Stony Brook University

Regulation of Fungal Morphogenesis

A Thesis Presented by

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To

The Graduate School

In Partial Fulfillment of the Requirements

For the Degree of

Master of Arts

In

Biological Sciences

Stony Brook University

December 2007

Stony Brook University

The Graduate School

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Abstract of the Thesis

Regulation of Fungal Morphogenesis

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Candida albicans is an opportunistic fungal pathogen in humans. It typically lives as a harmless commensal as part of the human gut and skin flora, but under some conditions it causes mucosal infections. In immunocompromised patients it can also cause life-threatening systemic infections. *C. albicans* is a multimorphic organism, and the ability to undergo morphological transition from budding to hyphal growth is associated with its virulence. This morphological switch was reported to be regulated by different signaling pathways. One key pathway is cAMP-PKA signaling pathway. The single adenylyl cyclase in *C. albicans* is CaCdc35, which was reported to interact with plasma membrane associated protein Ras1. However, the localization of CaCdc35 remains unclear. We proposed that adenylyl cyclase is plasma membrane associated. The GFP tagging technique was used to study the spatial localization of the adenylyl cyclase CaCdc35. Bacterial expression of CaCdc35 and protein purification were performed for further study of this protein. G protein-coupled receptor Gpr1 is an important plasma membrane

sensor in *C. albicans* and the deletion of *GPR1* produces defects in hyphal formation and morphogenesis in *C. albicans.* Gpr1 interacts with Gα protein Gpa2 and activates the cAMP signaling pathway. However, the nature of the ligand for Gpr1 is still unclear. Previous studies pointed to the possibility that amino acids are ligands of Gpr1. Thus, GFP tagging technique was used to visualize the protein on plasma membrane. Internalization experiment was performed to test the possibility that methionine is the ligand of Gpr1. Technique problems appeared to limit the conclusions. Future perspectives were brought up for solving the problems and exploring new topics in the study of *C. albicans*.

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List of Abbreviations

AC	adenylyl cyclase
Amp	ampicillin
cAMP	cyclic adenosine monophosphate
CYCc domain	adenylyl cyclase catalytic domain
DOC	sodium deoxycholate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KD	kilo Dalton
PCR	polymerase chain reaction
PKA	protein kinase A
RA domain	Ras association domain
SD Medium	synthetic defined medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Acknowledgements

I have been so lucky to work in such a happy lab that I can survive the short but challenging graduate study.

First of all, I would like to express my sincere thanks to people in Konopka lab. My advisor, James. Konopka, is a nice but rigorous person. I really appreciated that he provided me with the chance of performing my thesis study in his lab. Although I am a clumsy student, he is always patient. Javier is the one gave me most help no matter in experiments or in life. He always made fun of me, putting a brick in my bag, putting lotions in my gloves, drawing funny faces on the papers I was reading, etc. Thanks to his "bad behaviors", I felt relaxed in the stressful research. His friendship always cheered me up. Louis has helped me a lot with experiments. She is a very nice and reliable person. And I always borrowed her stuff. Also I want to express my thanks to Stacy, Doug, and Chengda (especially thank Doug for the beers).

Secondly, I would like to thank my committee members: Dr. Aaron Neiman and Dr. Nicolas Nassar. I did the protein purification experiments in Dr. Nassar's lab. It is very kind of Dr. Nassar to provide me with equipments and advice. Also, the beautiful graduate student Christina Chen in his lab gave me a lot of help. Only with their help, I could finish the experiments and this thesis. I also want to thank Dr. Neiman for being my committee member and giving many useful comments on my thesis.

Thirdly, I appreciate the help from Dr. Futcher's lab across the hall. Especially, I always borrowed plates, media or other solutions from Ying when ours were used up. I also appreciate the friendship from Ying, especially when I felt frustrated or had some problems in experiments.

The last and most important, I want to thank my family. My husband is also a graduate student in MIT. In the past two years, he came visiting me through 7~8 hours traffic every other weekend, never stop. With his love and support, I can finish my study. He is such a smart and sweet person that my life is lightened up with him. I do not have enough word to express my thanks to my beloved parents. They raised me, provided me with the best they had, and trusted me all these years. With their life-long love and support, I feel strong and confident.

Chapter 1

Introduction

1. *Candida albicans* pathogenesis

Candida albicans is an opportunistic fungal pathogen that typically grows as a commensal in the gastrointestinal and genitourinary tracts of humans and other warm-blooded animals. It is the most common fungal pathogen present in oral and vaginal infections (candidiasis). When the immune system is weak (for example, as a result of organ transplantation or cancer chemotherapy procedures), *C. albicans* increasingly colonizes and invades host tissues [1].

In immunocompetent people, candidiasis can usually only be found in skin and soft tissues, such as the oral cavity, the vagina, or other exposed and moist parts of the body. In immunocompromised patients, *C. albicans* can cause life-threatening systemic infections (Candidaemia) when it crosses into the blood stream and infects internal organs, such as the kidneys, leading to organ failure and death.

Candida albicans is also the single most common fungal species causing hospital-acquired infections in the United States, the treatment of which is estimated to cost more than US $1 billion annually [2]. Because *C. albicans* is eukaryotic and shares many of its biological processes with humans, most antifungal drugs cause deleterious side effects and usually act to inhibit the growth of fungi (fungistatic) rather than kill fungi (fungicidal) [1]. Most antifungal drugs interfere with the biosynthesis of ergosterol or the integrity of membranes containing ergosterol, the major sterol in the fungal plasma

membrane. Other drugs cause disruption of the fungal cell wall. Based on their mechanism of action, the major agents can be grouped into five classes: polyenes; azoles; allylamines; echinocandins; and other agents, including griseofulvin and flucytosine [3]. Polyenes, such as Amphotericin B, are fungicidal for a broad range of invasive fungal infections because they act by forming pores in ergosterol-containing membranes. Fluconazole is the first generation of triazole drugs that inhibit ergosterol synthesis. Caspofungin is a newer echinocandin agent that acts by blocking cell wall synthesis. In spite of these new antifungal agents, resistance to the current treatments is on the rise and the mortality rate from candidiasis is still 30%~40% [4]. Therefore, there is a need for novel, more powerful antifungal therapies.

