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Candida albicans Int1p Interacts with the Septin Ring in Yeast and Hyphal Cells

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The ability to switch between yeast and hyphal morphologies is an important virulence factor for the opportunistic pathogen *Candida albicans.* Although the kinetics of appearance of the filamentous ring that forms at the incipient septum differ in yeast and cells forming hyphae (germ tubes) (Soll and Mitchell, 1983), the molecular mechanisms that regulate this difference are not known. Int1p, a *C. albicans* gene product with similarity in its C terminus to *Saccharomyces cerevisiae* Bud4p, has a role in hyphal morphogenesis. Here we report that in *S. cerevisiae*, Int1p expression results in the growth of highly polarized cells with delocalized chitin and defects in cytokinesis and bud-site selection patterns, phenotypes that are also seen in *S. cerevisiae* septin mutant strains. Expression of high levels of Int1p in *S. cerevisiae* generated elaborate spiral-like structures at the periphery of the polarized cells that contained septins and Int1p. In addition, Int1p coimmunoprecipitated with the Cdc11p and Cdc12p septins, and Cdc12p is required for the establishment and maintenance of these Int1p/septin spirals. Although Swe1p kinase contributes to *INT1*-induced filamentous growth in *S. cerevisiae*, it is not required for the formation of ectopic Int1p/septin structures. In *C. albicans*, Int1p was important for the axial budding pattern and colocalized with Cdc3p septin in a ring at the mother-bud neck of yeast and pseudohyphal cells. Under conditions that induce hyphae, both Cdc3p and Int1p localized to a ring distal to the junction of the mother cell and germ tube. Thus, placement of the Int1p/septin ring with respect to the mother–daughter cell junction distinguishes yeast/pseudohyphal growth from hyphal growth in *C. albicans*.

INTRODUCTION

Candida albicans is a multimorphic opportunistic fungal pathogen of humans. The ability to change morphology between ovoid yeast forms and several filamentous forms (germ tubes, pseudohyphae, and true hyphae) contributes to *C. albicans* virulence (Odds, 1988, 1994). Switching between growth forms is influenced by many factors including temperature, pH, carbon source, nitrogen source, and cell concentration (Odds, 1988). Analysis of the molecular mechanism(s) of morphogenesis has been difficult in *C. albicans* because it is asexual, and thus not amenable to genetic analysis, and because its codon usage is nonstandard, interfering with heterologous gene expression (De Backer *et al.*, 2000).

INT1 encodes a protein (Int1p) that functions in morphogenesis. *C. albicans int1/int1* strains have a reduced ability to form hyphae on Milk-Tween and Spider media but form apparently normal hyphae in the presence of serum (Gale *et al.*, 1998). In addition, *int1/int1* strains have attenuated virulence in a mouse model of systemic candidiasis (Gale *et al.*, 1998). When *INT1* is expressed in *S. cerevisiae*, it causes a morphological switch to highly polarized filamentous cells that resemble *C. albicans* germ tubes and hyphae (Gale *et al.*, 1996). In *S. cerevisiae*, filamentous growth (termed "pseudohyphal growth" and most similar in morphology to *C. albicans* pseudohyphae) occurs in some diploid strains under nitrogen deprivation conditions (Gimeno and Fink, 1994) and in some haploid strains in rich medium (Roberts and Fink, 1994). Several *C. albicans* homologues of genes required for *S. cerevisiae* pseudohyphal growth (e.g., *STE20*

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Abbreviations used: CFP, cyan fluorescent protein; DIC, differential interference contrast; GFP, green fluorescent protein; HA, hemagglutinin epitope; *I*-IFG, *INT1*-induced filamentous growth; YFP, yellow fluorescent protein

and *STE12*) are also required for filamentous growth in *C. albicans* (Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996; Lo *et al.*, 1997); however, unlike pseudohyphal growth, *INT1*-induced filamentous growth (*I*-IFG) of *S. cerevisiae* is independent of *STE20* and *STE12* (Gale *et al.*, 1996, 1998) and occurs in all haploid and diploid strains grown in rich or minimal medium. Because Int1p alters *S. cerevisiae* morphology, we hypothesized that it interacts with proteins conserved between the two fungi, so that studies of Int1p function in *S. cerevisiae* would provide insights into its function in *C. albicans*.

In *S. cerevisiae*, morphogenesis depends on the actin cytoskeleton, bud-site selection proteins, the cell cycle machinery, and cytokinetic structures at the bud neck. For example, cytokinesis requires septin proteins, components of a filament ring that localizes to the cytoplasmic face of the plasma membrane in the mother-bud neck (Longtine *et al.*, 1996) and functions as a scaffold (Field and Kellogg, 1999) for proteins involved in bud-site selection (Chant *et al.*, 1995; Sanders and Herskowitz, 1996), chitin deposition (DeMarini *et al.*, 1997), cell cycle regulation (Barral *et al.*, 1999; Longtine *et al.*, 2000), and cytokinesis (Bi *et al.*, 1998; Lippincott and Li, 1998a,b). Loss of any one of four septins (Cdc3, Cdc10, Cdc11, or Cdc12) results in delocalization of the septins and associated proteins from the mother-bud neck and in the formation of elongated buds that cannot complete cytokinesis (reviewed in Longtine *et al.*, 1996).

Many of the *S. cerevisiae* proteins involved in morphogenesis are also found in *C. albicans*. For example, the *C. albicans* genome sequence includes sequences with similarity to *CDC3*, *CDC10*, *CDC11*, and *CDC12*, and the *C. albicans* homologues of *CDC3* and *CDC10* complement the morphogenesis defects of *S. cerevisiae cdc3* and *cdc10* strains (DiDomenico *et al.*, 1994). In classic studies of *C. albicans* morphogenesis, the site of yeast cell septation was found to be characterized by a filament ring that appears at the mother-bud neck at the time of bud emergence (Soll and Mitchell, 1983). In contrast, a filament ring (detected by electron microscopy and Calcofluor staining) appears in hyphae ~30 min after bud emergence and distant from the mother-bud junction. These studies suggested that the localization of septin proteins may be different in yeast and hyphal cells.

