Chemistry & Biology
Article

Inhibition of the Pathogenically Related Morphologic Transition in *Candida albicans* by Disrupting Cdc42 Binding to Its Effectors

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DOI 10.1016/j.chembiol.2007.10.008

SUMMARY

Morphologic transition from the yeast to the hyphal state in *Candida albicans* is associated with pathogenicity of this human pathogen. Such invasive transition of *C. albicans* cells is regulated by numerous cell signal transduction pathways, one of which involves a small GTPase, the *C. albicans* Cdc42 (CaCdcd42), with specific binding to downstream effectors, e.g., CaCla4 and Cst20, containing CRIB domains. Here, we report that in vivo inhibition of CaCdc42 by peptide-mediated transduction of the CRIB polypeptides can inactivate and even reverse the pathogenically related morphologic transition of *C. albicans*. The current work provides a promising strategy for disease intervention through disrupting protein-protein interactions in signal transduction pathways and brings the concept of signal transduction therapy into the front line of antifungal design as well as therapy for other signal transduction-related diseases.

INTRODUCTION

Cellular signal transduction mediates the conversion of external signals (hormones, growth factors, neurotransmitters, and cytokines) to a specific internal cellular response (gene expression, cell division, or even apoptosis), and this transduction plays an important role in cell growth and differentiation. The checks and balances of these signal transduction pathways can be thought of as overlapping networks of interacting molecules that control "go–no go" control points [1, 2]. As almost all known diseases exhibit dysfunctional aspects in these networks, there has been a great deal of enthusiasm for the prospect of identifying novel drug targets based on knowledge of key signal transduction components and their links to human diseases [3–5]. Signal transduction pathways are typically regulated by protein-protein interactions, which are mediated by specific modular domains and form an important mechanism for controlling signal transduction pathways [6]. Conversely, aberrant protein-protein interactions can disturb cellular function and/or proliferation, contributing to disease processes. Proteins encoded by pathogenic bacteria and viruses, or chimeric cellular oncoproteins, frequently exert their effects by disrupting normal protein-protein interactions, and thus reorganizing cellular behaviors [7, 8]. Recently our understanding of cellular signal transduction has been enhanced by rewiring signal pathways through scaffold engineering [9] and synthetic biology [10]. As such, one can use the prevalent interaction domains to facilitate the formation of new connections between existing signaling proteins, and thus to create new signaling pathways during the course of cellular proliferation [11].

Candida albicans is an opportunistic fungal pathogen that is found in the gastrointestinal flora of most healthy humans. This organism is the cause of most "yeast infections" in humans. *C. albicans* can proliferate to produce blood-borne infections that often cause death in immunocompromised patients. *C. albicans* can exist in three morphologic forms as yeast cells, pseudo hyphal cells, and hyphal cells [12]. The pathogenicity of *C. albicans* is contingent upon its ability to switch from yeast to hyphal growth in response to environmental signals [13–16]. This transition is regulated by numerous cell signal transduction pathways, one of which involves *C. albicans* Cdc42 (CaCdcd42), a small GTPase of the Rho family, with specific binding to downstream kinase effectors containing CRIB (Cdc42/Rac interactive binding) domains (e.g., CaCla4 and Cst20). CaCdc42 is required for polarized growth of both the yeast and hyphal forms of *C. albicans* [17], suggesting that CaCdc42 is probably related to the establishment of a polarized actin cytoskeleton during hyphal induction. As an essential gene, the deletion of both CaCdc42 alleles can completely abolish the yeast-to-hyphal transition of *C. albicans* cells. Furthermore, signal transduction from CaCdc42 to its two downstream kinase effectors, Cst20 and CaCla4, is required for hyphal growth under all experimental conditions examined [18, 19], through the interactions of Cdc42 with their CRIB domains. Biochemical assays revealed that the extended polypeptides (eCRIBs), containing the CRIB consensus sequence and a short segment C-terminal to the CRIB domain, have high-affinity binding to CaCdc42 in apparent low-nanomolar concentrations [20]. Significantly, these

results suggest that CaCdc42 can be a potential target for curbing candidiasis, and that *Candida* eCRIB peptides can function as potent inhibitors of CaCdc42 in vivo. Administration of such inhibitors can block CaCdc42 activity and interrupt the yeast-to-hyphal transition. However, one of the challenges is that *C. albicans* is a eukaryote, as are other fungal pathogens, possessing biological machinery similar to that of human cells. This similarity complicates the development of more selective antifungal therapies.

Recently, many signal peptide sequences have been thoroughly investigated to identify transducing molecules enabling protein entry into bacteria and yeast [21, 22]. Of particular interest for antifungal drug design is a peptide with an amino acid sequence of VLTNENPFSDP, called a NPF domain, which can trigger protein transduction into bacteria and, more interestingly, into *C. albicans* [23]. In the current work, we constructed NPF-eCRIB fusion peptides as putative antifungal agents that can interrupt CaCdc42-linked signal transduction pathways. The delivery of Cdc42-specific eCRIB peptides via the NPF domain into *C. albicans* cells should inactivate the CaCdc42 protein, and subsequently inhibit the virulent yeast-to-hyphal transition. Our results show that the eCRIB domains derived from CaCla4 and Cst20 specifically inhibit hyphal growth of *C. albicans* cells in vivo only after being conjugated to the cell-internalizing NPF sequence. Therefore, our work provides a promising approach to the design of new antifungal agents through specific intervention of cell signal transduction.

RESULTS

Design of Specific eCRIB-Based Peptide Inhibitors of CaCdc42

C. albicans eCRIB peptides cannot be directly used as antifungal agents because of two challenging reasons. First, *C. albicans* eCRIB domains are highly homologous to their human counterparts, e.g., the eCRIB domain of PAK-family kinases (Figure 1A); thus, they may not be specific to CaCdc42. Second, the cell wall of *C. albicans* may limit peptide diffusion across the cell membrane. As such, in an attempt to utilize *Candida* eCRIB peptides to design specific inhibitors, it is necessary to examine whether *C. albicans* eCRIB peptides can interact with human Cdc42 (HsCdc42). Binding assays of *C. albicans* eCRIB peptides to HsCdc42 were determined by monitoring the change of fluorescence signal intensity of sNBD, conjugated to HsCdc42, as a function of peptide concentration. As shown in Figure 1A, the two *C. albicans* eCRIBs exhibit micromolar binding affinities for HsCdc42, although they are much weaker than those for CaCdc42, indicating that *C. albicans* eCRIB peptides are somewhat specific to CaCdc42.