2. Candida morphogenesis.

An important feature of *C. albicans*, relevant to its pathogenesis, is its ability to switch between different morphological forms. *C. albicans* can grow in a single-celled, budding yeast form (blastospore), a filamentous form (including both pseudohyphae and true hyphae) and chlamydospores (Figure 1). The round buds resemble *Saccharomyces cerevisiae* buds, although they are a little more elongated. Similar to *S. cerevisiae*, *C. albicans* bud formation is also mediated by the actin cytoskeleton [5]. The formation of pseudohyphae occurs by polarized cell division in which cells form chains of elongated buds that do not detach from adjacent cells. True hyphal cells are long and highly polarized, with parallel sides and no obvious constrictions between cells [1]. Chlamydospores are asexual, thick-walled spores that are formed off of hyphae under certain environmental stress conditions; but their role in *C. albicans* biology remains

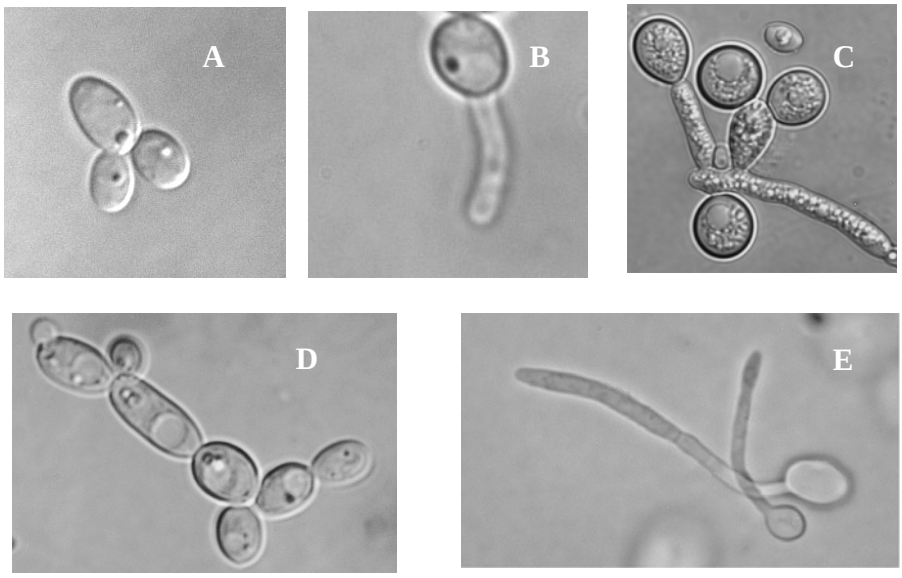

Figure 1. Examples of *C. albicans* distinct morphologies. A, budding cells; B, germ tube; C, chlamydospores; D, pseudohypha; E, true hyphae. Pictures were taken with different magnifications. (Figure 1-C, 1-E are kindly provided by Dr. Francisco Alvarez)

unclear. Morphological switching in *C. albicans* is important for its virulence, as will be described below in more detail.

Hyphae constitute the most common morphology in *C. abicans* cells found during infection. The hyphal growth pattern is thought to allow this organism to grow invasively in the host, penetrating deep into tissues of the organs. The term "germ tube" refers to the elongating structure that evaginates from a round yeast cell at the initial stage in the formation of the true hyphae, when the short filament has not yet undergone septation to form a separate cell.

The morphological transitions often represent a response of the fungus to changing environmental conditions and may permit adaptation to a different biological niche. In *C. albicans*, the yeast-to-hyphae transition has been studied extensively *in vitro*, and it has been shown to be regulated by various environmental cues. For example, serum is the most potent hyphal inducer. Certain sugars, such as *N*-acetylglucosamine (GlcNAc), can also stimulate hyphal growth. In recent years, many *in vivo* and *in vitro* experiments point to a prominent role for amino acids as nitrogen sources and as ligands for membrane receptors involved in the regulation of cellular morphology and virulence [6]. Other environmental conditions that affect the morphological switch are growth at 37^{o}C, change to a more alkaline pH, and/or 5% CO_2, starvation, and adherence to a matrix.

C. albicans also exhibits a morphological variation known as white/opaque (w/o) switching that is under epigenetic control [7]. W/O has recently been associated with the mating process, and also has been implicated in pathogenesis. Both white and opaque cells can become homozygous for the mating locus, but only opaque cells homozygous

for that locus can mate [7]. Meiosis and sporulation have never been observed in this organism, so it is unclear how the tetraploid cells that result from conjugation become diploid again.

The finding that mutant strains defective in hyphal growth are avirulent [8, 9] has implicated the yeast–hyphal transition in *C. albicans* pathogenicity. It will be explained in more detail in the coming section.

3. Hyphal signal pathway.

C. albicans' hyphal growth is induced by environmental stimulation of the organism and is repressed by the quorum sensing molecule farnesol [10]. Many transcription factors that regulate hyphal genes have been identified, but the role of the plasma membrane sensors that are involved in transmitting different signals to downstream effectors is poorly understood. The few identified upstream components of the hyphal signaling pathway include a variety of plasma membrane sensory proteins. Many signaling pathways that induce hyphal growth act in part by stimulating adenylyl cyclase (summarized in Figure 2).

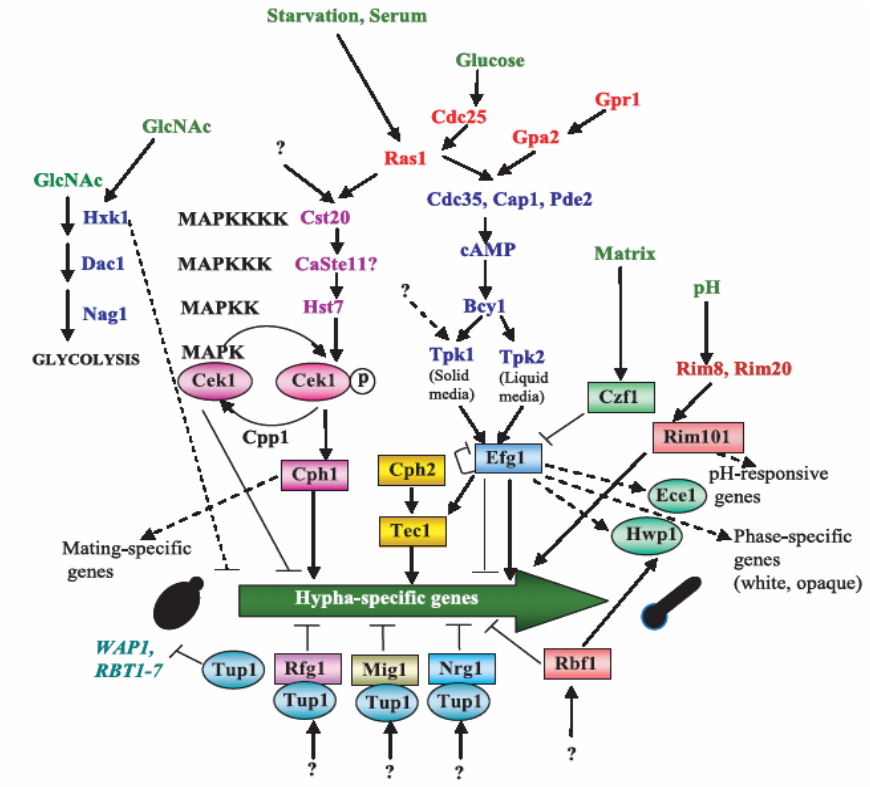

Figure. 2 Regulation of hyphal growth in *C. albicans* by multiple signaling pathways (Adapted from ref [11]).