In a BLAST search, *C. albicans* Int1p is most similar to *S. cerevisiae* Bud4p and is the only predicted protein with significant similarity to Bud4p in the Candida genome sequence (http://sequence-www.stanford.edu/group/candida/search.html). Although the two proteins are only 24% identical over their entire sequence, they are 35% identical and 45% similar in the C-terminal ~375 amino acids. In *S. cerevisiae*, Bud4p is found with the septin ring at the mother-bud neck and is required for the haploid-specific axial budding pattern, but it is not required for septin ring localization (Sanders and Herskowitz, 1996). Specific factors required for bud site selection in *C. albicans*, an asexual diploid, have not been studied. *C. albicans* cells exhibit a predominantly bipolar budding pattern at temperatures >30°C and an increasing predominance of the axial budding pattern at temperatures <30°C (Chaffin, 1983; Herrero *et al.*, 1999).

In this study, we analyzed the effects of Int1p expression in *S. cerevisiae* to identify proteins that interact with Int1p to effect changes in bud morphology. We then used these insights to develop (and test) hypotheses regarding Int1p localization and interactions in *C. albicans*.

MATERIALS AND METHODS

Strains, Growth Conditions, and Plasmids

S. cerevisiae and *C. albicans* strains used in this study are described in Table 1. Yeast media (rich medium [YPAD], synthetic complete medium, and synthetic minimal medium lacking specific nutrients) have been described previously (Sherman, 1991). *S. cerevisiae* was grown at 30°C except where noted. *C. albicans* strains were grown in YPAD at 30°C to promote yeast form growth and at 37°C in YPAD containing 20% serum to promote hyphal growth. In Figure 1B, 10 μg/ml α-factor mating pheromone (Sigma, St. Louis, MO) was added to YJB5763 cells growing exponentially in YPAD + 2% glucose and incubation was continued until >90% of cells were forming mating projections (~3 h).

Plasmid p*GAL1-INT1-URA3* (pCG01 [Gale *et al.*, 1996]) drives expression of *INT1* from the *GAL1* promoter from vector pBM272 (Johnston and Davis, 1984). To achieve high-level expression of proteins from the *GAL1* promoter, strains pregrown in glucose-containing medium were subcultured into medium containing raffinose and galactose. p1227 (*CDC12-GFP-LEU2)* was provided by J. Konopka (State University of New York, Stony Brook, NY). pML726 (*CDC3-GFP-URA3*) was provided by J. Pringle (University of North Carolina, Chapel Hill, NC). pMM19 (*CDC3-GFP-LEU2*) was constructed from pML726, and p*GAL1-INT1-TRP1* was constructed from pCG01 (Gale *et al.*, 1996) by marker swapping *URA3* to *LEU2* (for pMM19) or *URA3* to *TRP1* (for pCG01) with the use of pUL7 and pUT11, respectively (Cross, 1997).

Morphological Observations

Chitin/bud scars were stained by adding 100 μg/ml Calcofluor white (Sigma) to the growth medium for 15–30 min. Bud scar patterns were scored for cells with 3–5 bud scars and were considered "axial" if all bud scars were in a single chain, "bipolar" if at least two bud scars were at opposite ends of a cell, and "random" if the pattern was neither bipolar nor axial. The scorer was blinded as to the identity of the strains being analyzed. The χ^2 test of goodness to fit (Snedecor and Chochran, 1980) was performed by taking the distribution of wild-type into three classes as the null model and testing each strain against this model. Similarly, pairs of mutant strains were tested against one another. Samples were considered significantly different at the $P < 0.005$ level. Nuclear DNA was detected with DAPI (Sigma).

Differential interference contrast (DIC) and epifluorescence microscopy were performed with the use of a Nikon Eclipse E800 photomicroscope equipped with standard UV and FITC filter sets. Cyan fluorescent protein (CFP) (excitation filter 380–400 nm, barrier 435–485 nm) and yellow fluorescent protein (YFP) (excitation filter 490–510 nm, barrier 520–550 nm) filter sets were obtained from Chroma Technology Corporation (Brattleboro, VT). Digital images were collected with the use of a CoolCam liquid-cooled, three-chip color CCD camera (Cool Camera Company, Decatur, GA), captured to a Pentium II 300 MHz computer with the use of Image Pro Plus version 4.1 software (Media Cybernetics, Silver Spring, MD), and processed with the use of Adobe Photoshop. Images for YFP and CFP were converted to red and green, respectively. In merged images, overlapping YFP and CFP signals appear yellow or orange.

Indirect immunofluorescence was performed as described previously (Pringle *et al.*, 1991), with the use of mouse monoclonal anti-hemagglutinin (HA) antibody (1:250) (Berkeley Antibody Co., Richmond, CA) and rabbit polyclonal anti-Cdc3p antibody (1:10) (Kim *et al.*, 1991). Secondary antibodies (goat anti-mouse IgG tagged with CY3 and goat anti-rabbit IgG tagged with CY2; both from Jackson ImmunoResearch, West Grove, PA) were used at 1:300 dilution.

