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Original Article

Asc1, a WD-repeat protein, is required for hyphal development and virulence in *Candida albicans*

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Candida albicans **is a human pathogenic fungus which can undergo a morphological transition from yeast to hyphae in response to a variety of environmental stimuli. We analyzed a *C. albicans* Asc1 (Absence of growth Suppressor of Cyp1) protein which is entirely composed of seven repeats of the WD domain, and is conserved from fungi to metazoan. Deleting the *ASC1* in *C. albicans* led to a profound defect in hyphal development under hypha-inducing conditions examined. Furthermore, deletion of the *ASC1* attenuated virulence of *C. albicans* in a mouse model of systemic infection. These data strongly suggested that the conserved WD-repeat protein Asc1 is required for morphogenesis and pathogenesis of *C. albicans*.**

Keywords *Candida albicans*; Asc1; hyphal development; virulence

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Introduction

Candida albicans is an opportunistic pathogen that poses a considerable public health problem, with an estimated 40% mortality rate for systemic candidiasis [1,2], causing life-threatening systemic infections in immunocompromised patients as well as a range of superficial infections [3]. *Candida albican* has a complex life cycle that involves changes in morphology, cell physiology, and adherence as the cells pass through a variety of different environments. It can undergo reversible morphogenetic transitions between budding yeast, pseudohyphal, and hyphal growth forms [4]. The ability to grow in and reversibly switch among multiple growth forms has been shown to be essential for its pathogenicity, and accordingly, loss of switching capacity results in decreased virulence or avirulence [5–9]. The yeast-to-hyphae transition of *C. albicans* can be triggered *in vitro* by a variety of factors, including carbohydrates, amino acids, salts, pH changes, high temperature, starvation, serum, and growth within a matrix [10]. These various hyphal inducers trigger a wide range of signal transduction pathways involved in morphogenesis.

All cells have the ability to sense extracellular stimuli and environmental changes, and then respond appropriately to these signals. Receptor of activated protein C kinase (RACK1) is a highly conserved protein in eukaryotic cells. It is a member of the family of proteins with WD repeats [11]. The individual WD40 repeat can simultaneously interact with different signaling molecules, allowing RACK1 to integrate inputs from distinct signaling pathways. Cryo-electron microscopy and X-ray crystallographic data indicate that RACK1 displays a β-propeller structure, with seven blades corresponding to the WD-repeat domains, in a general conformation closely resembling the β-subunit structure of heterotrimeric G proteins [12–14]. RACK1 was initially identified by its ability to interact with protein kinase C isoforms, and later studies demonstrated that it also binds *in vivo* other proteins related to signal transduction pathways, leading to the suggestion that it might act as a scaffold to recruit elements involved in cell signaling [11,12]. RACK1 was found to be a core component of the eukaryotic 40S ribosomal subunit [15–17], suggesting that its signaling functions might directly influence the efficiency and specificity of translation. All these investigations raised the exciting possibility that RACK1 connects the signaling and translation machinery in the cell. RACK1 also functions in diverse developmental processes, such as sexual differentiation in *Schizosaccharomyces pombe* [18] and control of cell proliferation in *Drosophila melanogaster* [19].

In *Saccharomyces cerevisiae,* the orthologue of RACK1 is Asc1 (Absence of growth Suppressor of Cyp1) [20]. *Saccharomyces cerevisiae* Asc1 functions as a G-protein β subunit coupled to glucose responsiveness [21] and required for Flo11-dependent adhesive growth and dimorphism [22]. Asc1 binds directly to GDP-Gpa2 and inhibits Gpa2