The cAMP-PKA pathway plays a very important role in filamentous growth of *S. cerevisiae*, *C. albicans*, and other fungi [12]. In *S. cerevisiae*, formation of pseudohyphae is dependent on the activation of cAMP pathway [13]. In *C. albicans*, an increase in cAMP levels accompanies the yeast-to-hypha transition, and inhibition of the cAMP phosphodiesterase induces this transition [14]. *C. albicans* has a single gene homologous to the *S. cerevisiae* adenylyl cyclase gene (*CYR1/CDC35*). The cyclase is not essential for growth in *C.albicans* but is required for hyphal development [15]. Some upstream components of the cAMP signal pathway have been identified, but how they transmit different signals to downstream effectors is not clear.

Ras1 is the single Ras homologue in *C.albicans*. Mutants lacking Ras1 are viable but have a severe defect in hyphal growth in response to serum and other inducers [16]. *Ras1* mutants are defective in cAMP induction in response to glucose and serum stimulation, but this defect can be suppressed by exogenous cAMP. Mutants lacking the guanine nucleotide exchange factor Cdc25 show a similar phenotype, suggesting that the Cdc25-Ras1 pathway functions upstream of the cAMP signaling pathway [6]. This is supported by recent research in which the clear interaction between Ras1 and the Cdc35 RA domain (Ras association domain) is demonstrated by *in vitro* binding assays [17]. However, an important gap remains in understanding the upstream components: the receptor activating Cdc25 has not been identified.

In *S.cerevisiae*, the cAMP-PKA pathway is activated by a G protein-coupled receptor system consisting of the G protein-coupled receptor ScGpr1 and the Gα protein ScGpa2. This GPCR system is required for pseudohyphal and invasive growth induction and *ScGPR1* or *ScGPA2* mutants can be suppressed by the addition of cAMP [18, 19].

Genes similar to *ScGPA2* and *ScGPR1* have been identified in *C. albicans*. *GPA2*, which encodes an α subunit homologue of the heterotrimeric G-protein, was proposed to function in the regulation of cAMP levels. *GPR1* encodes a protein that is predicted to contain seven membrane-spanning helices, a feature characteristic of G protein-coupled receptors. It has been confirmed that the Gpr1 protein is an important plasma membrane sensor, and deletion of *GPR1* produces defects in hyphal formation and morphogenesis in *Candida albicans* [20]. The defect can be reversed by exogenous addition of cAMP or by overexpression of downstream components in the pathway. Epistasis analysis revealed that Gpa2 acts downstream of Gpr1 (the receptor) in the same signaling pathway, and a two-hybrid assay indicated that the carboxy terminus of Gpr1 interacts with Gpa2 [20]. However, the nature of the ligand for Gpr1 is still unclear. Research showed that induction of cAMP accumulation by glucose and serum is independent of CaGpr1. However, Gpr1 is rapidly internalized upon addition of some amino acids, specifically methionine [6]. This points to the possibility that an amino acid is the ligand of CaGpr1 and induces hyphal morphogenesis through Gpr1-cAMP pathway.

The adenylyl cyclase CaCdc35 is also involved in CO_2 sensing in *C. albicans*. The fungus is present in various parts of the body where the CO_2 concentration is more than 150-fold higher (5%) than in atmospheric air (0.033%). The presence of 5% CO_2 strongly induces filamentous growth and invasion of the underlying agar [21], a response that requires the catalytic domain of adenylyl cyclase (Cdc35) but not Ras1. In the cell, CO_2 is present in its hydrated form, bicarbonate. In *C. albicans*, *NCE103* encodes a carbonic anhydrase that greatly accelerates bicarbonate formation. Nce103 is required for *C. albicans* cells to grow in air but not under high CO_2 concentrations (>0.5%). It is

not the case that CO_2 is sensed through aquaporin water channels, as in plants and mammalian cells, since *C. albicans* Aqy1 has been ruled out as CO_2 sensor. Whether there is indeed a specific CO_2 sensor protein on the plasma membrane remains to be clarified [21].

The downstream components of the Cdc35-cAMP pathway include cAMP-dependent protein kinases [21] and the transcription factor Efg1. In *C.albicans*, the PKA catalytic subunit is encoded by two paralogues (*TPK1* and *TPK2*) [22] and both isoforms have a positive, stimulatory function on the formation of true hyphae. The regulatory subunit of PKA in *C. albicans* is encoded by *BCY1* [23]. *C. albicans* Bcy1 may tether the PKA catalytic subunit to the nucleus and thereby perform a pivotal role in regulating the enzymatic activity and availability of PKA in response to growth phase-related nutritional requirements [23]. The *C. albicans EFG1* gene encodes a basic helix-loop-helix transcription factor and plays a central role in morphogenesis. The activated Efg1 initiates hyphal formation by inducing genes involved in hyphal formation and/or repressing genes directing the yeast form [24].

4. Goals of this thesis

The first goal of this thesis was to learn more about spatial distribution of the important components of cAMP signal pathway in *C. albicans*. Adenylyl cylcase (Cdc35) is an important component of the cAMP signal pathway. Previous studies have showed that the Ras association domain (RA) of Cdc35 is critically required for hyphal morphogenesis, and proved the direct interaction between RA domain and Ras protein [17]. But where Cdc35 is localized in the cell remains unclear. Unlike the adenylyl

cyclase in mammalian cells that has transmembrane domains, Cdc35 is not a membrane spanning protein. Based on the fact that G proteins like Gpa2 and Ras1 are attached to plasma membrane, we wanted to test the hypothesis that Cdc35 is recruited to the plasma membrane. We were especially interested in whether there is any difference in Cdc35 localization between yeast cells and hyphal cells.

We were also interested in further study of Cdc35 in both genetic and biochemical ways, such as testing different mutants of Cdc35 in hyphal growth, interaction between Cdc35 and Gpa2, the relationship between localization change and morphology switching, etc. With the help of Dr. Nassar's lab, we were able to express and purify three Cdc35 fragments. With these proteins, we aimed at obtaining antibody to Cdc35 and studying the three dimensional structure by crystallization.

Another goal of the thesis was to study Gpr1. Previous studies have shown that Gpr1 is an important sensor in plasma membrane and functions upstream of cAMP signal pathway in induction of hyphal morphogenesis. Interestingly, glucose and serum are inducers of Ras1-Cdc25, but do not appear to act as ligands for Gpr1 in *C. albicans*. Research aimed at identifying Gpr1 ligands has implicated several types of amino acids. We were interested in studying whether methionine is the ligand for Gpr1 and the nature of other possible ligands.

It is imperative to study plasma membrane sensors and critical components of cAMP signal pathway since they can provide us with a better understanding of how environmental changes affect the morphology of *Candida* as well as related pathogens. More importantly, these plasma membrane proteins would be potential drug targets in the development of novel therapies to treat fungal infections.

Chapter 2

Materials and Methods

Strains and Media

The *C. albicans* and *S. cerevisiae* strains used in this study are described in Table 1.

The *C. albicans CDC35-GFP* strain was constructed by homologous recombination [25]. PCR was used to add ~70 bp of sequence which is homologous to the 3' end of the *CaCDC35* open reading frame to a cassette that contains GFP and a *URA3* selectable marker (Figure3). The Ura^+ colonies resulting from the transformation of the PCR product into *C. albicans* were then grown on SD-ura medium and screened for GFP-positive cells by PCR and fluorescence microscopy.