Table 1. Yeast strains constructed for this study

Name	Genotype
S. cerevisiae	
M-652[a]	**a**/α *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 GIN4-GFP:kan/GIN4-GFP:kan*
M-912[a]	**a**/α *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 HSL1-GFP:kan/HSL1-GFP:kan*
M-768[b]	**a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-5 SEP7-GFP:kan*
M-780[b]	**a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-5 MYO1-GFP:kan*
YJB1843[c]	**a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* + [p*GAL1-INT1-URA3*]
YJB1844[c]	**a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* + [pBM272]
YJB1875[d]	**a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* + [p*GAL1-INT1-URA3*]
YJB1876[d]	**a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1* + [pBM272]
YJB2681	as M-768 except + [p*GAL1-INT1-URA3*]
YJB3362[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [p*GAL1-INT1-GFP-URA3-TRP1*]
YJB3363[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [p*GAL1-INT1-HA-HIS3-URA3*]
YJB3527[f]	**a** *leu2-Δ1 ura3-52 can1 ade2 his3-Δ200 trp1-Δ63 ssd1 ho cla4::URA3 Δclb1 clb3::TRP1 clb4::HIS3 bar1* + [p1227]
YJB3542[g]	**a** *ade2-10 cry1 his4 leu2-Δ1 trp1-Δ63 ura3-52 SUP4-3ts cdc12-6ts* + [pMM19] + [p*GAL1-INT1-URA3*]
YJB3958[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [p*GAL1-INT1-3HA-HIS3-URA3*] + [p1227]
YJB4840[g]	**a** *ade2-10 cry1 his4 leu2-Δ1 trp1-Δ63 ura3-52 SUP4-3 cdc12-6* + [p*GAL1-INT1-GFP-URA3-TRP1*]
YJB5001[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [p1227] + [p*GAL1-INT1-URA3*]
YJB5170[h]	**a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-5 MYO1-GFP:kan* + [p*GAL1-INT1-URA3*]
YJB5172	as M-652 except + [p*GAL1-INT1-URA3*]
YJB5174	as M-912 except + [p*GAL1-INT1-URA3*]
YJB5505[j]	**a**/α *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 CDC12/CDC12-YFP:TRP1* + [p*GAL1-INT1-CFP-URA3-TRP1*]
YJB5763[k]	**a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-5 2* + [p*GAL1-INT1-URA3*]
YJB5765[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [p*GAL1-INT1-URA3*]
YJB5766[e]	**a** *leu2-Δ1 trp1-Δ63 ura3-52 prb1-1122 pep4-3 prc1-407 gal2* + [pMM19] + [p*GAL1-INT1-URA3*]
YJB6003[i]	**a** *bar1 leu2-Δ1 ura3-52 trp1-Δ63 his2 ade1 swe1::LEU2* + [p*CDC3-GFP-URA3*] + [p*GAL1-INT1-TRP1*]
C. albicans	
CAF2[l]	*URA3/ura3::imm434*
CAG3[m]	*ura3::imm434/ura3::imm434 int1::hisG-URA3-hisG/int1::hisG*
CAG5[m]	*ura3::imm434/ura3::imm434 int1::hisG/int1::INT1:URA3*
YJB5850[n]	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG CDC3/CDC3-CFP:HIS1 INT1/INT1-YFP:URA3*

[a] Longtine *et al.*, 2000.
[b] Provided by John Pringle (University of North Carolina, Chapel Hill, NC).
[c] Derived from YPH499 (Sikorski and Hieter, 1989).
[d] Derived from YPH501 (Sikorski and Hieter, 1989).
[e] Derived from YJB365 (Berman strain collection).
[f] Derived from HT9 (Tjandra *et al.*, 1998).
[g] Derived from JKY81-5-1 (provided by James Konopka (SUNY, Stonybrook, NY).
[h] Derived from M-780 (Bi *et al.*, 1998).
[i] Derived from DLY1028 (McMillan *et al.*, 1998).
[j] Derived from YEF473 (Bi and Pringle, 1996).
[k] Derived from YEF473A (Bi and Pringle, 1996).
[l] Fonzi and Irwin, 1993.
[m] Gale *et al.*, 1998.
[n] Derived from BWP17 (Wilson *et al.*, 1999).

Construction of Fluorescent Protein and HA Fusions

Protein tags were introduced by PCR-mediated gene modifications (Wach *et al.*, 1994, 1997; Longtine *et al.*, 1998b; Gerami-Nejad *et al.*, 2001) with the use of synthetic oligonucleotides (IDT, Coralville, IA). PCR products were used to transform yeast strains directly, and transformants were screened by PCR for correct insertion of the tag. Plasmids and genomic sequences were isolated (Hoffman and Winston, 1987) and sequenced (University of Minnesota Microchemical Facility) to verify that fusions were in-frame.

To tag the 3′-end of sequences in *S. cerevisiae*, we used pFA6a-*GFP*(S65T)-*TRP1* (Longtine *et al.*, 1998b), pDH3 (Yeast Resource Center, University of Washington, Seattle, WA), or pFA6a-*3HA-TRP1* (Longtine *et al.*, 1998b) as template with primers A Forward and A Reverse (Table 2) to generate *INT1-GFP*, *INT1-CFP*, and *INT1-HA* transformation cassettes for integration into the plasmid contained in strain YJB5765. pYFP-URA3 (Gerami-Nejad *et al.*, 2001) was used as the template with primers C Forward and C reverse (Table 2) to generate a *CDC12-YFP* transformation cassette for YEF473.

Table 2. Oligonucleotide primer sequences used in this study

Name	Sequence (5′ → 3′)
A Forward	ACAACAACAACAACAACAACAACAACAAAGCTCCCAACAGCGGATCCCCGGGTTAATTAA
A Reverse	GCATATATTTGCCAATTAAAATTAAAAAAATCAAAAGTAGGAATTCGAGCTCGTTTAAAC
C Forward	AAAACTAGAAGAGCAGGTCAAAAGCTTGCAAGTAAAAAAATCCCATTTAAAAGGTGGTGGTTCTAAAGGTGAAG-AATTATT
C Reverse	GAAGAGACAAAGAGATAGGCGTTGAAATTCACGAGACAAAGAGGAAGACATTAACGACGGCCAGTGGAATTC
D Forward	ACAAAAATTATTACCACAAGACCCACCAGCACAACCAGCTCCACAAAAGAGTCGTAAAGGATTTTTACGTGGTG-GTGGTTCTAAAGGTGAAGAATTATT
D Reverse	AATTAAACAAACAGATTAACAAACAAATAAACTAAATTAAGTTACATACTATTTAGCTATACCTCGGCCCTCTAGA-AGGACCACCTTTGATTG
E Forward	ACGTCAACCATGGGTAAATTTGATGCTTCAACAACAACAACAACAACAACAACAACAAAGCTCCCAACAGGGTG-GTGGTTCTAAAGGTGAAGAATTATT
E Reverse	AAAACGCTATTAGACTAAAAGATAATACAAAATGGGCATATATTTGCCAATTAAAATTAAAAAAATCAAAAGTAG-GAATTCCGGAATATTTATGAGAAAC

To tag sequences in *C. albicans*, we used pCFP-HIS1 and pYFP-URA3 (Gerami-Nejad *et al.*, 2001) as templates with primers D Forward and D Reverse and E Forward and E Reverse (Table 2) to generate *CDC3-CFP* and *INT1-YFP* transformation cassettes for use with strain BWP17.