In this work, we would like to utilize NPF-mediated protein transduction to enhance the specificity of eCRIB peptides to CaCdc42 rather than to optimize the selectivity of eCRIB peptides, because the former strategy will localize CaCdc42 binding to within *C. albicans* cells. As mentioned previously, the NPF domain was proven to be functionally unique to bacteria and yeasts. Particularly, the NPF domain has been extensively examined and shown to efficiently transduce small-molecule compounds (e.g., FITC) and proteins (e.g., Rectin A and Green fluorescent protein [GFP]) into *S. cerevisiae* and *C. albicans* cells [22, 23]. Previous investigations also indicated that the NPF peptide-mediated internalization into yeast cells was independent of its position of incorporation in the host fusion protein [23]. Therefore, in the current work, the NPF domain was arbitrarily placed at the N terminus of designed fusion proteins or peptides. Figure 1B lists two NPF-conjugated eCRIB peptides, defined as NPF-eCaCla4 (I) and NPF-eCst20 (II), respectively. These two NPF-eCRIB peptides were prepared through a well-developed recombinant peptide expression system (see Experimental Procedures). The conjugated peptides were overexpressed as fusion proteins in *Escherichia coli* BL21(*DE3*) cells (Figure 1Ba). The peptides were released from the fusion proteins by chemical cleavage via CNBr and purified by HPLC with a C_{18} reverse-phase chromatographic column. The purity of the final products was determined to be ~95%–99% by using a 20% SDS-PAGE gel (Figure 1Bb) as well as an analytical HPLC assay (data not shown). The identity of the purified peptides was confirmed by electrospray mass spectrometry, as shown in Figure 1C for the NFP-eCaCla4 peptide.

Importantly, the two NPF-eCRIB peptides were found to retain high-affinity binding to CaCdc42 comparable to the eCRIBs alone (Figure 1B). These two NPF-eCRIB peptides also strongly inhibit the Cdc42-mediated activation of two *C. albicans* kinases (i.e., Cst20 and CaCla4) in in vitro assays (see Figure S1 in the Supplemental Data available with this article online). However, the NPF domain itself was confirmed to have no significant binding affinity to CaCdc42. Moreover, the NPF-eCRIB peptides were found to exhibit similar conformational flexibility as those of the CRIB peptides alone [20, 24], as indicted by the narrow dispersion of their ^{1}H-^{15}N-NMR HSQC peaks (see Figure 1D for NPF-eCaCla4 peptide).

Specificity of Putative Antifungal Peptides to *Candida* Cells

C. albicans cells grow in the yeast form at 25°C in Lee's medium or at 30°C in YPD medium (Figures 2Aa and 2Ac). As shown in Figures 2Ab and 2Ad, the yeast cells can readily be triggered to grow into the hyphal form by simply increasing culture temperature and/or the addition of serum (e.g., 37°C in Lee's medium or 37°C in YPD medium with 10% bovine serum). To examine whether the NPF-eCRIB peptides could penetrate into *C. albicans* cells, Lee's medium was primarily used in the current work because of simplicity.

Initially, the internalization of two NPF-eCRIB peptides into mammalian cells and *C. albicans* cells was compared by monitoring GFP fluorescence. The GFP protein was fused to the C terminus of the NPF-eCRIB peptides, and schematic representations of two GFP fusion proteins are shown in Figure 2B. Apparently, one of the GFP-conjugated NPF-eCRIB fusion proteins, i.e., NPF-eCaCla4-GFP,

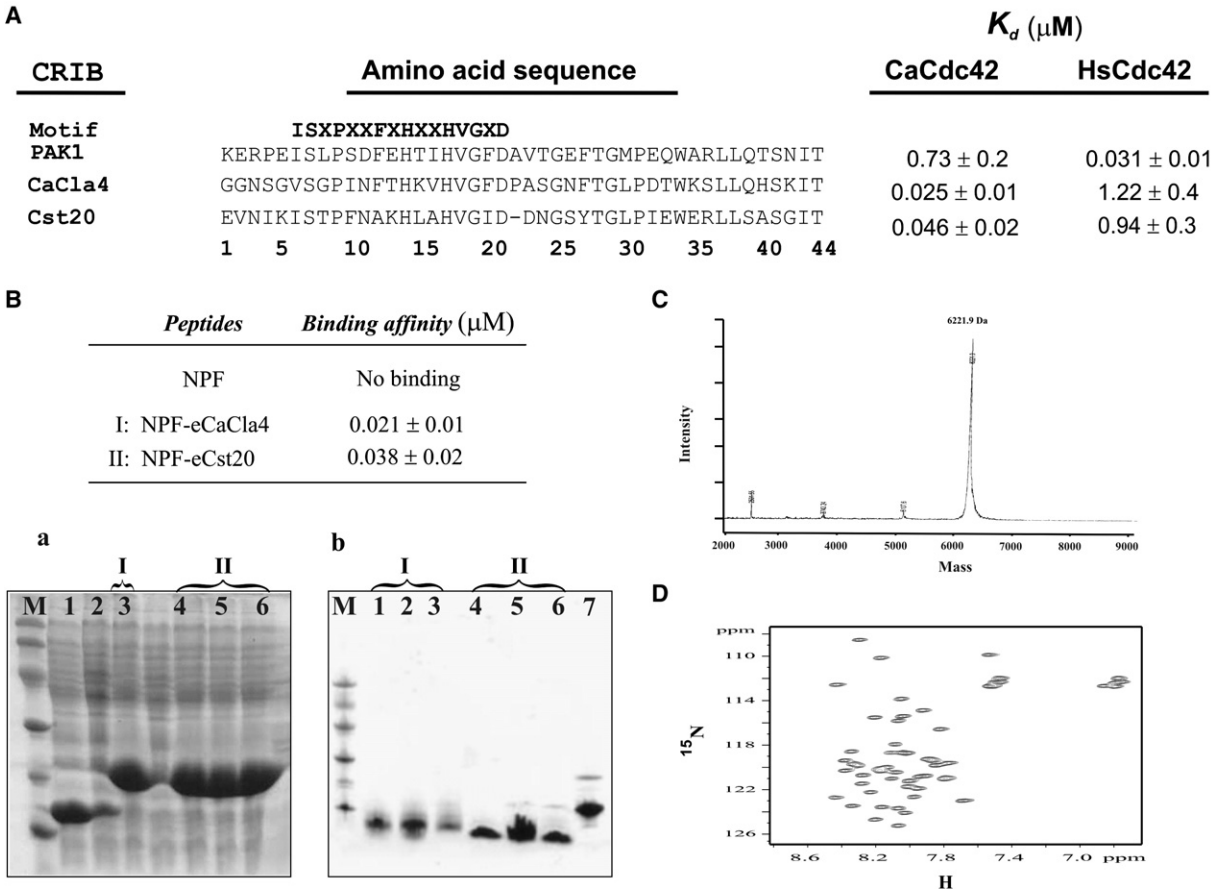

Figure 1. Design of Peptide-Mediated eCRIB Peptides as Putative Antifungal Agents

(A) The extended CRIB peptides (eCRIBs) of two *C. albicans* kinases, CaCla4 and Cst20, are highly homologous to that of human PAK1 kinase.