Gap Repair

The *C. albicans pADH1-GPR1-GFP* strain was constructed by gap repair. The open reading frame of *GPR1* was amplified by PCR, adding ~70bp of sequence homologous to 3' of the pADH1 promoter and 5' of *GFP* gene respectively (Figure 4). The *pADH1 - GFP* plasmid was digested with EcoRI at the unique site between promoter and *GFP* open reading frame. Both linearized plasmid and *GPR1* gene were transformed into *S.cerevisiae* strain DB1033 (Kindly provided by Dr. Rolf Sternglanz). Positive colonies were identified by PCR and plasmids were recovered by transformed into *E. coli*. Miniprep constructed *pADH1-GPR1-GFP* plasmid from *E. coli* and transformed it into *C.*

albicans strain CAI4. Positive colonies were identified by PCR and visualized under microscope.

Microscopy

Cdc35-GFP and pADH1-Gpr1-GFP fluorescence was analyzed in cells that were grown overnight in synthetic complete medium containing glucose, but lacking uracil. Cells were washed with ddH_2O, and analyzed immediately by microscopy. Cells were analyzed by fluorescence microscopy to detect GFP and by Differential Interference Microscopy (DIC) to detect cell morphology.

Internalization

CAI4 strain carrying *pADH1-GPR1-GFP* plasmid was grown overnight in minimal medium with 2% glucose and 3% glycerol respectively. After inoculation into fresh minimal medium, the cells were grown at 30℃ with shaking for 3-4 hours until OD_{600} is 0.6-0.8. Cycloheximide was added into both media to a final concentration of 10μg/ml. Methionine (10mM) was only added into minimal medium with glycerol. Cells were incubated at 30℃ with shaking and then samples were collected every 30 minutes. Cells were washed with ddH_2O and analyzed immediately under microscope. Images were captured using an Olympus BH2 microscope equipped with a Zeiss AxioCam digital camera.

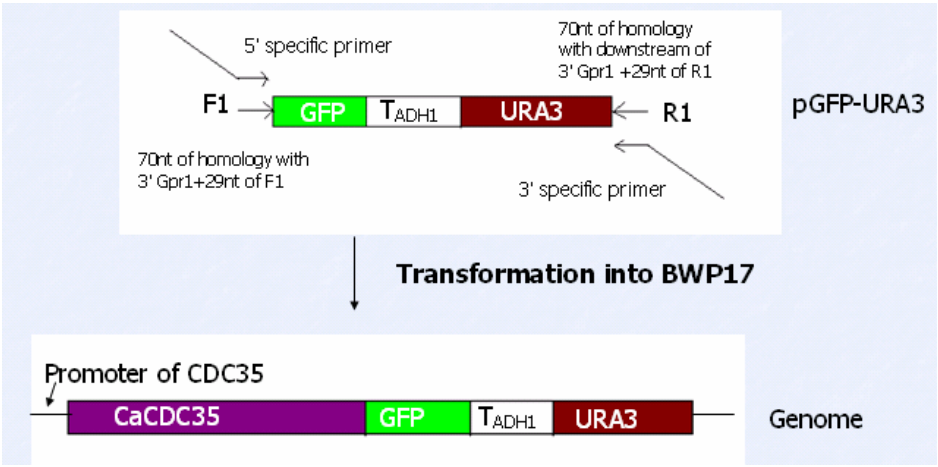

Figure 3. Schematic overview of GFP tagging *CaCDC35* by homologous recombination. See text for more details.

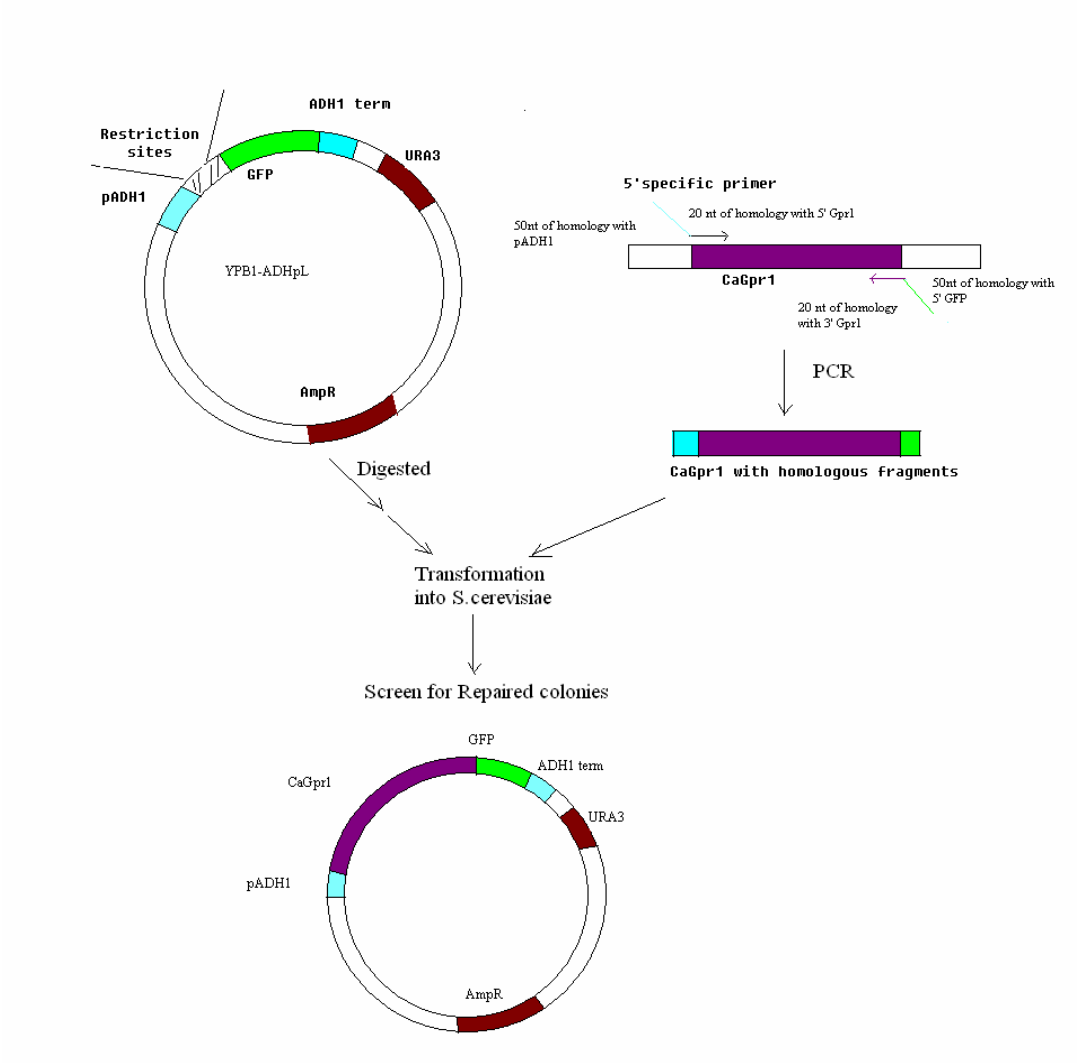

Figure 4. Schematic overview of gap repair strategy for GFP tagging *CaGPR1*. See text for more details.