Immunoprecipitation

Cells expressing *INT1* for 8 h were lysed by the method of Frazier *et al.* (1998), except that cell debris was removed by three 10 min centrifugations at 12,000 × *g* followed by three 5 min centrifugations at 12,000 × *g*. Lysates were precleared by mixing for 1 h at 4°C with a combination of protein A- and protein G-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Each lysate was split into four aliquots to which were added rabbit anti-Rap1 antiserum (Enomoto *et al.*, 1997), rabbit anti-Cdc11 IgG (Santa Cruz Biotechnology), mouse anti-HA IgG (12CA5) (Roche Molecular Biochemicals, Indianapolis, IN), or mouse anti-green fluorescent protein (GFP) IgG (Roche). After mixing for 1.5 h at 4°C, Protein A-Agarose beads or Protein G-Agarose beads were added and incubated for 1 h at 4°C. The beads were then washed by centrifugation four times with cold lysis buffer, and protein was eluted by boiling in reducing protein electrophoresis buffer (Laemmli, 1970) for 5 min. Samples were separated on a 7.5% acrylamide gel (Laemmli, 1970), blotted to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and detected with peroxidase-conjugated anti-HA antibody (12CA5) (Roche) and "Supersignal" chemiluminescent substrate (Pierce Chemical Company, Rockford, IL).

RESULTS

INT1-induced Filaments Are Highly Polarized Buds with Defects in Cytokinesis

INT1 expression in *S. cerevisiae* causes the formation of a high proportion (>50–90%, depending on the strain background) of filamentous cells (*INT1*-filaments) (Gale *et al.*, 1996). If *INT1* expression is subsequently repressed, round buds emerge from the *INT1*-filaments (Figure 1A), indicating that the elongated cells were not in a terminal physiological state. To determine whether *I*-IFG is due to a cell cycle block that inhibits nuclear division, we analyzed the number of nuclei present in *INT1*-filaments. We observed an average of three nuclei per filament (range one to eight nuclei per filament) after ~18 h of growth in galactose. Cells not expressing *INT1* and growing in galactose-containing medium would have gone through approximately eight cell divisions. To determine whether *I*-IFG is restricted to a particular stage of the cell cycle, *INT1* was expressed in *MAT***a** yeast cells arrested in G1 by treatment with α-factor. These cells did not produce filaments (Figure 1B, center panel). In contrast, cells expressing *INT1* formed filaments in the absence of α-factor (Figure 1B, right panel). Thus, *INT1*-filaments, like normal buds, do not emerge until the cell cycle has traversed START. In addition, *I*-IFG is dependent on the ability to polarize the cytoskeleton: a $cdc24\text{–}4^{ts}$ strain did not form *INT1*-induced filaments at the restrictive temperature. Thus, *INT1*-filaments appear to be elongated buds that can progress through the nuclear cell cycle, albeit with an apparent delay.

To determine whether *INT1*-filaments contain septa, chitin was stained with Calcofluor. No distinct septa or chitin rings were observed even in very elongated *INT1*-filaments (Figure 1C). This diffuse localization of chitin appeared similar to that in *S. cerevisiae* strains with mutations in the septins or proteins involved in chitin deposition that localize to the mother-bud neck (DeMarini *et al.*, 1997). The absence of septa and the presence of multiple nuclei suggest that *INT1*-expressing cells are defective in cytokinesis. To distinguish between a defect in cytokinesis and a defect in cell separation, cells expressing *INT1* were fixed and treated after fixation with lyticase, which digests yeast cell walls and separates cells that have undergone cytokinesis (Hartwell, 1971; Pringle and Mor, 1975). We compared *INT1*-filaments with the aggregates of cells formed in a *myo1* strain that is defective in cell separation but not in cytokinesis (Bi *et al.*, 1998). In contrast to *myo1* strains, which separated into individual cells, >95% of the *INT1*-filaments were not affected by lyticase treatment.

Unusual Septin Structures in Cells Expressing INT1

Septin localization to the mother-bud neck (Figure 2A) is disrupted in septin mutants. Because cells expressing *INT1* resemble septin mutants, we analyzed septin localization in *INT1*-filaments with the use of functional GFP-tagged septins. All proteins that were examined localized

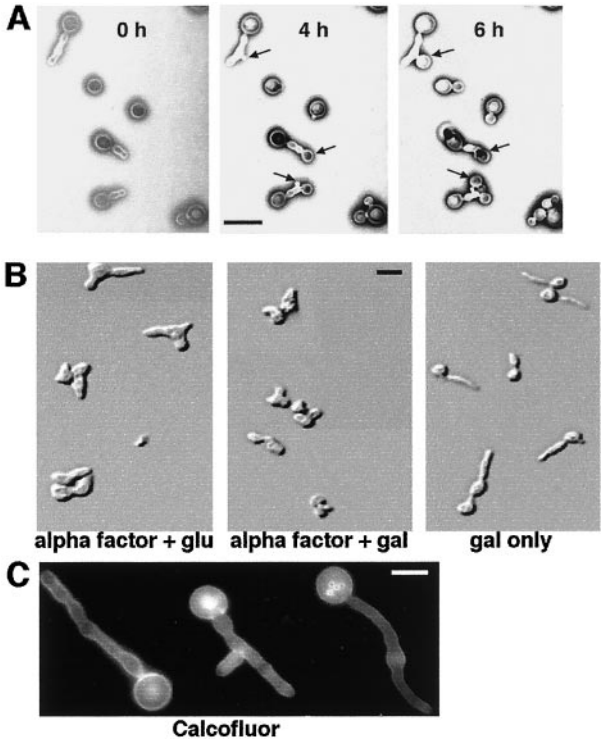

Figure 1. Characterization of *INT1*-filaments in *S. cerevisiae*. (A) The filaments induced by *INT1* expression are not in a terminal physiological state. YJB5763 was grown in galactose to induce *INT1*-filaments and then immobilized onto agar containing glucose to repress *GAL1-INT1*. Images were then obtained at the times indicated. Sites of new bud growth are indicated by arrows. Bar, 10 μm. (B) *INT1*-filaments do not form in α-factor–arrested cells. DIC micrographs of YJB5763 were arrested with α-factor as described in MATERIALS AND METHODS, washed once with sterile water, and resuspended in medium containing α-factor with glucose (glu) or galactose (gal), or galactose without α-factor, as indicated. Bar, 10 μm. (C) Chitin localization is diffuse in *INT1*-induced filaments. Shown is fluorescence micrograph of strain YJB5763 expressing *INT1* and stained with Calcofluor white. Bar, 5 μm.