guanine nucleotide exchange activity. Asc1 can bind to the downstream effector Cyr1, resulting in diminished cyclic AMP or 3′-5′-cyclic adenosine monophosphate (cAMP) production. Asc1 can also interact with a second downstream effector Ste20, which results in repression of basal signaling via the MAP kinase branch of the glucose signaling apparatus [21]. Moreover, deletion of the *ASC1* abolished amino acid starvation-induced adhesive growth and impaired basal expression of *FLO11* and its activation upon starvation in haploid cells. In addition, Flo11-dependent pseudohyphal growth during nitrogen limitation is *ASC1*-dependent in diploid cells [22]. The *ASC1* gene encodes a pre-mRNA which is spliced and constitutively expressed in the presence or absence of amino acids. Asc1 represses the transcriptional activator Gcn4 in the absence of amino acid starvation. The Asc1-mediated transcriptional repression requires the Gcn4 transcriptional activator and a Gcn4 recognition element in the target promoter [23]. Like the mammalian RACK1, the Asc1 is also identified as a core component of the small (40S) ribosomal subunit. The purified Asc1-deficient ribosomes have increased translational activity compared with wild-type yeast ribosomes. The *asc1* null mutant increases the levels of specific proteins *in vivo*, suggesting that one of Asc1's functions is to repress gene expression [24].

By searching *C. albicans* genome database (http://www.candidagenome.org), a protein designated as Asc1 (orf19.6906) was found to share highest similarity with *S. cerevisiae* Asc1 (RACK1). Genome-wide transcription profiling analysis by DNA microarrays and expression profiling analysis by two-dimensional gel electrophoresis revealed that the expression of *Candida albicans* Asc1 (CaAsc1) was iron-, temperature-, and Gcn4-dependent; and was downregulated by amino acid starvation (3-aminotriazole), caspofungin, and farnesol [25–29]. In this report, we provided experimental evidence that deletion of the *CaASC1* caused defects in filamentous growth of *C. albicans*. We also showed that the *asc1/asc1* null mutant attenuated virulence in a mouse model of systemic infection. We proposed that *C. albicans* Asc1, the counterpart of mammalian RACK1, plays a role in morphogenesis and pathogenesis of *C. albicans*.

Materials and Methods

Strains and culture conditions

Candida albicans strains used in this study are listed in **Table 1**. Routine growth was on YPD (1% yeast extract, 2% peptone, 2% glucose) medium or SD (SC supplemented with 2% glucose) medium at 30°C. Media were used for yeast and hyphal growth as described previously [30–35]. SLAD (synthetic low-ammonia) medium, SCLD (SC supplemented with 0.1% glucose) medium, GlcNAc medium, Lee's medium, or YPD containing 10% serum was used for hyphal induction.

Table 1 ***Candida albicans*** **strains and plasmids used in this study**

Strains or plasmids	Genotype	Reference
SC5314 strain	Wild type	[31]
CAF2-1 strain	*URA3/ura3:: λimm434*	[31]
CAI4 strain	*ura3:: λimm434/ura3:: λimm434*	[31]
BWP17 strain	*ura3:: λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	[36]
CLX1a-1 strain	*ura3:: λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG ASC1/asc1::HIS1*	This study
CLX1a-2 strain	*ura3:: λimm434/ura3:: λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG asc1:: ARG4/asc1:: HIS1*	This study
pGEM-*HIS1*	pGEM-T carrying a 1.0-kb *CaHIS1* fragment	[36]
pRS-*ARG4*ΔSpeI	pRS314 carrying a 2.3-kb *CaARG4* fragment	[36]
pGEM-*URA3*	pGEM-T carrying a 1.2-kb *CaURA3* fragment	[36]
pBA1	*C. albicans ADH1* promoter in pBES116	[44]
pBA1-ASC1	1-kb full length *C. albicans ASC1* without intron in pBA1	This study

Plasmid and strain construction

All plasmids used in this study are listed in **Table 1**. Methods for genomic DNA isolation and Southern blot hybridization were as previously described [30]. All probes were randomly labeled with Random Primers DNA Labeling System (Invitrogen, Carlsbad, USA) with [α-32P]-dATP. The genomic DNA from *C. albicans* wild-type strain SC5314 was used as a template for PCR amplifications [31]. All constructs were verified by DNA sequencing. Since the *C. albicans ASC1* contains a 258-bp intron in its genome, to construct an intron-less expression vector for complementation analysis, SC5314 mRNA was extracted and reverse-transcribed to cDNA. A cDNA fragment containing the *ASC1* open reading frame (ORF) was amplified by PCR and subcloned into pBA1 to generate pBA1-ASC1. The primers used for PCR are listed in **Table 2**.