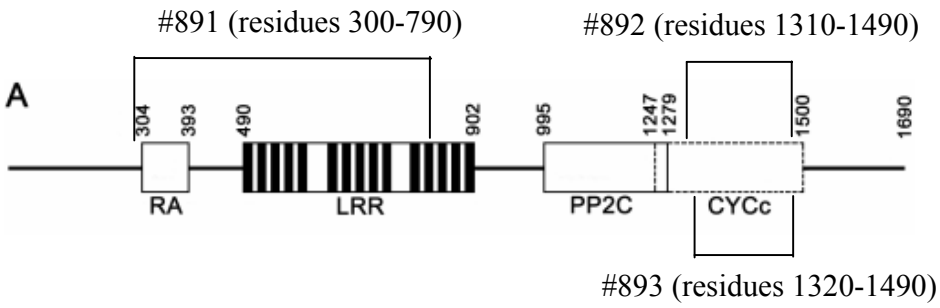

Figure 5. Schematic overview of CaCdc3 domains. #891 fragment is corresponding to Ras association (RA) domain and most part of a leucine rich repeats (LRR). #892 and #893 are just 10 amino acids different, corresponding to most part of the adenylyl cyclase catalytic (CYCc) domain (Adapted from [17]).

Table 1. Strains and plasmids used in this thesis project.

Strain	Parent	Genotype
C. albicans strains		
Sc5314		Wild Type
CAI4	Sc5314	*ura3Δ:: imm434/ura3Δ:: imm434*
BWP17	Sc5314	*ura3Δ::imm434/ ura3Δ::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*
ZQY1	CAI4	*ura3Δ::imm434/ura3Δ::imm434 pADH1-GPR1-GFP-URA3/GPR1*
ZQY2	BWP17	*ura3Δ::imm434/ ura3Δ::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CDC35-GFP-URA3/CDC35*
S. cerevisiae strain		
DB1033		*MATa ura3Δ::imm434*
C. albicans transformation vectors		
pYPB1-ADHPL		*pADH1-GFP-URA3* cassette
pZQV1		*GPR1::GFP* fusion construct
E.coli expression vectors		
pProEX-HTb		expression vector for N-terminal His tag construct
p891		300-790 amino acid of Cdc35 with His tag
p892		1310-1490 amino acid of Cdc35 with His tag
p893		1320-1490 amino acid of Cdc35 with His tag

Protein purification

The three fragments of *CDC35* were coloned into expression vector *pProEX-HTb*. The protein fragments are #891 (from 300-790 aa), #892 (from 1310 to 1490 aa), #893 (from 1320 to 1490 aa) (Figure 5). These proteins were produced as fusions containing 6×His to facilitate purification. *E. coli* strain BL21+ carrying expression vectors were grown at 37℃ with shaking over night. Cells were inoculated into 1 liter LB-Amp+ medium, grown at 37℃ for another 3 hours until OD_{550} is 0.6 to 0.8. IPTG (0.5 mM) was added to induce protein expression. Cells were grown at 18℃ for 20 hours. Cells were then harvested by centrifuged at 5,000 rpm at 4℃ for 30 minutes. Cold lysis buffer (50 mM $KHPO_4$, 150 mM KCl, pH 7.5) was used to resuspend pellet and added to a final volume of 30mL. PMSF (500 μM), βME, $MgCl_2$ and DNase were added, and cells were incubated on ice for 1 hour. French Press was used to lyse cells. Doc (0.5%) and Tween20 (1%) were added and cells were incubated on ice for 30 minutes. Samples were centrifuged at 21,000rpm at 4℃ for 20 minutes, and then proteins in the supernatant were purified using a Nickel Column. The column was equilibrated with Buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole, pH 8.0) and proteins were washed with Buffer B (20 mM Tris-HCl, 500 mM NaCl, 20 mM Imidazole, pH 8.0). Fractions were collected and column was washed with Buffer C (20 mM Tris-HCl, 500 mM NaCl, 250 mM Imidazole, pH 8.0). SDS-PAGE was used to detect proteins.

Chapter 3

Localization of Adenylyl Cyclase (Cdc35)

Introduction

Previous studies have shown that the cAMP signaling pathway is essential for hyphal morphogenesis. In *C. albicans*, adenylyl cyclase (Cdc35) was reported to be activated by the plasma membrane protein Ras1, but its localization is not known. In many signal transduction pathways, spatial localization of signaling proteins is tightly related to activity regulation. Therefore, I attempted to examine the localization of CaCdc35 protein by fusing it to fluorescent protein GFP in order to clarify the molecular mechanism in cAMP signal pathway regulation.

Results

A GFP tag was introduced at 3' end of adenylyl cyclase *CDC35* ORF as described in Chapter 2. The colonies with proper integration were identified by PCR. We obtained two independent colonies. The two strains carrying the *CDC35-GFP* fusion gene were grown to log phase and analyzed by microscopy. Cdc35-GFP did not give a readily detectable signal. However, in some cases cells were observed with fluorescence at the plasma membrane, as expected (Figure 6). I tried to confirm production of Cdc35 by Western Blotting, but the signal was also too weak to be detected with anti-GFP antibody (not shown).

I then examined Cdc35-GFP in cells induced to form hyphae by growing cells at

37℃ in medium containing *N*-acetylglucosamine (GlcNAc). The rationale for this experiment was to test whether Cdc35-GFP was easier to detect when the cAMP pathway was activated. Unfortunately, the signal was still very weak.

Discussion

Cdc35-GFP signal was very weak and could not be reliably detected in all cells. This is not too surprising, since similar results were observed for the *S. cerevisiae* Cdc35-GFP (GFP database http://yeastgfp.ucsf.edu/getOrf.php?orf=YJL005W). One reason for this is that there does not appear to be much adenylyl cyclase protein in cells, consistent with it acting as a sensitive signal transducer.

Although the Cdc35-GFP signal was weak, it appeared to be detectable in some cells, suggesting that even a small increase in signal would be beneficial. One approach I tried was to tag both alleles with GFP protein in the diploid *C. albicans*, but I was not successful in identifying the double transformant. We did not consider trying to overexpress *CDC35-GFP,* because this could activate the cAMP-PKA pathway.

Another alternative we started to examine is to use new GFP variants that are designed to fold more efficiently and give a brighter signal. This is our preferred approach, since we would be able to study Cdc35 at normal protein levels and avoid problems due to Cdc35 mislocalization by over production.