as multiple rings and elaborate spiral-like structures that often appeared to be connected (Figure 2, C–E) and, like the normal septin ring, were localized near the cell periphery (Figure 2, C and D, asterisks). Similar structures were observed by indirect immunofluorescence with the use of septin-specific antibodies (Figure 2H). Thus, with the use of either fusion proteins or native proteins, we detect elaborate septin structures at the periphery of the polarized buds induced by *INT1* expression. These structures were sharply distinct from the septin structures observed in other types of elongated *S. cerevisiae* cells such as *cla4*Δ cells and cells expressing high levels of *SWE1* (Sia *et al.*, 1998; Tjandra *et al.*, 1998; Longtine *et al.*, 2000) (Figure 2B). In addition, overexpression of Bud4p (the closest *S. cerevisiae* homologue of Int1p; see INTRODUCTION) only rarely (<1% of cells) induced elongated buds, and these had normal-looking septin rings at the mother-bud neck (our unpublished results).

Colocalization and Interaction of Int1p with Septins in S. cerevisiae

Induction of an Int1-GFP or an Int1p-HA fusion protein also produced many (~85% of the cells) *INT1*-filaments. Int1-GFP was first visible ~6 h after induction, when it localized primarily to the mother-bud neck (Figure 2F, left panel). After 6–8 h, elongated buds became apparent, and the majority of the Int1-GFP localized to the junction between the mother cell and the elongating bud. At later times, Int1-GFP appeared as rings and spiral-like structures similar to the septin structures in *INT1*-filaments (Figure 2F, right panel). Indeed, double-label experiments with the use of Int1-CFP and Cdc12-YFP (Figure 2G) or immunofluorescence on Int1p-HA–expressing cells (Figure 2H) revealed a high degree of colocalization of Int1p and the septins in the rings and spirals.

This colocalization suggested that Int1p may physically interact with one or more septin proteins. To test this, we immunoprecipitated septins and associated proteins from *INT1*-filaments with the use of antibodies directed against native Cdc11p or against GFP (for Cdc12-GFP). Int1p coprecipitated in both cases (although more effectively with Cdc11p), but not in a control experiment with the use of antibodies to Rap1p, an abundant nuclear protein (Figure 3). Although Int1p may have a higher affinity for Cdc11p than for Cdc12p, this result may also reflect different affinities of the anti-Cdc11p and anti-GFP antibodies used. Thus, Int1p and the septins appear to interact specifically in the "Int1p/septin spirals."

To determine whether septin function is required for the establishment and/or maintenance of the Int1p/septin spirals, we used a *cdc12–6* strain that forms normal septin rings at 23°C but delocalizes all septins after a brief incubation at 37°C (Kim *et al.*, 1991; Longtine *et al.*, 1996; Barral *et al.*, 2000). In one experiment, cells were pregrown at 37°C for 1 h before inducing *INT1* expression. Under these conditions, no bud neck or spiral localization of Int1-GFP or Cdc3-GFP was observed. Rather, both proteins appeared diffuse or formed ectopic aggregates (Figure 4A). In contrast, wild-type cells formed and maintained spirals at 37°C. In a second experiment, *INT1* was induced for 12 h at 23°C so that elongated buds and spirals were evident, and then the cells were shifted to 37°C. The Cdc3-GFP structures disappeared, and the Int1-GFP structures appeared to become fragmented (Figure 4B). Thus, both the establishment and maintenance of intact spirals required functional Cdc12p. The fragmented appearance of Int1-GFP in *cdc12–6* cells suggests that existing spirals remained partially intact in the absence of Cdc12p, perhaps because some of the Int1p within the spirals is stabilized by Int1p–Int1p interactions rather than by interactions only with the septins.

Even low levels of *INT1* expression (that generated <5% filamentous cells) could apparently produce subtle disorganization of the septins. We observed that such expression disrupted both axial and bipolar bud-site selection patterns (Table 3) as previously observed for other alterations of septin organization (Flescher *et al.*, 1993; Chant *et al.*, 1995).

Int1p/Septin Spirals Do Not Contain Other Bud-neck Associated Proteins

To determine whether expression of *INT1* affects the localization of other bud-neck–associated proteins, we analyzed the localization of Myo1-GFP, Gin4-GFP, and Hsl1-GFP (Bi