A PCR-based homologous recombination method was used to disrupt the *ASC1* in *C. albicans* strain BWP17 [36]. Primers ASC1-5DR and ASC1-3DR (**Table 2**) were used to amplify *C. albicans HIS1*, *URA3*, and *ARG4* from plasmids pGEM-*HIS1*, pGEM-*URA3*, and pRS-*ARG4*ΔSpeI,

Table 2 Primers used in this study

Primers	Sequence (5′−3′)	Purpose and features
ASC1F	GCTATCGATATGGCTGATCAAGAAGTTTTAG	pBA1-ASC1
ASC1R	GTCGGTACCTTAAGCAGATGGAGTCATAACT	
ASC1-5DR	GTTAATTTCATTTCCCTTCAATTTCTTTTCTTTTCTTCTTT; TTTAAAAAACAAACAATCAATTATCTACGTTTTCCCAGT; CACGACGTT	*CaASCI* disruption
ASC1-3DR	TTTCCCACAAAAAAACAAAATCTATACAAAAAAAAACT; TCTTGTTGTATGTTAAATTTAGAATTAAATTTGTGGAATT; GTGAGCGGATA	
ASC1PF	CAAATGAGCCAATATCTGACC	ASC1 probe
ASC1PR	ATCAATGCCAAGATTCATCAG	
asc1-5′	GGACGCGAGTAAACTTAGGCAC	*asc1* mutants verification
asc1-3′	GTCGGTACCTTAAGCAGATGGAGTCATAACT	
his1-3′	TTCTCCAACGAAAACTGGGATATC	
arg4-3′	TTGAAGCTAGTGTGGAAAGAAGAG	

The underlined sequences refer to the corresponding restriction sites.

respectively. The first copy of *ASC1* was disrupted by transformation of *C. albicans HIS1* into BWP17. The second copy of *ASC1* was subsequently replaced by *C. albicans ARG4*. Generated mutants (CLX1a-1 and CLX1a-2) were confirmed by PCR and Southern blot analysis. The primers used for PCR verification are listed in **Table 2**. Restriction endonucleases used above were provided by Invitrogen (Carlsbad, USA).

Virulence assay

Virulence assay was performed as described previously [30]. The newly plated *C. albicans* strains were grown in liquid YPD at 30°C overnight, suspended in physiological saline solution, counted in a hemacytometer and adjusted to a concentration of 5×10^7 cells/ml or 5×10^6 cells/ml. Eight ICR male mice (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, SIBS, Shanghai, China) weighing 18−21 g, for each strain, were injected into lateral tail veins with 0.1 ml cell suspension. The survivals of mice were observed and recorded continuously for at least 25 days after injection.

Results

Sequence analysis of *C. albicans* Asc1

Saccharomyces cerevisiae RACK1 was identified as a recessive extragenic suppressor of a hap1$^-$hem1$^-$ strain and designated as Asc1 [20]. By searching *C. albicans* genome database (http://www.candidagenome.org), a protein designated Asc1 (orf19.6906) was found to share the highest similarity with *S. cerevisiae* Asc1 (RACK1) [**Fig. 1(A)**]. The identities of the primary sequences between *C. albicans* Asc1 (CaAsc1) and *S. cerevisiae* Asc1 (ScAsc1) were 66%. The *ScASC1* ORF is interrupted by an intron of 273 bp that shelters the U24 small nucleolar RNA (snoRNA) [20]. Sequence analysis of the *CaASC1* reveals that its ORF is also interrupted by an intron of 258 bp, at position 529 after ATG [**Fig. 1(A)**]. The *CaASC1* ORF was predicted to encode 317 amino acids. Like other RACK1 family members, CaAsc1p is entirely composed of seven repeats of the WD domain [**Fig. 1(B)**]. The WD-40 repeats (also known as WD or beta-transducin repeats) are short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, in which the repeating units serve as a rigid scaffold for protein interactions. The specificity of the proteins is determined by the sequences outside the repeats. Examples of such complexes are G proteins (beta subunit is a beta-propeller), TAFII transcription factor, and E3 ubiquitin ligase [37,38]. On the basis of sequence comparisons, CaAsc1p belongs to a highly conserved subgroup of the WD-repeat family, the RACK1 family. By using BLAST and ClustalX program, it was found that *C. albicans* Asc1 is the orthologue of Asc1 (RACK1) in *S. cerevisiae*, Cpc2 (RACK1) in *S. pombe*, and RACK1 in mammalian [**Fig. 1(C)**].