(B) The top shows two NPF-conjugated eCRIB peptides with high-affinity binding to CaCdc42. In (a), the expression of the fusion proteins containing NPF-eCRIB peptides in LBA medium was evaluated by a 12% SDS-PAGE gel. Lane M, molecular weight marker; lanes 1 and 2, the fusion carrier proteins; lane 3, NPF-eCaCla4 fusion proteins; and lanes 4–6, NPF-eCst20 fusion proteins. In (b), NPF-eCRIB peptides purified with HPLC were evaluated by a 20% SDS-PAGE gel. Lane M, molecular weight marker; lanes 1–3, NPF-eCaCla4 peptides; lanes 4–6, NPF-eCst20 peptides; and lane 7, the fusion carrier protein.

(C) Mass spectrum of NPF-eCaCla4 with an ES-TOF spectrometer indicates a correct molecular weight close to its theoretical value (i.e., 6221.7 Da).

(D) ^{1}H-^{15}N HSQC spectrum of the NPF-eCst20 peptide.

could not be internalized into HeLa cells, as shown in Figures 2Ba and 2Bb. The GFP-conjugated NPF-eCst20 fusion protein, i.e., NPF-eCst20-GFP, exhibited the same resistance to cellular uptake (see Figure S2). However, when the GFP fusion proteins were incubated with *C. albicans* cells composed of yeast and hyphal forms, the fusion proteins exhibited specific targeting to hyphal cells. The GFP fusion proteins not only penetrated into the hyphal cells (Figures 2Ca and 2Cc), but also accumulated on the surface of hyphal cells (see Figure 2Cc for NFP-eCaCla4-GFP). Contrary to our expectations, both fusion proteins had not been observed to penetrate into *C. albicans* yeast cells under the current experimental conditions (Figure 2C). GFP alone was unable to penetrate into *C. albicans* cells under the same conditions (data not shown), as reported previously [23].

Furthermore, the internalization of NPF-eCRIB peptides into hyphal cells was confirmed by quantitating radioisotope-labeled peptides in various cell fractions. As shown in Figure S3, 43%–72% of NPF-eCRIB peptides are mainly found in the cellular fraction composed of cell walls and membranous proteins. Peptides could be trapped in cell walls or be bound to Cdc42 because Cdc42 is a membrane-bound protein. On the other hand, 16%–42% of NPF-eCRIB peptides are identified from the cellular fraction containing cellular cytoplasm of hyphal cells. Nevertheless, each NPF-eCRIB peptide can maintain a high peptide concentration in both cellular fractions up to at least 25 hr.

Inhibition of Hyphal Growth by Putative Antifungal Peptides

In order to evaluate whether NPF-eCRIB peptides have antifungal activity against *C. albicans* hyphal growth, a relatively high concentration of peptides was arbitrarily selected (i.e., 100 μM). Initially, the effect of the peptides

