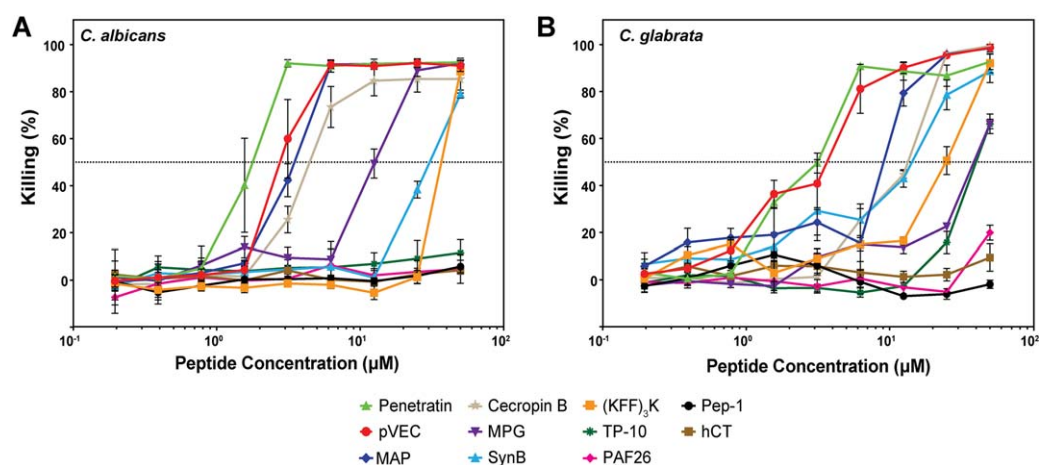

Figure 6. Toxicity of CPPs toward (A) *C. albicans* and (B) *C. glabrata*. Cells were incubated with serial dilutions of peptides (0.2–50 μM) for 1 h at 30°C. Samples were diluted, mixed with YPD medium, and incubated at 30°C for 16 h. Optical density (OD$_{600}$) of the cultures was measured and converted to killing percentage. Error bars represent the standard error of the mean for three separate experiments (*N* = 3). Dotted line represents 50% killing, which was used to determine the MIC50 (Table III).

Table III. *Antimicrobial activity of peptides*

	Minimum Inhibitory Concentration (MIC50, μM)[a]										
	Cecropin B	Penetratin	pVEC	MAP	SynB	(KFF)$_3$K	MPG	TP-10	PAF26	Pep-1	hCT
C. albicans	4	1	2	4	50	50	10	> 50	> 50	> 50	> 50
C. glabrata	25	2	4	8	25	50	50	32	> 50	> 50	> 50

[a] MIC50 values are defined as the minimum concentration of peptide required to reduce growth of cells by 50%. The highest concentration tested was 50 μM.

and strong toxicity toward fungal cells, suggesting these peptides would be promising candidates for delivering antifungal agents or could potentially serve as antifungal agents themselves.

Although toxicity of CPPs may be desirable in the case of delivering antifungal molecules, CPPs also have potential in delivering non-toxic bioactive cargo to study the effect of the cargo on cellular function.[55,56] In this case, toxicity from CPP vehicles would need to be as low as possible. While most of the peptides we studied would not be suitable for this type of application, SynB is the exception. At a concentration of 10 μM, essentially 100% of *Candida* cells were positive for peptide translocation [Fig. 1(C)], while the same concentration led to a loss of viability for only 10% of *C. albicans* and 5% of *C. glabrata* cells (Fig. 6). SynB also has a low level of toxicity toward mammalian cells,[57] further increasing its potential applications in studying the biology of *Candida* pathogens.

Our data for the toxicity of the CPPs highlight the importance of considering the goal of cargo delivery in selecting a CPP. The level of CPP translocation, along with the toxicity toward the target cells and any other cells that may be present, must be evaluated to identify a CPP with the desired delivery and toxicity profiles.

Conclusion

We have identified a number of CPPs that are able to translocate into the fungal pathogens *C. albicans* and *C. glabrata*. We also explored the intracellular distribution of the CPPs following translocation into fungal cells and found that vacuoles play a significant role in CPP trafficking. Our results suggest that translocation of CPPs into fungal cells may involve multiple mechanisms and that these mechanisms may lead to the toxicity observed for some peptides. Further work to explore the translocation mechanisms of CPPs could improve their efficacy and toxicity profiles to make CPPs viable therapeutic delivery agents. By increasing the translocation of bioactive cargo using CPPs, effectiveness of antifungal agents could be improved and new classes of molecules that currently lack the ability to cross cell membranes could be explored as antifungal agents.