Construction of *C. albicans asc1/asc1* mutants

RACK1 proteins are involved in a wide variety of regulatory functions, including signal transduction, translational regulation, and diverse developmental processes. To elucidate the function of CaAsc1 in *C. albicans*, we constructed an *asc1/asc1* null mutant by sequential gene disruption using a PCR-based homologous recombination method [36]. *Candida albicans* strain BWP17 was used as the

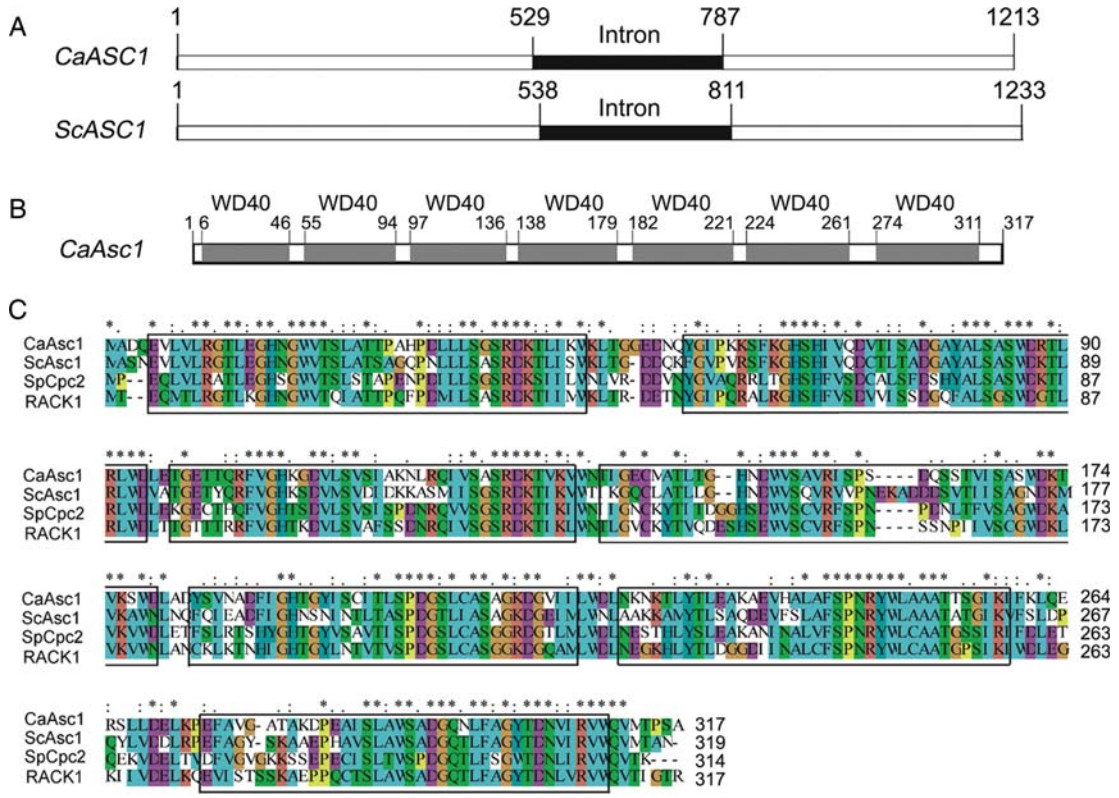

Figure 1 Sequence analysis of *C. albicans* Asc1 (A) Schematic representation of *C. albicans ASC1* gene (upper line). The white boxes represent the two exons found in *CaASC1*. The black box represents the intron. The positions of the ATG and of the beginning and end of the intron are indicated. The *S. cerevisiae ASC1* gene (*ScASC1*) is represented with the same symbols (bottom line). (B) Schematic representation of *C. albicans* Asc1 protein. The seven WD-40 repeats were predicted by ScanProsite (http://www.expasy.ch/tools/scanprosite/). (C) Sequence comparisons of CaAsc1, ScAsc1, SpCpc2, and RACK1 proteins. Sequence alignments were performed using ClustalX program.