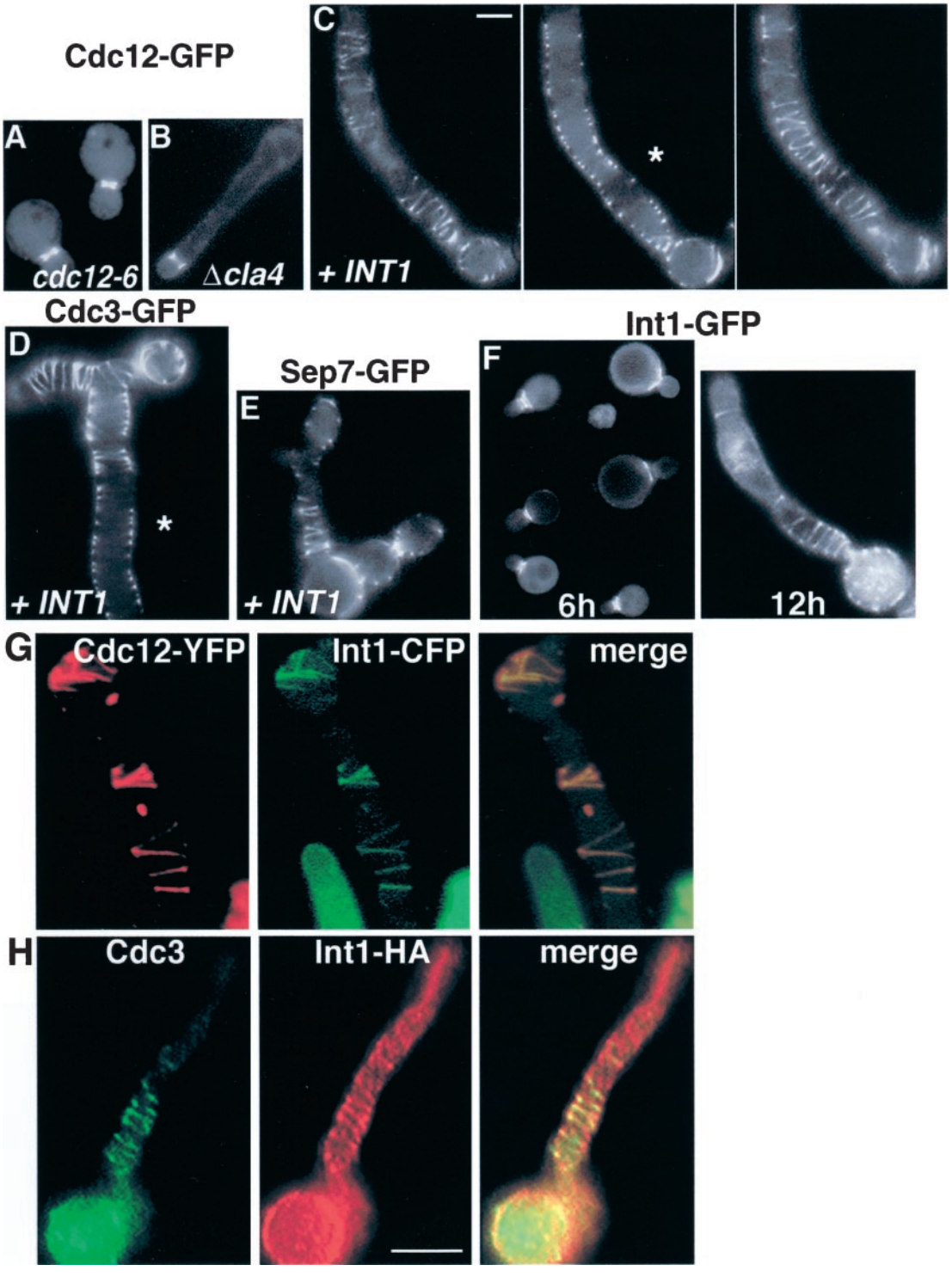

Figure 2. Septins and Int1p localize as spirals in *INT1*-filaments. Examples of images taken in the equatorial plane of a cell are noted by an asterisk (C and D). (A) YJB5001 grown in glucose and expressing Cdc12-GFP. (B) YJB3527 (*cla4*Δ) expressing Cdc12-GFP. (C) YJB5001 grown on galactose and thus expressing Cdc12-GFP and Int1p. Serial optical sections of one cell are shown. Bar (shown in C), 5 μm, is representative for A–F. (D) YJB5766 expressing Cdc3-GFP and Int1p. (E) YJB2681 expressing Sep7-GFP and Int1p. (F) YJB3362 expressing Int1-GFP for 6 or 12 h of growth on galactose, as indicated. (G) YJB5505 expressing both Cdc12-YFP and Int1-CFP after 12 h of *INT1* induction. (H) Indirect immunofluorescence micrographs of YJB3363, after 12 h of *INT1-HA* induction, with the use of anti-HA and anti-Cdc3p antibodies as indicated. Bar (shown in H), 5 μm, is representative of G–H.

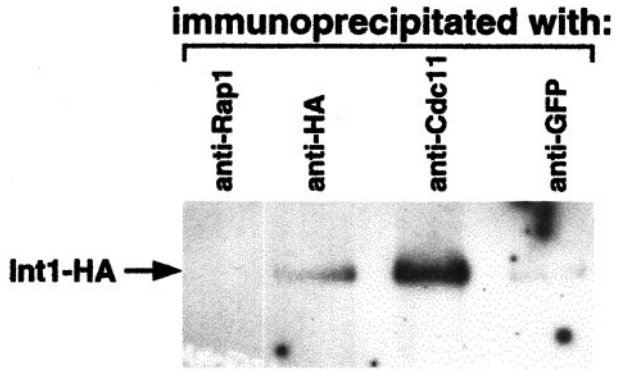

Figure 3. Int1p immunoprecipitates with Cdc11p and Cdc12p septins. Immunoblot of cell lysates from YJB3958 expressing both Int1-HA and Cdc12-GFP. Antibodies to Rap1p, HA, Cdc11p, or GFP were used to precipitate protein complexes as indicated. The precipitates were separated by SDS-PAGE, and Int1-HA was detected with anti-HA antibody.

et al., 1998; Lippincott and Li, 1998b; Barral *et al.*, 1999; Longtine *et al.*, 2000). In *INT1*-filaments, no detectable signal was observed for Myo1-GFP, Gin4-GFP, or Hsl1-GFP either at the mother-bud neck or in spirals; instead, these proteins appeared diffuse and cytoplasmic (Figure 5). In the subset of *INT1*-expressing cells that had normal buds (presumably because they did not respond to Int1p), these proteins were at the neck (Figure 5, arrows). Thus, perturbation of septin organization by *INT1* expression also disrupted the localization of Myo1p, Gin4p, and Hsl1p, consistent with previous reports that the localization of all three proteins requires normal septin structure (Bi *et al.*, 1998; Longtine *et al.*, 2000). Moreover, these results show that the Int1p/septin spirals do not contain all of the proteins normally associated with the septin ring.