Materials and Methods

Peptides

The peptides listed in Table I were commercially synthesized at >95% purity with an N-terminal 5-carboxyfluorescein (FAM) (Genscript, Piscataway, NJ). The lyophilized peptides were reconstituted in sterile, ultrapure H$_2$O and diluted to a final concentration of 10 mM Na$_2$HPO$_4$ buffer.

Strains and culture conditions

C. albicans strain SC5314 and *C. glabrata* strain ATCC2001 were purchased from American Type Culture Collection (ATCC, Manassas, VA). *Candida* cells were inoculated from yeast-peptone-dextrose (YPD) agar plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, and 2% w/v agar) into 5 mL of liquid YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) and grown overnight at 30°C while shaking at 230 rpm. The cells in the overnight culture were subcultured into 5 mL of fresh YPD medium at OD$_{600}$=0.1 (equivalent to $\sim$2 × 10^6 CFU/mL). The culture was then grown at 30°C to OD$_{600}$ = 0.5 (equivalent to $\sim$1 × 10^7 CFU/mL) while shaking. Cells were harvested by centrifugation at 4,000 × g for 10 min and washed twice with 10 mM Na$_2$HPO$_4$ buffer before use in downstream assays.

Fluorescence imaging

For each peptide, 100 μL of peptide solution (2–100 μM, depending on the experiment) was prepared in 10 mM Na$_2$HPO$_4$ buffer, mixed with 100 μL of cell suspension containing 5 × 10^5 cells in 10 mM Na$_2$HPO$_4$ and incubated at 30°C for 60 min. Cells were collected by centrifugation at 5000 × *g* for 10 min at 4°C and washed once with 10 mM Na$_2$HPO$_4$. The cell pellet was then incubated with 200 μL of 0.025% trypsin (Invitrogen, Waltham, MA) at 37°C for 10 min to remove surface-bound peptide.[30] Cells were collected and washed again with 10 mM Na$_2$HPO$_4$. For vacuole staining, 1 μM of CellTracker Blue CMAC (Invitrogen Molecular Probes, Waltham, MA) was added into the washed cell suspension and incubated at ambient temperature for 10 min. To prepare the cells for imaging, cells were collected and resuspended in 5 μL of 10 mM Na$_2$HPO$_4$. The suspension was transferred to a glass slide and imaged using an Olympus IX83 fluorescence microscopy system (Olympus, Center Valley, PA). PI (1 mg·mL^{-1}; Invitrogen, Waltham, MA) was added

immediately before imaging as needed to examine the membrane integrity. DIC, GFP fluorescence, vacuolar stain fluorescence, and/or PI fluorescence images were taken using the automatic process manager of the CellSens Dimension software (Olympus), and images were analyzed using NIH ImageJ software.[58]

Quantification of translocation

To prepare the *Candida* cells for quantification by flow cytometry, procedures analogous to those for microscopy were followed. Fresh cells were incubated with dilutions of each peptide (1–50 μM) and then treated with trypsin. After washing the cells with 10 mM Na_2HPO_4, the cells were resuspended in 150 μL of 10 mM Na_2HPO_4. Cell suspensions were analyzed for FAM and PI fluorescence using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Only single cells were selected for analysis, and the analysis was performed using FlowJo software (FLOWJO Inc., Ashland, OR).

Antifungal activity assay

In order to assess the antimicrobial activities of the peptides, a microdilution assay was performed. After subculturing cells and growing the culture to $OD_{600} = 0.5$, a 5×10^5 cells/mL cell suspension was prepared in 10 mM Na_2HPO_4. Serial dilutions (20 μL) of the peptides were prepared at 0.4–100 μM in 96-well plates. A control containing 100 μM of free FAM in 10 mM Na_2HPO_4 buffer was also added to the plate. The cell suspension (20 μL) was then added into each well, and the plate was incubated at 30°C with vigorous shaking for 60 min. Treated cells were diluted 20-fold in 10 mM Na_2HPO_4 buffer, and 100 μL of diluted cell suspension was added into 100 μL fresh YPD medium in a new 96-well plate. Plates were incubated at 30°C with vigorous shaking for 16 h, and the OD_{600} of the wells was measured using a 96-well plate reader (BioTek, Winooski, VT). The percentage of killing was calculated from

$$\text{Killing } (\%) = \left(1 - \frac{OD_{600, \text{peptide}}}{OD_{600, \text{control}}}\right) \times 100 \qquad (1)$$

Minimal inhibitory concentrations were determined as the minimum concentration resulting in a 50% reduction in cell viability (MIC50).

Acknowledgments

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