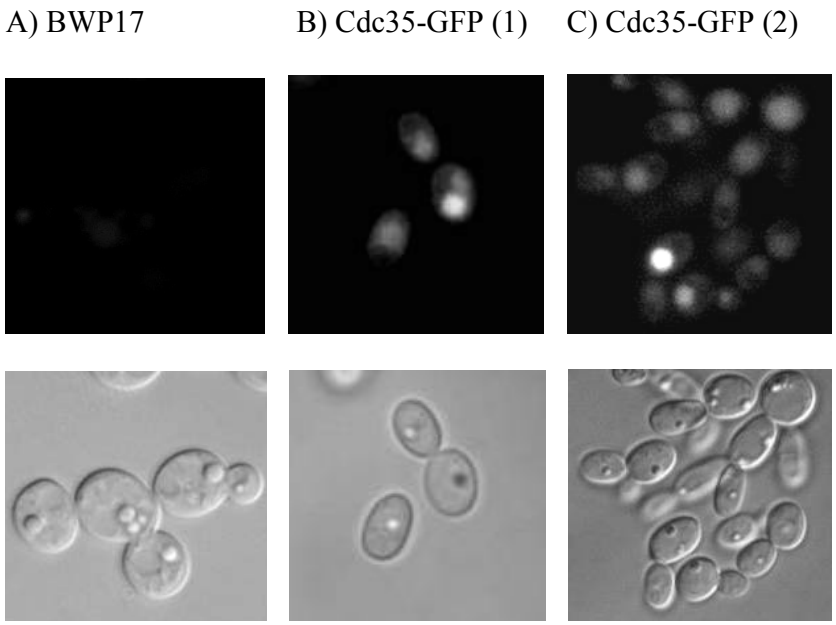

Figure 6. Cdc35-GFP in microscopy. *C. albicans* strain BWP17 was transformed with *GFP-URA3* cassette amplified by PCR. Two colonies were identified to carry *CDC35-GFP* fusion gene. The cells were grown in SD-ura medium until log phase. Uridine was added back to medium for BWP17. Then fluorescence and light microscope images were recorded. No signal was detected in BWP17 strain without any GFP fusion protein. In the two independent colonies of Cdc35-GFP, we can only detect very weak signal at the plasma membrane but strong signal in vacuole. Fluorescence images are shown on top panels and light microscope (DIC) images are shown below.

Chapter 4

Regulation of Gpr1 Localization

Introduction

Gpr1 is a plasma membrane sensor for nutrients that acts upstream of the cAMP signal pathway. Gpr1 contains 7 transmembrane helices and functions by activating a Gα subunit called Gpa2, which then activates adenylyl cyclase. Interestingly, Gpa2 does not associate with typical Gβγ subunits [26]. Recently, other investigators reported that Gpr1-GFP was down-regulated by methionine, which stimulated endocytosis of Gpr1-GFP [6]. Therefore, to further study the regulation of components of the cAMP signal pathway, we examined localization of Gpr1-GFP in the presence and absence of methionine.

Results

Gpr1 was tagged with GFP at the C terminus by integrating a cassette at the 3' end of *GPR1* in the genome. This work was done by a previous rotation student in the lab. We verified that no signal could be detected in this strain. Therefore, I tried new experiments to overexpress *GPR1-GFP*.

In order to overexpress *GPR1-GFP* we used the gap repair method in *S. cerevisiae* to place *GPR1-GFP* under control of *ADH1* promoter on plasmid YPB1-ADHpL (refer to chapter 2 for details). This plasmid can replicate as a multicopy plasmid in *S. cerevisiae*. Consistent with this, we saw some *S. cerevisiae* cells with very

strong Gpr1-GFP fluorescence at the plasma membrane. This proved that the fusion gene can be expressed. Also, it confirmed that Gpr1 is located at plasma membrane (Figure 7).

C. albicans does not replicate *S. cerevisiae* plasmids, so it was integrated into a random site in the genome, presumably as a single copy. Inspection of cells by microscopy revealed weak fluorescence in the plasma membrane. This was surprising, since *ADH1-STE2-GFP* and *ADH1-IST2-GFP* gave a strong signal in previous studies from our lab (Warenda, Ph.D. dissertation). This suggested Gpr1 is not stably produced in cells.

Although the ADH-Gpr1-GFP signal was weak, it was detectable. Therefore, I tried to repeat a previous report that Gpr1-GFP could be internalized by endocytosis in response to methionine, a putative ligand for Gpr1 [6]. Surprisingly, there was no difference in Gpr1-GFP localization in cells grown in the medium with methionine or without methionine (Figure 8). However, one problem is that there was a strong signal present in the intracellular vacuole. This is true even in minimal medium without any amino acids added. This strong intracellular signal may obscure Gpr1-GFP trafficking response to methionine.

To try to improve the sensitivity of the assay, I added cycloheximide to block new protein synthesis after adding methionine. But it is still not possible to detect Gpr1-GFP endocytosis from plasma membrane. Therefore, we could not reproduce the previous reports. This experiment should be repeated in the future with improved GFP detection methods.

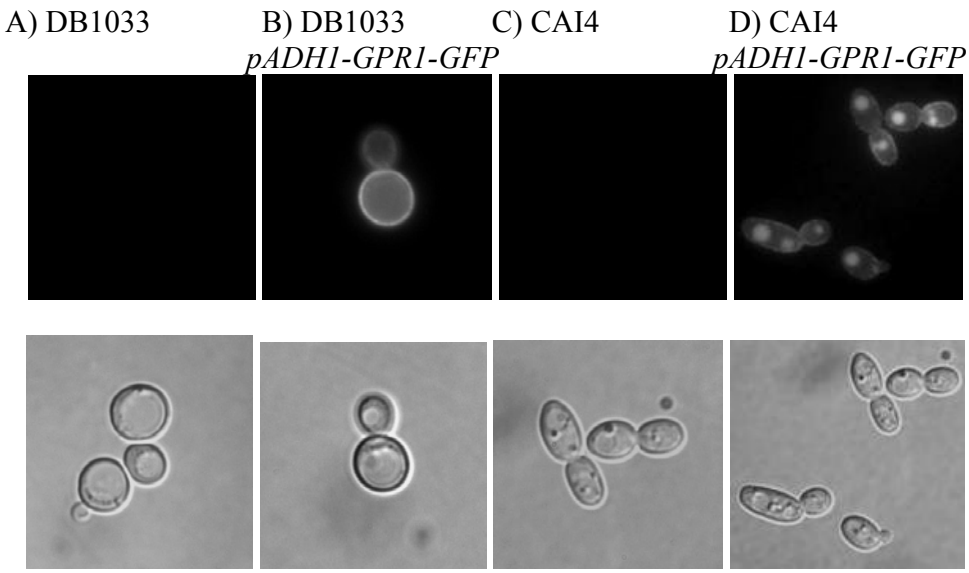

Figure 7. Visualization by fluorescence microscopy of *pADH1-GPR1-GFP* construct. Cells were grown in SD-ura medium at 30℃ until log phase. Then fluorescence and DIC images were recorded. In Figure 1-B, strong signal on plasma membrane was recorded in *S. cerevisiae* strain. Figure 1-D showed weak signal on plasma membrane in *C. albicans* strain.