The Role of Swe1p in I-*IFG and Int1p/Septin Spiral Formation*

Because alterations of the septin ring can activate the Swe1p kinase and thus produce a cell-cycle delay that results in the formation of elongated buds (Barral *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000), we asked whether Swe1p is necessary for the effects seen during *INT1* overexpression by inducing *INT1* in *swe1* and *SWE1* strains expressing *CDC12-GFP* or *INT1-GFP*. In the *swe1* strains, the proportion of cells forming polarized buds was significantly reduced but was not eliminated (<60% of the level in the *SWE1* strain). Furthermore, in some of the *swe1* cells with polarized buds, ectopic structures containing Int1-GFP and Cdc3-GFP were observed (Figure 6). In the *swe1* strain, just as in wild-type strains, the spiral-like Int1p/septin structures are observed only in cells that form elongated buds. Thus, expression of *INT1* generates polarized buds partly, but not entirely, through a Swe1-mediated delay of the cell cycle that presumably results from the alteration of septin structures; however, *SWE1* does not appear to be required for the formation of Int1/septin spirals.

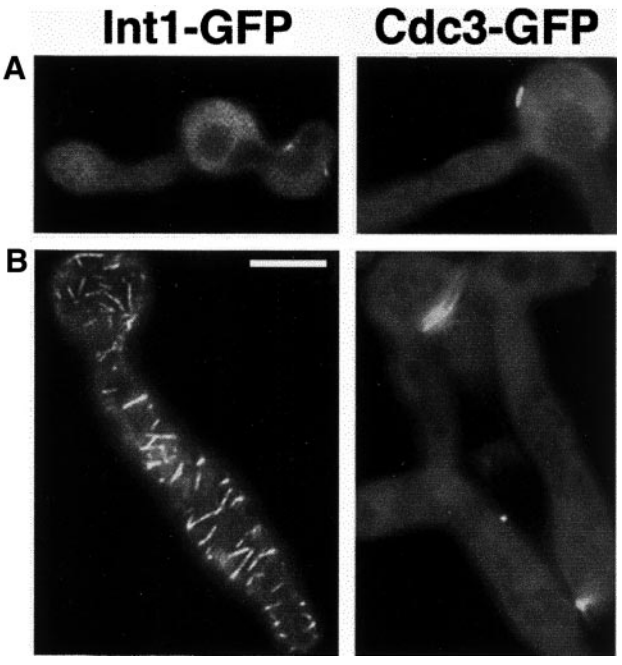

Figure 4. Cdc12p septin is required for the establishment and maintenance of spirals. Bar, 10 μm. Fluorescence micrographs of *cdc12–6* strains YJB4840 and YJB3542 expressing Int1-GFP (left) or Cdc3-GFP and Int1p (right). (A) Strains were pregrown at 37°C for 1 h to inactivate Cdc12–6p, subcultured to galactose-containing medium at 37°C to induce *INT1* expression, and photographed after 12 h of growth. (B) Strains were pregrown in galactose at 23°C to induce *INT1* and generate spirals, shifted to 37°C to inactivate Cdc12–6p, and photographed after 12 h at 37°C. Bar, 5 μm.

Localization of Int1p in C. albicans

In C. albicans, *INT1* only affects hyphal growth under a subset of hyphal induction conditions. To determine whether Int1p acts by interacting with the *C. albicans* septins, we first localized Int1p and septins in yeast, pseudohyphal, and hyphal cells by tagging one copy of *INT1* with *YFP* and one copy of *CDC3* with *CFP*. These cells formed yeast and hyphal cells at rates similar to those of the parental strains, indicating that the fluorescent protein tags did not interfere with septin or Int1p function. Both Int1-YFP and Cdc3-CFP localized to the mother-bud necks of small and large budded yeast cells (Figure 7A) and pseudohyphal cells (Figure 7C). Occasionally, we observed an Int1p signal without a corresponding Cdc3p signal on unbudded cells (Figure 7C, arrows). This may be caused by differing focal planes of localization or differing intensities of signals, or it may indicate that Int1p is present before Cdc3p at incipient bud sites. Additionally, Int1p colocalized with only one chitin-containing structure (Figure 7B; arrows indicate chitin signals without corresponding Int1p signals), suggesting that Int1p is present only at the necks of newly formed (or forming) buds.

In hyphal cells, Int1p localized to single or double rings that colocalized with Cdc3p and chitin (Figure 7, D and E) at a position distant from the junction of the mother cell and the germ tube. In general, the position of the first septin ring was observed 10–20 μm distant from the mother–daughter neck. In contrast to the solitary signal present in yeast cells,

Table 3. Effect of *INT1* expression on the bud site selection pattern of *S. cerevisiae* strains

Strain	Ploidy	Plasmid	% Axial	% Bipolar	% Random
YJB1876	Haploid	pBM272	84	14	2
YJB1875	Haploid	p*GAL1-INT1*	40	8	52
YJB1844	Diploid	pBM272	3	84	13
YJB1843	Diploid	p*GAL1-INT1*	<1	5	94

The indicated strains were grown overnight to saturation in minimal medium without uracil, lacking glucose, but containing raffinose (2%). Growth in raffinose is sufficient to induce a small amount of *INT1* expression from p*GAL1-INT1*. Cells were subcultured 1:10 into fresh medium containing galactose to fully induce *INT1* expression, grown for 5 h, and stained with Calcofluor white to visualize bud scars. A total of 200 cells was scored for each strain.

we observed Int1p and Cdc3p remaining at multiple septa within the growing hypha (Figure 7E, arrows, and unpublished observations). Thus, in *C. albicans*, septins and Int1p colocalize in a ring that becomes the site of septation and that probably corresponds to the filamentous ring observed by Soll and coworkers (Soll and Mitchell, 1983). Furthermore, the number and position of Int1p/septin ring(s) distinguish yeast/pseudohyphae from true hyphae.