parent strain for *ASC1* deletion [**Fig. 2(A)**]. First copy of the *ASC1* was disrupted by replacement of *C. albicans HIS1*. *Candida albicans ARG4* was used to substitute for second copy of the *ASC1*. The homozygous *asc1/asc1* mutants were confirmed by PCR and Southern blot analysis [**Fig. 2(B,C)**]. Deletion of the *ASC1* gene was examined by loss of *ASC1* PCR products (with asc1-5′+asc1-3′ primer set) and gain of *HIS1* PCR products (with asc1-5′+his1-3′ primer set) or *ARG4* PCR products (with asc1-5′+arg4-3′ primer set) [**Fig. 2(B)**]. Southern hybridization showed that two copies of the *ASC1* genes in the genome were disrupted [**Fig. 2(C)**]. The *ASC1* coding region including all seven WD domains was replaced with a *HIS1* or *ARG4* insertion.

ASC1 is required for hyphal development of *C. albicans*

Candida albicans can develop into hyphae in response to various environmental stimuli, such as serum, *N*-acetylglucosamine (GlcNAc), high temperature, neutral pH, and starvation. To determine the role of Asc1 in hyphal development, we analyzed the phenotypes of the *asc1/asc1* mutant strains in several hypha-inducing media, including serum containing media, Lee's, SCLD, and SLAD medium [30–32]. In liquid YPD + 10% serum medium, which is one of the most effective hypha-inducing conditions, wild-type cells developed long hyphae after 3.5 h incubation at 37°C, whereas *asc1/asc1* mutant cells displayed shorter hyphae [**Fig. 3(A)**, first row)]. On solid serum-containing agar, the *asc1/asc1* mutant strain displayed a more severe defective phenotype in filamentous growth and formed small downy colonies without long filaments, although the wild-type strain produced florid filamentous colonies [**Fig. 3(A)**, second row]. The defects in filamentous growth observed in the *asc1/asc1* mutants were caused by the *ASC1* deletion, as the phenotype was reversed by re-introducing pBA1-ASC1, an intron-less *ASC1* expression vector under the control of the *ADH1* promoter. Interestingly, *ASC1* exerted its effects on filamentation in a dose-dependent manner, since the *ASC1/asc1* heterozygous mutants were partially impaired in filaments formation and reintegrating a single copy of *ASC1* could not fully restore the ability of *asc1/asc1* mutants to form filaments [**Fig. 3(A)**, second row]. In liquid Lee's medium, the *asc1/asc1* mutant showed a similar phenotype to that observed in liquid serum-containing medium, and developed stunted hyphae [**Fig. 3(A)**, third row]. On solid

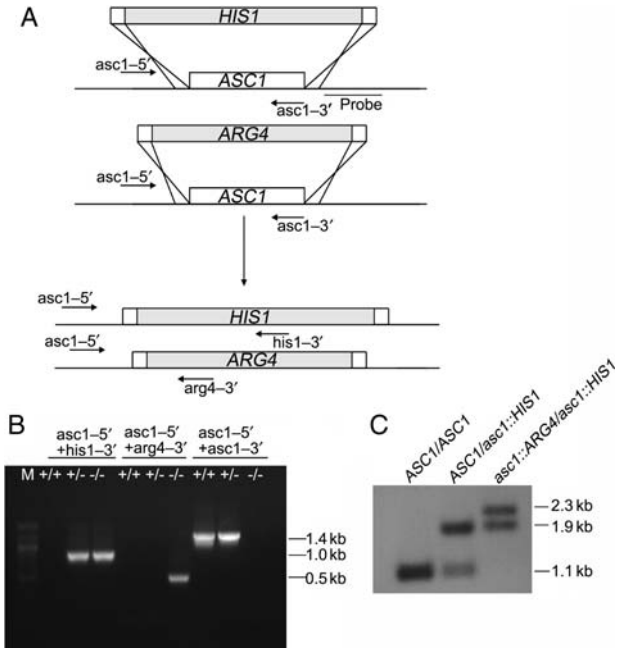

Figure 2 Disruption of *C. albicans ASC1* gene (A) Strategy for knocking out *ASC1* gene in *C. albicans* strain BWP17. Full length *CaASC1* ORF was replaced through homologous recombination by a PCR-amplified *HIS1* or *ARG4* gene with two *ASC1* flanking regions. (B) PCR analysis of *asc1* mutants by using primers showed in (A) and **Table 2**. (C) Southern blot analysis of *asc1* mutant strains. Genomic DNA from wile-type strain (BWP17), *ASC1/asc1* mutant (CLX1a-1) and *asc1/asc1* mutant (CLX1a-2) were digested with *Bgl*II/*Eco*RV and hybridized with a 0.6-kb probe of downstream region fragment of *ASC1* as indicated in (A).