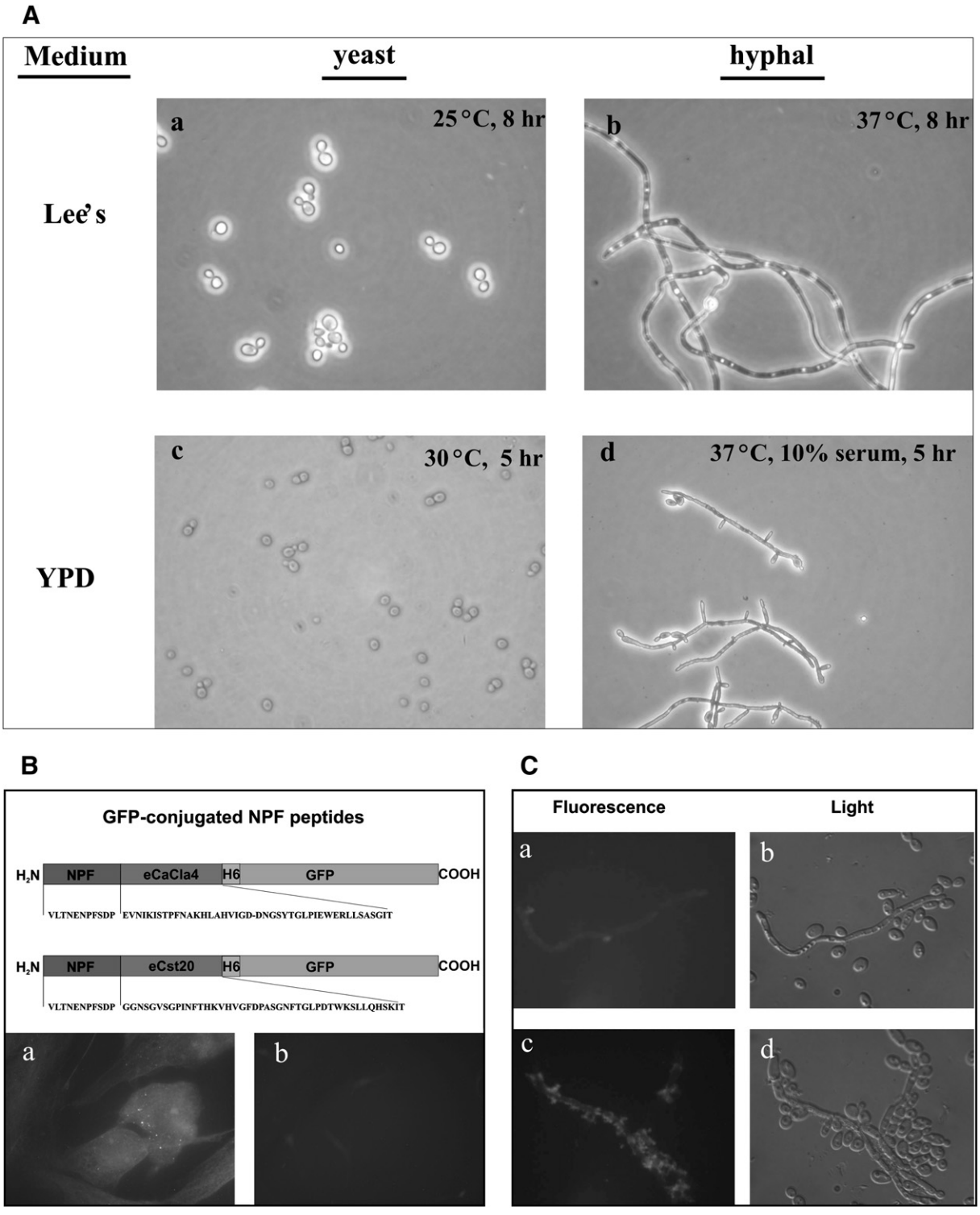

Figure 2. NPF-eCRIB Peptides Preferentially Bind to *Candida* Hyphal Cells

(A) Dimorphism of *C. albicans* cells.

(B) The top shows schematic representations of two NPF-eCRIB-GFP fusion proteins. (a) and (b) show micrographs of HeLa cells after they were incubated with NPF-eCla4-GFP protein.

(C) Micrographs of *C. albicans* cells after they were incubated with NPF-eCst20-GFP protein ([a] and [b]) or NPF-eCaCla4-GFP protein ([c] and [d]).

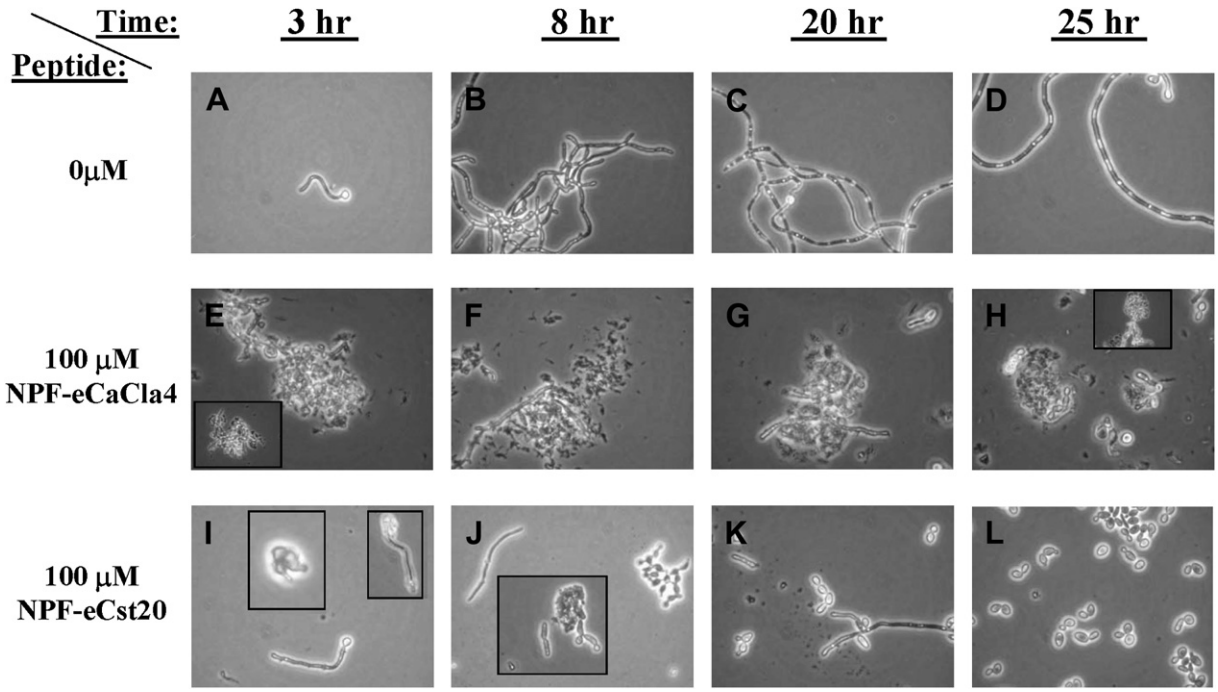

Figure 3. Inhibition of the *C. albicans* Yeast-to-Hyphal Transition by NPF-eCRIB Peptides
(A–L) Each peptide was added into fresh yeast-cell cultures at a final concentration of 100 μM. Different types of cells are shown in the insets.

on the yeast-to-hyphal transition was examined. *C. albicans* yeast cells grown overnight in Lee's medium at 25°C were transferred into fresh Lee's medium and cultured at 37°C, and the cells began development of hyphae within 3 hr (Figure 3A). If the NPF-eCaCla4 peptide was added to the above-described fresh Lee's medium (the culture temperature was 37°C), most of the cells still remained in yeast form. Yeast cells were prone to aggregation with increasing culture time (Figures 3E–3H). Although some hyphal cells were observed in the cultures, it seems that hyphal cells are fragmentized through cell lysis (Figures 3E and 3F). In contrast, if NPF-eCst20 peptide was added into the fresh medium and the culture temperature was adjusted to 37°C, no cell aggregation and cell lysis were observed (Figures 3I–3L). Within 8 hr, *C. albicans* cells remained in either yeast forms or pseudo hyphal forms. The amount of yeast cells increased with increasing culture time, implying that both of the peptides can block the morphologic transition of *C. albicans* cells, but not its yeast-cell proliferation. Taken together, both the peptides dramatically reduced the amounts of hyphal cells in culture, compared to cultures in the presence of NPF-CRIB peptides (Figures 3A–3D). However, it was unclear whether yeast cells were reversed from hyphal form or were simply maintained in the yeast-cell form in the presence of peptides in medium.