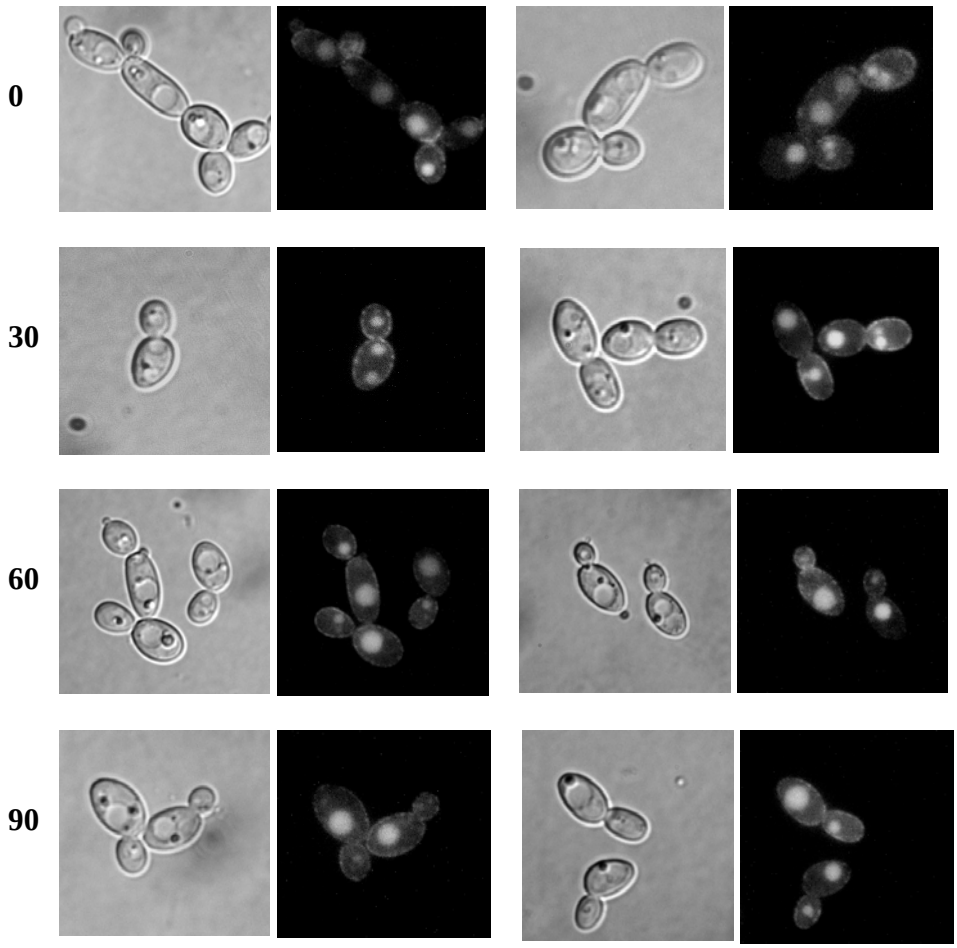

Figure 8. Internalization of Gpr1 induced by methionine. Cells were grown in SD medium and Sgc medium respectively. After grown overnight at 30℃, the cells were diluted and grown for another 4 hours until log phase. We added methionine to a final concentration of 10 mM, and cycloheximide (10 μg/ml) for blocking new protein expression. The cell samples were taken at the time points as indicated on the left of the figure and visualized by fluorescent microscopy.

Discussion

Unfortunately, the Gpr1-GFP signal is too weak to be reliably detected. In this chapter, we showed that fusing the *GPR1* open reading frame to *ADH1* strong promoter improved sensitivity and gave a weak but detectable signal at plasma membrane. This result is similar to that of Cdc35 adenylyl cyclase protein described in Chapter 3. Therefore, improved methods are needed to study the localization of cAMP signal pathway components as will be described in Chapter 6.

Chapter 5

Bacterial Expression of Cdc35

Introduction

Little structural information is known about fungal adenylyl cyclase because it is very different from mammalian adenylyl cyclase. Therefore, we expressed three fragments of *C. albicans* adenylyl cyclase CaCdc35 in bacteria: one fragment corresponding to the Ras association (RA) domain and two fragments corresponding to the adenylyl cyclase catalytic (CYCc) domain. Another potential use of the bacterially produced fragments of CaCdc35 is to raise antibodies that can be used for more sensitive detection than GFP-tagging.

Results

Three regions CaCdc35 were chosen for study. The first one (#891) is corresponding to amino acid 300-790 (RA domain). The other two regions (#892: amino acid 1310-1490 and #893: amino acid 1320-1490) were chosen in the catalytic domain (Figure 5 in Chapter 2). The latter two are very similar, but Dr. Nassar's lab was interested in expressing both of them to help find a fragment that folds well and will work for structural studies.

The expression plasmids carrying these three fragments were constructed by former lab members, but they had not been tested for protein purification. The proteins

were designed to be expressed with a 6×Histag at the N terminus to facilitate rapid purification on a Nickel Column.

The plasmids were transformed into the *E. coli* $BL21^{+}$ strain. Cells were grown and induced with IPTG (0.5 mM) at 18℃ for 20 hours as described in Chapter 2. Cells were harvested, lysed and then the cell extracts were purified on a Nickel Column. Samples of each steps of purification were then tested by SDS-PAGE (Figure 9, 10 and 11).

Analysis of a Commassie Blue stained gel showed that all of the three fusion proteins were well produced. However, all proteins appeared to be insoluble as they mainly pelleted with the insoluble fraction. It is unfortunate because inclusion bodies are not useful for crystallization and structural studies. But, these aggregated proteins may still be usable for raising antibodies that should cross-react with CaCdc35 protein.

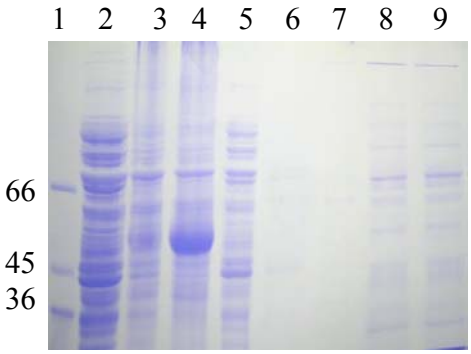

Figure 9. SDS-PAGE analysis of the CaCdc35-RA domain expressed in *E. coli* (#891). Protein was purified by Nickel column. 1. Protein Ladder; 2. Supernatant after ultrasonic lysis; 3. Pellet from inner layer; 4. Pellet from outer layer; 5. Flowthrough; 6. Collection of Column Wash; 7. Fraction 4 of Nickel Column purification; 8. Fraction 5; 9. Fraction 6. The molecular weight of the RA domain is 58KD. Protein is in the pellet from outer layer (see lane 4). The protein is inclusion body.