Lee's medium, the *asc1/asc1* mutant also impaired hyphal development, and formed wrinkle colonies with short filaments [**Fig. 3(A)**, fourth row]. We also examined the phenotype of *asc1/asc1* mutant strains in GlcNAc medium which is used for hypha-induction [33,34]. In liquid GlcNAc medium, the *asc1/asc1* mutant cells developed stunted hyphae at 37°C, similar to that observed in liquid Lee's medium (data not shown). Contrastingly, in some nutrient-limited media, such as liquid SCLD or SLAD medium, the *asc1/asc1* mutant cells failed to form hyphae and grew in yeast-like form [**Fig. 3(B)**]. The defects of *asc1/asc1* mutant in hyphal development could be rescued by re-introducing the *ASC1* expression vector pBA1-ASC1 [**Fig. 3(B)**]. Consistently, deletion of the ASC1 prevented the filaments growth and formed smooth colonies in solid SCLD or SLAD medium (data not shown). Blocking hyphal development of the *asc1/asc1* mutants in SCLD, a glucose depletion medium, and in SLAD, a nitrogen starvation medium, suggested that *C. albicans* Asc1 is essential for hyphal development in responding to certain extracellular stimuli.

We also analyzed the role of *C. albicans* Asc1 in yeast growth. In contrasting to the profound defect in hyphal development, the *C. albicans asc1/asc1* mutant had little defect in yeast morphology. The *asc1/asc1* mutant cells exhibited similar cell morphology to that of wild-type cells, grew in yeast form and showed normal yeast cell size when cultured in YPD or SD media at both 30°C and 25°C (data not shown). On the other hand, deletion of *ASC1* caused a general growth defect. The *C. albicans asc1/asc1* mutant cells grew slower than wild-type cells (120- and 80-min doubling times during log phase, respectively, in YPD at 30°C). The results suggest that *C. albicans* Asc1 plays a role in general cell growth.

Deletion of *ASC1* attenuates virulence of *C. albicans*

The ability of *C. albicans* to undergo a reversible morphological transition between yeast and hypha is important for its pathogenicity. Non-filamentous strains are avirulent [8], and constitutive filamentous strains also show decreased pathogenicity [6,39]. Deletion of the *ASC1* impaired the ability of cells to switch between yeast and hyphal forms. We examined the virulence of the *asc1/asc1* mutant strains in a systemic model of infection. Cells of wild-type, *ASC1/asc1* heterozygous mutant and *asc1/asc1* homozygous mutant strains were inoculated into each mouse by tail vein injection. Injection with an inoculum of 5×10^6 wild-type cells caused death of all mice within 8 days [**Fig. 4(A)**], and a smaller inoculum of 5×10^5 cells killed all mice within 12 days [**Fig. 4(B)**]. The *ASC1/asc1* heterozygote showed slightly decreased virulence compared with wild-type strain. The *asc1/asc1* null mutant cells were dramatically less virulent at both inoculum doses: 50% mice survived for more than 19 days after injection with 5×10^6 cells [**Fig. 4(A)**] and all mice survived for more than 20 days after injection with 5×10^5 cells [**Fig. 4(B)**]. The results showed that deleting *ASC1* in *C. albicans* reduced its virulence in a mouse model of systemic infection. The reduced virulence of *asc1/asc1* null mutant may correlate with its defective abilities of yeast-hypha transition and general growth.