In the next step, the same peptide concentration as that in the aforementioned experiments was used to examine whether the NPF-eCRIB peptides can inhibit hyphal growth. *C. albicans* hyphal cells, which were prepared from overnight cultures in Lee's medium at 37°C, were inoculated into fresh Lee's medium and continued to grow in hyphal proliferation in the absence of peptides (Figures 4A–4D). If the NPF-eCaCla4 peptide was added into the fresh medium during hyphal growth, it appeared that the hyphal cells were fragmented and switched to yeast-like forms (Figures 4E–4H). The population of yeast cells increased with increasing culture time, although hyphal cells were observed (insets in Figure 4H). If NPF-eCst20 peptide was added to the fresh medium during hyphal growth, the numbers of yeast cells markedly increased with time in culture, whereas the numbers of hyphal cells dramatically decreased (Figures 4I–4L). These observations suggested that hyphal cells can be reversely transitioned into yeast forms. Conversely, in control experiments in the absence of peptides, no yeast form of *C. albicans* cells was populated (Figures 4A–4D). These experiments provide evidence that both of the NPF-eCRIB peptides cannot only inhibit the yeast-to-hyphal transition of *C. albicans* cells, but can also reverse hyphal cells into yeast form.

Dose Dependence of Peptide Inhibition on the Yeast-to-Hyphal Transition of *C. albicans*

Yeast cells were prepared by the same procedure as that in the aforementioned experiments and were used to examine the effect of peptide concentrations on the inhibition of the yeast-to-hyphal transition of *C. albicans* cells. Initially, *C. albicans* yeast cells were incubated with different concentrations of peptides (~0–100 μM) for 3 hr at room temperature, as suggested previously [23], before culture temperature was increased to 37°C. The cell

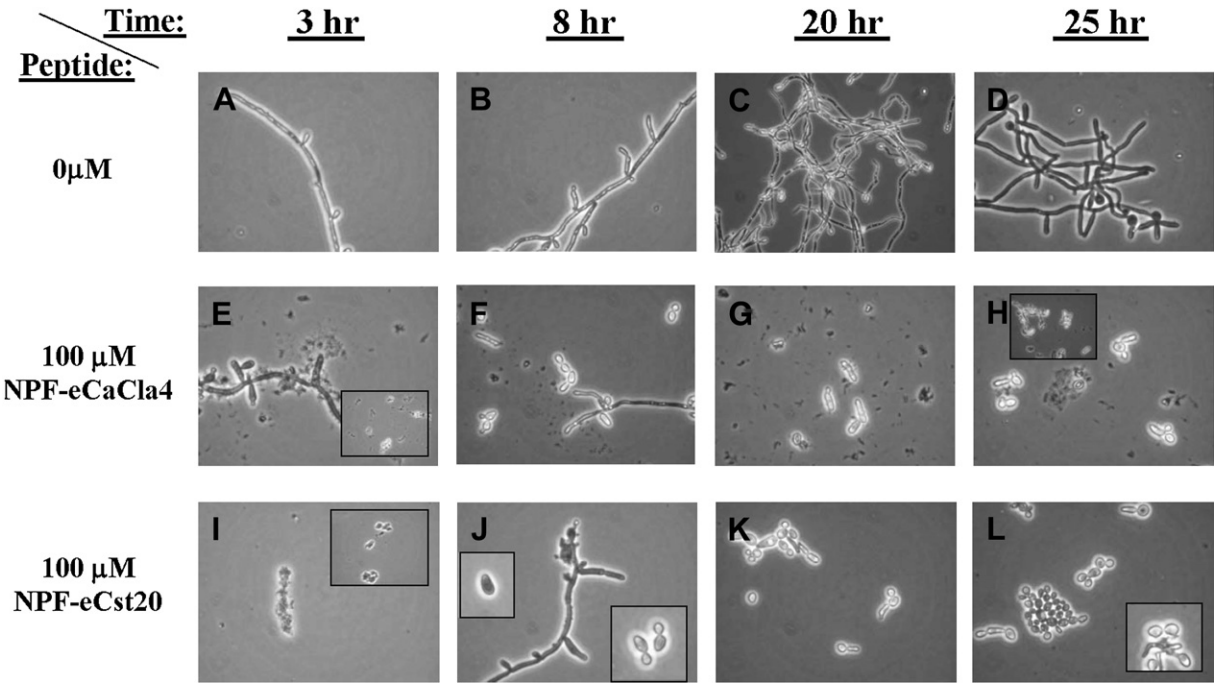

Figure 4. Inhibition of *C. albicans* Hyphal Growth by NPF-eCRIB Peptides
(A–L) Each peptide was added into fresh hyphal-cell cultures at a final concentration of 100 μM. Different types of cells are shown in the insets.

cultures were then shaken for 5 hr at 37°C, and an aliquot of the culture broth was taken out for examination by optical microscopy. As shown in (A)–(C) for NPF-eCaCla4 and (E)–(G) for NPF-eCst20 in Figure 5, cells can still proliferate into the hyphal form, suggesting that there was no significant inhibition in the presence of peptides of less than 25 μM. When the peptide concentration increased to 25 μM, *C. albicans* cells were observed to either remain in the yeast form (NPF-eCaCla4 in Figure 5D) or develop into the pseudo hyphal form (NPF-eCst20 in Figure 5H). When peptide concentration further increased to more than 25 μM, more yeast cells were populated in the media (Figures 5I and 5J and Figures 5M and 5N for NPF-eCaCla4 and NPF-eCst20, respectively). After cells were cultured for 16 hr, the inhibition of peptides during the yeast-to-hyphal transition became more significant, even in the presence of a low concentration of peptides (see Figure 6). In cell cultures in which the initial peptide concentration was as low as 12 μM, the majority of *C. albicans* cells were observed to remain in yeast form (Figure 6C for NPF-eCaCla4 and Figure 6G for NPF-eCst20). In control experiments, no apparent inhibition was observed for cultures in the presence of 100 μM CRIB peptide (Figures 5K, 5N, 6K, and 6N). The similar phenomenon was also observed for cultures in the presence of 100 μM NPF peptide (Figures 5O and 6O). Quantitative analysis showed that the population of yeast cells increased as a function of peptide concentration (Figures 5P and 6P).