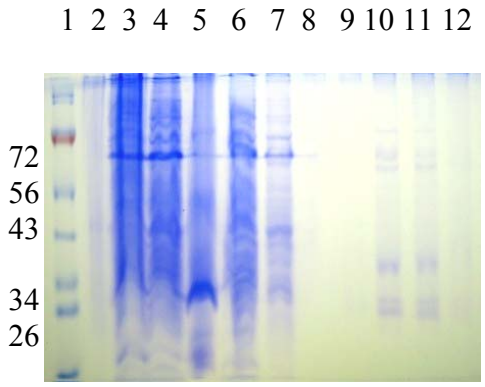

Figure 10. SDS-PAGE analysis of the Cdc35 catalytic domain (#892). Protein was purified by Nickel column. 1. Protein Ladder; 2. Before IPTG induction; 3. After IPTG induction; 4. Supernatant after ultrasonic lysis; 5. Pellet after ultrasonic lysis; 6. Flowthrough; 7. Collection of Column Wash; 8-12. Fractions of Nickel Column purification. The molecular weight of #892 fragment is 24KD. Protein is in the pellet (see lane 5). The protein is inclusion body.

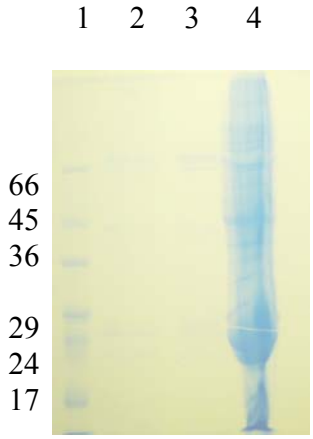

Figure 11. SDS-PAGE analysis of the CaCdc35 catalytic domain (#893). Protein was purified by Nickel column. 1. Protein Ladder; 2. Whole cell lysis; 3. Supernatant after ultrasonic lysis; 4. Pellet after ultrasonic lysis. The molecular weight of #893 fragment is 23KD. Protein is in the pellet (see lane 4). Conclusion: the protein is inclusion body.

Discussion

Initial studies showed that the three fragments of CaCdc35 adenylyl cyclase are insoluble. At this point, there appears to be two options. One is to try denaturing and refolding the proteins; alternatively, we can select new fragments for expression and purification. For example, we can use much larger fragments of the catalytic domain for protein purification. At least some portions of CaCdc35 are soluble, because a previous study by Klengel et al. [21] was able to study the catalytic activity of a bacterially expressed fragment of CaCdc35 in *in vitro* enzyme studies. This fragment contained residues 1166-1571 [21]. However, the tradeoff is that a larger fragment may contain disordered domains that would not crystallize and permit structural studies.

Chapter 6

Future Perspective

Introduction

This thesis project was aimed at studying the localization and regulation of cAMP signal pathway components in *C. albicans*. The cAMP signal pathway is essential for hyphal morphogenesis in *C. albicans*, but the molecular mechanism that regulates this pathway is not clear. In this thesis, I studied the spatial localization of adenylyl cyclase CaCdc35 and plasma membrane sensor CaGpr1 using GFP fusions. Also, I tried to study the structure of CaCdc35 by purifying His-tagged fragments. However, technical problems limited the conclusions. A summary of perspectives for future research will be provided in this chapter.

GFP Signaling

One technical problem in the study of *C. albicans* is the detection of GFP tagged proteins. In my study of CaCdc35 and CaGpr1, the GFP signal was very weak and it prevented me from further study of their regulation. Although the signal of Cdc35-GFP and pADH1-Gpr1-GFP is weak, it is detectable. Therefore, a better folding GFP protein may help a lot. One possible way to improve the GFP signal I am trying is to test different GFP variants in *C. albicans*. The GFP protein we used in the experiment is codon-optimized and fluorescence-enhanced version of GFP [27]. After analyzing the key amino acids that affect the protein folding in GFP (Figure 12), we designed three different variants. Future studies can finish the cloning of new GFP mutants and test them in *C. albicans*.

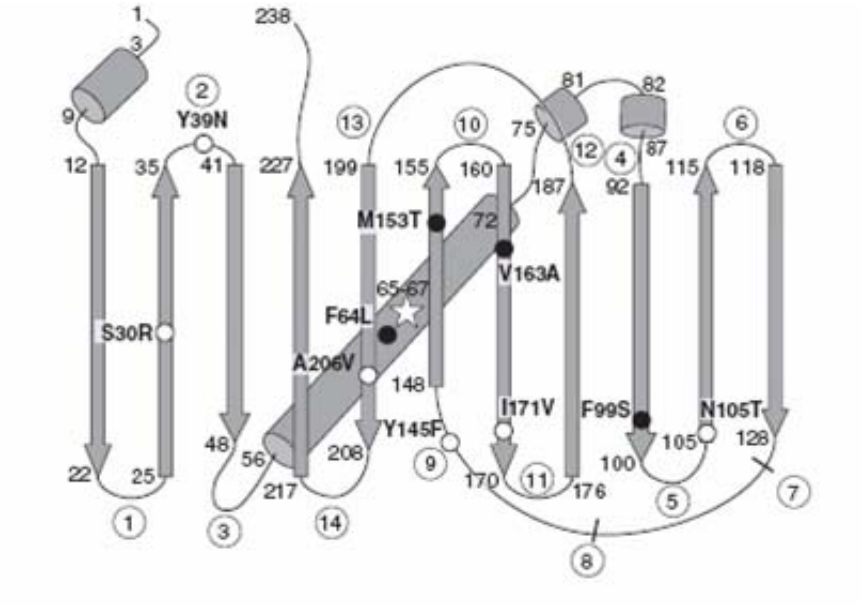

Figure 12. Schematic representation of the GFP scaffolding (cited from [28]).

Protein purification

The three fragments of CaCdc35 expressed in *E. coli* were insoluble. To improve solubility, I may lower the IPTG concentration or increase salt concentration of the buffer. Also, we can denature the inclusion body and try to obtain soluble protein by refolding it. One option is to denature the protein with urea (8-10 M) or guanidine hydrochloride (6-8 M), and then allow the proteins to refold by dialysis against buffers with lower salt concentrations. This can be done in a step by step approach, or by column purification. No information about refolding CaCdc35 protein is known. Therefore, it may be necessary to do many tests before obtaining a soluble protein with high activity. Alternatively, we can design new fragments of Cdc35 as described in Chapter 5. Furthermore, we can try other expression systems, for example, yeast cells or baculovirus.

Other interesting topics

Although *C. albicans* cAMP signaling pathway has been studied for years and found as key pathway for hyphal morphogenesis, many gaps remain to be filled. We observed the weak signal of CaCdc35 protein at plasma membrane, which indicates that CaCdc35 is recruited to the membrane, but we do not know how it associates with the membrane. Cdc25-Ras1 and Gpr1-Gpa2 are two important signaling pathways that activate CaCdc35 in *C. albicans* to stimulate hyphal development. It is reported that CaCdc35 directly interacts with Ras1; however, how it interacts with Gpa2 is unknown. Another major question is the identity of the factors that activate CaCdc25-the guanine nucleotide exchange factor (GEF) that activates Ras1. Ligands of the plasma membrane

sensor CaGpr1 are also not characterized well. Amino acids, especially methionine, are thought to be one candidate. Other possibilities remain to be tested. Structure studies of plasma membrane receptors and the critical components of the signal pathway will be an important challenge to address. The answers to the above questions will be very useful in designing new inhibitors of *C. albicans* signal pathways and developing new strategies of antifungal therapies.

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