Discussion

In this work, we analyzed the role of Asc1 in morphogenesis and pathogenesis of *C. albicans*. We found that cells lacking Asc1 have a defect in hyphal development in response to several environmental stresses, especially glucose and nitrogen starvation. Moreover, *asc1/asc1* null mutant displays dramatically reduced virulence during the course of systemic infections. CaAsc1 is a RACK1 family protein, contains seven WD repeats, which are highly conserved throughout the eukaryotic kingdom. More than a decade of research has established RACK1 as a key player in multiple signaling pathways. As a core component of the 40S ribosomal subunit, it raised a possibility that

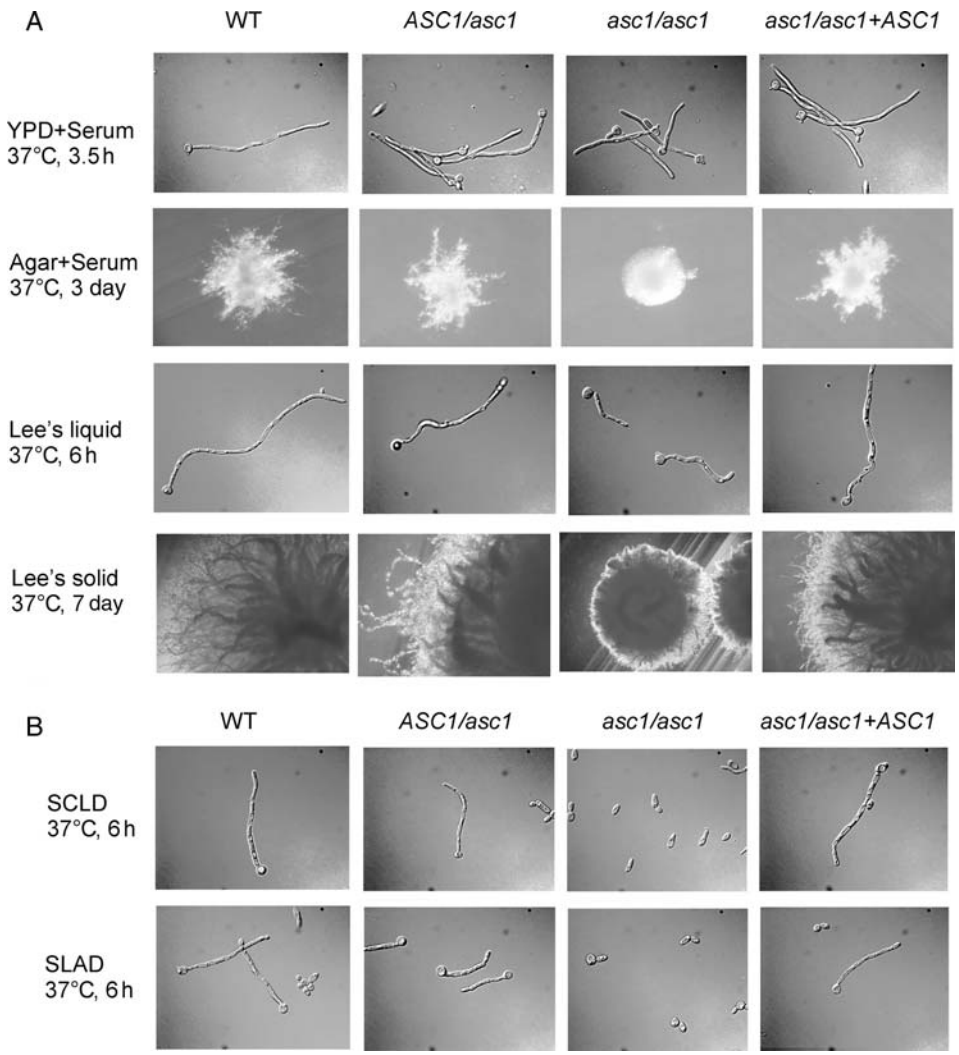

Figure 3 Deletion of *ASC1* impairs hyphal development in *C. albicans* For cell morphology observation in liquid media, overnight cultures of the wild-type (SC5314), *ASC1/asc1* heterozygote (CLX1a-1 + pBA1), *asc1/asc1* null mutant (CLX1a-2 + pBA1), and *ASC1* revertant (CLX1a-2 + pBA1-ASC1) strains were diluted in YPD+10% serum at 37°C for 3.5 h or in Lee's, SCLD, SLAD media at 37°C for 6 h. For colony morphology observation in solid media, the strains were streaked onto the plates and incubated at 37°C for 3 or 7 day. (A) *asc1/asc1* mutants were impaired in hyphal growth in liquid and solid serum containing media or Lee's media. (B) *asc1/asc1* mutants were impaired in hyphal development in liquid SCLD or SLAD media.