However, when the peptide concentration was 50 μM or higher, two NPF-eCRIB peptides exhibited different effects on cell growth. The NPF-eCaCla4 peptide caused cell lysis (Figures 5I, 5J, 6I, and 6J). In contrast, no cell lysis was observed in cultures in the presence of the NPF-eCst20 peptide (with a peptide concentration of up to 100 μM) (Figures 5G, 5M, 6G, and 6M) or in control experiments ([K] for eCaCla4, [N] for eCst20, and [O] for NPF in Figures 5 and 6). Cell lysis had been assayed with RT-PCR to confirm that cell fragments were generated from *C. albicans* cells (see Figure S4), although it is not clear whether they are from yeast cells or hyphal cells. It seems that the cell fragmentation is related to some unknown mechanism by which the NPF-eCaCla4 peptide can interfere with hyphal growth. Given the complexity of Cdc42-linked signal transduction, the significant inhibition with a low-micromolar concentration of peptides (e.g., 12 μM) suggests that the NPF-eCRIB peptides are promising potent antifungal agents. Moreover, similar inhibitions were also observed in other culture media including YPD and YNB media (data are not shown). Thus, the inhibition of *C. albicans* hyphal growth by both NPF-eCRIB peptides is a time- and dose-dependent process but is medium independent.

DISCUSSION

Peptide-mediated protein transduction into mammalian cells is becoming a widely accepted tool that complements genetic methods for the study of gene and protein functions [25–27]. Cargo delivery into microbial cells is a relatively new area of research, and differential delivery into pathogenic microbes can be of enormous value for the development of more effective antimicrobial agents.

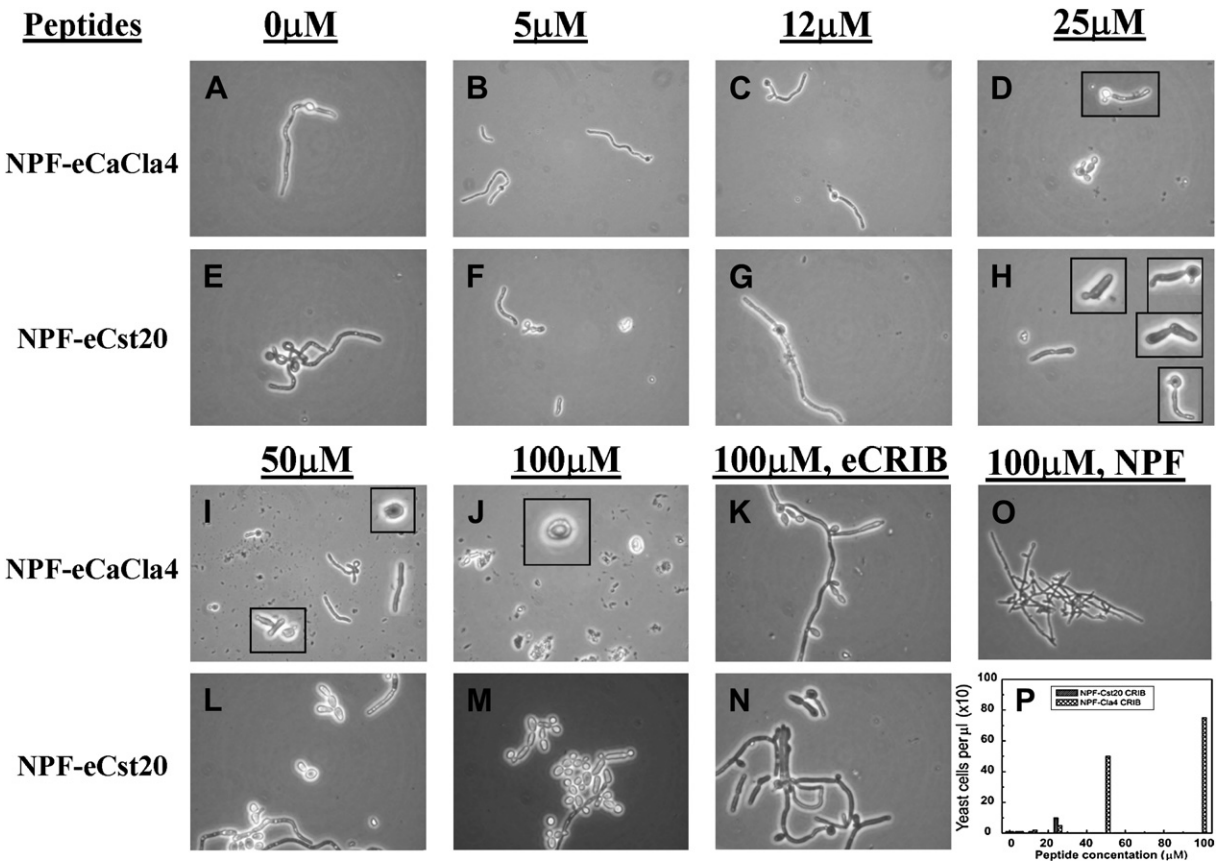

Figure 5. Concentration Dependence of Peptide Inhibition on the *C. albicans* Yeast-to-Hyphal Transition

(A–P) An 8 hr yeast-cell culture at 25°C was washed three times with 1× PBS buffer before the peptide was added, and the reaction was incubated for 3 hr at room temperature. Then fresh Lee's medium was added, and the reaction was shaken at 37°C for 5 hr. The starting concentration of cells was 0.01 OD_{540}, except for in the control experiments with eCRIB peptides ([k] and [m]) or NPF peptide ([o]). Different types of cells are shown in the insets.

In this work, we used NPF peptide [22], a previously well-characterized peptide-transduction domain specific to microbes, to selectively transduce *C. albicans* eCRIB domains of Cdc42-binding kinase effectors (i.e., CaCla4 and Cst20) into *C. albicans* hyphal cells. Our results revealed that these eCRIB peptides could specifically inhibit *C. albicans* hyphal growth in vivo only when they were transduced into the cells by using the NPF peptide, implying that NPF-mediated transduction of CRIB peptides (i.e., NPF-eCRIBs) can be potent antifungal agents against candidiasis (i.e., yeast infection).

Interestingly, under the current experimental conditions, no inhibition of *C. albicans* yeast growth was observed for the two NPF-eCRIB peptides. Our results show that NPF peptides do not target *C. albicans* yeast cells but do target hyphal cells (Figure 2). A possible explanation is that hyphal cells may have a higher population of NPF receptors on their surfaces compared to yeast cells, which needs to be clarified with a biochemical assay in the future. Nevertheless, the current work suggests that the signal transduction through the CaCdc42/CaCla4 or Cst20 interaction can be a key checkpoint for the yeast-to-hyphal transition or for hyphal growth of *C. albicans* cells, implying that the inhibition of these protein-protein interactions by the eCRIB peptides may not affect the interaction of CaCdc42 with other signaling proteins. Previously, genetic studies showed that the deletion of both Cdc42 alleles could completely abort *Candida* cell growth [17]. Therefore, the current results raise new questions regarding whether or how CaCdc42 regulates the growth of yeast and hyphal cells differentially in *C. albicans*. By comparison, the functional aspects of Cdc42 regulating cell polarity have been extensively explored [28]. To answer this question, further genetic and biochemical investigations are needed. Two previous genetic models of *C. albicans*, which overexpress Cdc42 and is haploinsufficient for Cdc42 [17], would be useful for dissecting Cdc42-linked signal transduction pathways, in combination with our chimeric inhibitor peptides.

The modulation of protein-protein interactions has provided the molecular basis for our understanding of many biological and cell signaling pathways [29]. Manipulation of protein-protein interactions by rewiring signal pathways [30, 31] has not only opened up a new area for synthetic biology in studying the functional complexity of cells [32, 33], but it has also brought a novel concept of signaling transduction therapy into the front line of modern drug research [34]. These investigations can enhance our understanding

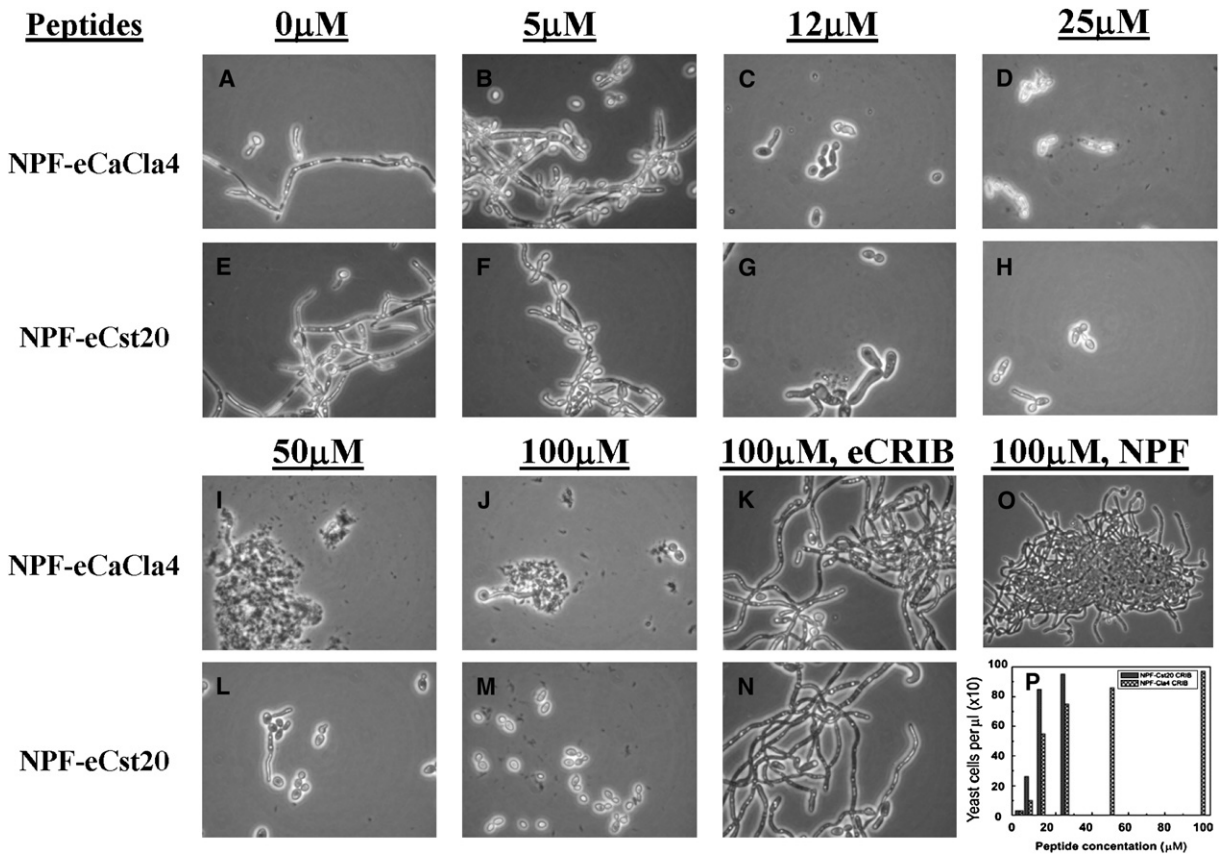

Figure 6. Concentration Dependence of Peptide Inhibition on the *C. albicans* Yeast-to-Hyphal Transition
(A–P) The conditions were the same as those in Figure 5, except that samples were taken from the culture after 16 hr.

of the molecular mechanisms of human diseases, because most of these diseases are related to dysregulation of intra- and intercellular signaling communication pathways. The current work provides an important and timely example of the concept by preventing pathogenic cell growth through selectively interrupting protein-protein interactions relevant to signal transductions.

SIGNIFICANCE

Protein-protein interactions regulating biological and cell signaling are the coming wave of drug target discovery in the postgenomics era because many diseases, such as cancers and diabetes, are related to aberrant signal transduction. Disruption of these protein-protein interactions is still a major challenge for the discovery of small-molecule drugs (SMDs) due to the existence of a large interface in these interactions. On the contrary, peptides or peptidomimetics have unique advantages over SMDs in inhibiting protein-protein interactions with respect to the increased time efficiency of discovery efforts. *C. albicans* is an opportunistic fungal pathogen, and its morphologic transition from yeast to hyphae is pathogenesis relevant. The transition is regulated by multiple cellular signaling transduction pathways, one of which involves *C. albicans* Cdc42 (CaCdc42), a small GTPase, interacting specifically with the extended CRIB (eCRIB) domains of Cdc42-binding effectors. In the current study, these eCRIB peptides were utilized to inhibit the morphologic transition through disrupting protein-protein interactions engaged by CaCdc42. Our results demonstrate that specific inhibition of the interactions of CaCdc42 with its downstream effectors by internalized eCRIB peptides can inactivate and even reverse the pathogenically related morphologic transition of *C. albicans*. Therefore, the current work provides a glimpse into a promising new generation of anticandidiasis agents, because *C. albicans* cells would be arrested in nonpathogenic yeast forms and eventually be killed by host factors such as macrophages. Most significantly, the work brings the concept of manipulating signaling transduction pathways into the front line of drug design for other signal transduction-related diseases. As well, these peptides are excellent templates that can be utilized for the rational design of peptidomimetics or SMDs. Consequently, the strategy described in this work may render it applicable to a broad range of human diseases caused by aberrant protein-protein interactions.