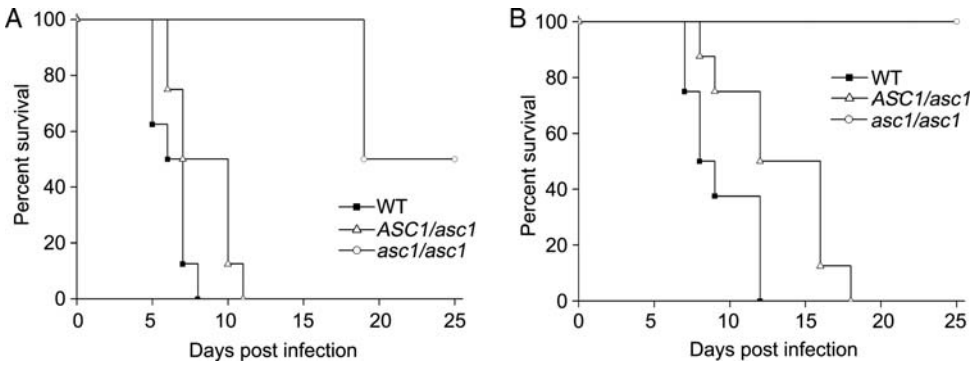

Figure 4 Virulence assay ICR male mice were injected with wild-type (CAI4 + pBA1), *ASC1/asc1* heterozygote (CLX1a-1 + pBA1), *asc1/asc1* null mutant (CLX1a-2 + pBA1) strains. The mice were injected with 5×10^6 (A) and 5×10^5 (B) *Candida albicans* cells.

RACK1 connects the signaling and translation machinery in the cell.

Two G-protein signaling pathways were identified in *S. cerevisiae*. The first one is the pheromone-promoted mating pathway [40–42], and the second one mediates pseudohyphal differentiation in diploids and invasive growth in haploids. In diploids, cells undergo pseudohyphal differentiation upon limitation of nitrogen [32], whereas in haploids, the cells undergo invasive growth upon limitation of glucose [43]. *Saccharomyces cerevisiae* Asc1 contains seven WD-repeat domains and displays a β-propeller structure in a general conformation closely resembling the β-subunit structure of heterotrimeric G proteins. The abilities of binding to GDP-Gpa2 or downstream effectors allow the Asc1 to integrate inputs from distinct signaling pathways and regulate diverse developmental processes.

Candida albican Asc1 shared high sequence similarity with *S. cerevisiae* Asc1. Blocking of *asc1/asc1* mutant in hyphal development responding glucose and nitrogen starvation may reflect its functions as a G-protein β subunit resembling the functions of ScAsc1 in Gpa2 coupled signaling. On the other hand, *C. albicans* cAMP/protein kinase signaling pathway plays a key role in responding to extracellular stimuli in serum, Lee's medium and GlcNAc medium. Defect of *asc1/asc1* mutant in hyphal development responding to serum, GlcNAc and the components in Lee's medium may reflect its functions mediated by interacting with other signaling proteins. Indeed, RACK1 was initially identified by its ability to interact with protein kinase C isoforms, and we cannot rule out the possibility that the *C. albican* Asc1 mediates multiple cellular processes by interacting with different proteins via WD domain.

In summary, we have described a conserved WD protein Asc1 and its functions in hyphal development and virulence in *C. albicans*. We conclude that Asc1 is important for *C. albicans* morphogenesis and pathogenicity. CaAsc1 has highly conserved characteristics with the sequences from other *Candida* species. The CaAsc1 is identical to *Candida dubliniensis* Asc1 (CdAsc1) (GenBank CAX40664.1). Only seven different amino acids were found between the CaAsc1 and *Candida tropicalis* Asc1 (CtAsc1) (GenBank EER31126.1). All these imply that Asc1 may be very important for the *Candida* sp. Because Asc1 is conserved among fungal species, we propose that Asc1 is a strong candidate target for therapeutic intervention against fungal pathogens, not limited to *C. albicans*.

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