EXPERIMENTAL PROCEDURES

Cultures of *C. albicans* Cells

The *C. albicans* SC5314 strain was maintained by periodic transfer to slants of YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Overnight cultures were inoculated from a fresh colony and were grown in YPD (pH 6.0–6.5) at 30°C. The overnight cultures were diluted to an OD_{540} of 0.05–0.1 in YPD or YPD plus 10% fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA) and were grown at 30°C for yeast and 37°C for hyphal cells. Cultures grown in Lee's medium containing 10% glucose were started from an overnight culture in 1 l temperature-adjusted medium and grew at either 37°C to induce hyphae or at 25°C to maintain yeast growth.

Peptide Preparation

All of the peptides, including NPF, CRIB domains (eCaCla4 and eCst20), and NPF-conjugated CRIB domains (NPF-eCaCla4 and NPF-eCst20), used in the current study were prepared by using a recombinant peptide expression system as described previously [35]. A detailed procedure is described in the Supplemental Data. The identity of the final products was verified by electrospray mass spectrometry.

Preparation of Cdc42 Proteins

The expression plasmid for *Candida* Cdc42 (CaCdc42) was described previously [20]. A similar protocol was used to construct expression vector for human Cdc42 (HsCdc42). Briefly, DNA fragments encoding human Cdc42 protein (residues 1–178) were amplified from a GST fusion protein expression plasmid [36] by a standard PCR method by using *pfu* polymerase. Through PCR, two restriction sites, *Nde I* and *BamH I*, were generated at the 5′ end and 3′ end, respectively. A stop codon, TAG, was placed immediately after the codon for residue 178. The PCR fragment was subcloned into pET-15b vector (Novagen, Madison, WI), and the resulting construct was defined as pHsCdc42.

Both CaCdc42 and HsCdc42 proteins were expressed in the *E. coli* BL21 strain as hexahistidine fusion proteins. The proteins were purified and nucleotide exchanged as described previously [20].

Preparation of GFP Fusion Protein

The NPFSD-GFP expression plasmid (the gift of Dr. Liam Good, Karolinska Institute) [23] was used as a template to construct expression vectors for NPF-CRIB-GFP fusion proteins. To facilitate protein purification, a hexahistidine tag was inserted between the peptide and GFP (see Figure 2B). The fusion proteins were purified by Ni-NTA agarose affinity chromatography with a native condition purification procedure, provided by the supplier (QIAGEN, Mississauga, ON). The proteins were eluted with a buffer containing 200 mM imidazole and were dialyzed against 50 mM phosphate buffer (pH 6.5) overnight to remove imidazole.

Peptide-Binding Assay

Peptide binding affinity was measured for the interaction of the peptides with sNBD-labeled CaCdc42 or HsCdc42, and extrinsic fluorescence enhancements were measured with a Hitachi F-2500 fluorescence spectrophotometer as essentially described in previous publications [20]. The K_D values for the binding were determined by fitting the fluorescence titration data to a simple bimolecular association model as described by Leonard et al. [37]. The fluorescence intensity (F) is related to the dissociation constant (K_D), the protein concentration (P), and the peptide concentration (L) as follows

$$F = F_0 + (F_t - F_0)\left[\frac{(K_d + L_T + P_T) - \sqrt{(K_d + L_T + P_T)^2 - 4P_T L_T}}{2P_T}\right], \quad (1)$$

where F_0 and F_t are the fluorescence intensities at the starting and end points of the titration, respectively. P_T is the total concentration of sNBD-labeled Cdc42, and L_T is the total concentration of the peptide at any point in the titration. Fitting of the data was carried out by using the computer program Microcal Origin 6.0 (Northampton, MA). Average K_D values were determined from five independent measurements.

Mammalian Cell Culture

HeLa cells were maintained in Minimum Essential Medium with Earle's Salt (E-MEM) supplemented with 2 nM L-glutamine, 10% (vol/vol) fetal bovine serum (GIBCO-BRL), 1% (vol/vol) penicillin-streptomycin (GIBCO-BRL), and 0.2% (vol/vol) fungizone amphotericin B (GIBCO-BRL). The cells were cultured in a humidified incubator at 37°C and in the presence of 5% CO_2.

When the mammalian cells were more than 80% confluent, ~5–20 μl GFP fusion proteins was added to the medium. Cells adhered on the plate were washed three times with PBS and were visualized by using the phase-contrast function on a Nikon PCM2000 confocal microscope at 0, 3, 5, and 8 hr after posttransduction.

Peptide Inhibition of *C. albicans* Cells

An overnight cell culture at 25°C in Lee's medium was used to generate yeast cells, and an overnight cell culture in Lee's medium at 37°C was used to generate hyphal cells. The overnight cell culture was used to inoculate fresh Lee's medium, and the starting concentration of cells was an OD_{540} of 0.01. Each peptide was added into cell cultures with a final concentration between 0 and 100 μM. A total of 3.5 μl culture liquid was taken for light microscopic observation of cell morphogenesis.

NMR Measurements

NMR spectra were acquired with a Bruker 600 MHz NMR spectrometer at 25°C by using standard pulse sequences [38]. The NMR samples were prepared by dissolving the peptides in 50 mM phosphate buffer (pH 6.5). Spectral processing, display, and analysis were performed by using the XwinNMR software package (Version 3) supplied with the spectrometer system.

Supplemental Data

Supplemental Data include information regarding in vitro peptide inhibition of kinase activation and the cell lysis assy and are available at http://www.chembiol.com/cgi/content/full/14/11/1273/DC1/.

ACKNOWLEDGMENTS

We thank the Yeast Genetic Group of Biotechnology Research Institute for the access to its *Candida* facility, Andrea Ruas and Shiqian Li for their technical assistance, and Nicole Sukdeo for helpful discussion and proofreading the manuscript. This work was funded by the Genomics and Health Initiative of the National Research Council of Canada (NRCC Publication No. 49517), was sponsored by the Government of Canada, and was partially supported by the Guangdong Key Laboratory of Biopharmacy (2002B60119) and the Guangzhou Scientific and Technological Fund (2006Z3-E4152) to H.L. and Z.S.

Received: June 8, 2007
Revised: September 13, 2007
Accepted: October 9, 2007
Published: November 26, 2007

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