Role of Int1p in C. albicans Bud Site Selection

Because Int1p localizes to septin rings and has similarity to *S. cerevisiae* Bud4p, we asked whether Int1p has a role in *C. albicans* bud site selection by comparing the bud site selection patterns of an *int1/int1* strain (CAG3), an *int1/int1::INT1* reintegrant strain (CAG5), and the parental strain (CAF2) at 28°C. CAF2 and CAG5 displayed approximately equal numbers of cells with axial and bipolar budding patterns. In contrast, the *int1/int1* strain displayed a significant reduction in axial budding and an increase in bipolar budding (Table 4) when compared with both CAF2 and CAG5. Thus, like Bud4p, *INT1* contributes to the axial bud-site selection pattern in budding *C. albicans* cells.

DISCUSSION

Int1p and Septins in S. cerevisiae

C. albicans is an asexual diploid organism that forms budding yeast and pseudohyphal cells as well as elongated hyphal cells that generally remain attached to one another. Because the molecular mechanisms of morphogenesis are better understood in *S. cerevisiae*, and because Int1p expression in *S. cerevisiae* generated a highly filamentous phenotype, we used the analysis of Int1p in *S. cerevisiae* to guide our study of Int1p function in *C. albicans*. The effects of Int1p on yeast cell shape are almost certainly distinct from the pathways regulating pseudohyphal growth in *S. cerevisiae* or hyphal growth in *C. albicans*. Indeed, in *S. cerevisiae*, *I*-IFG occurs in the absence of genes required for pseudohyphal growth (Gale *et al.*, 1996, 1998). Despite this, study of Int1p in *S. cerevisiae* provided us with insights into the potential role and function of Int1p in *C. albicans*, specifically, that Int1p interacts with the septin ring at the mother-bud neck. Although Int1p interacts with at least a subset of the septins, bud-neck proteins such as Myo1p, Gin4p, and Hsl1p do not associate with the Int1p/septin spirals. This implies that the spirals are ectopic septin structures rather than elaborate versions of essentially normal septin rings. Further study of the unusual spiral-like nature of the Int1p/septin structures in *S. cerevisiae* may provide additional information for models of septin organization.

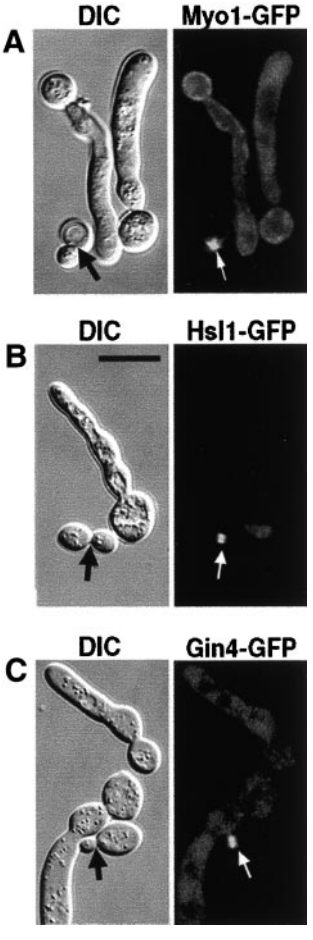

Figure 5. Expression of *INT1* alters the localization of other bud-neck–associated proteins. DIC and fluorescence micrographs of strains expressing *INT1* and Myo1-GFP (YJB5170) (A), Hsl1-GFP (YJB5174) (B), or Gin4-GFP (YJB5172) (C). Cells not forming polarized buds in response to *INT1* expression (black arrows) have normal localization of the GFP-fusion proteins at the mother-bud neck (white arrows). Bar, 10 μm.

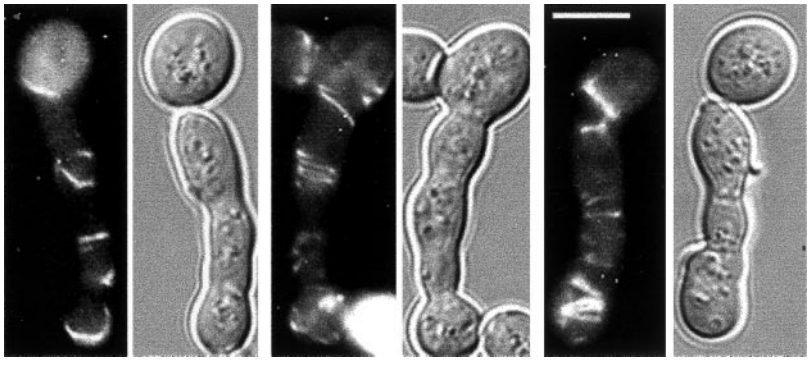

Figure 6. The formation of Int1p spirals does not require *SWE1*. DIC and fluorescence micrographs of *S. cerevisiae* strain YJB6003 (*swe1*) expressing Cdc3-GFP and Int1p are shown. Bar, 5 μm.

Despite the similarity between Int1p and Bud4p, high levels of Bud4p did not change cell morphology or septin structure in the dramatic way that Int1p expression did. Lord *et al.* (2000) reported that very high levels of Bud3p induced a proportion (~40%) of filamentous cells, some of which contained ectopic septin structures. Similarly, overexpression of the polo-like kinase Cdc5p, which may have a role at the bud neck in the initiation of cytokinesis (Lee *et al.*, 1998, 1999; Song *et al.*, 2000), results in hyperpolarized buds containing ectopic septin ring structures, although these do not appear as elaborate as the Int1p/septin spirals that we have observed. In addition, overexpression of Afr1p, a bud-neck–localized protein required for the formation of mating projections, also causes hyperpolarized bud growth (Konopka *et al.*, 1995). Thus, high-level expression of some, but not all, bud-neck components can cause hyperpolarized bud growth and formation of ectopic septin structures.

Alteration of the septin ring can activate Swe1p and thus delay the transition from polarized to isotropic growth that normally occurs during activation of Clbp/Cdc28p kinase complexes (Barral *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000). Because Swe1p contributes to *INT1*-induced filamentous growth (Asleson *et al.*, 2001), disruption of the septin ring by Int1p probably prolongs the polarized growth phase of the cell cycle via Swe1p activation; however, Sla2p, a component of actin cortical patches, contributes to *INT1*-induced filamentous growth even in the absence of Swe1p (Asleson *et al.*, 2001), indicating that the activation of Swe1p does not account for all of the polarized growth observed in cells expressing Int1p. In addition, we observed ectopic sep-

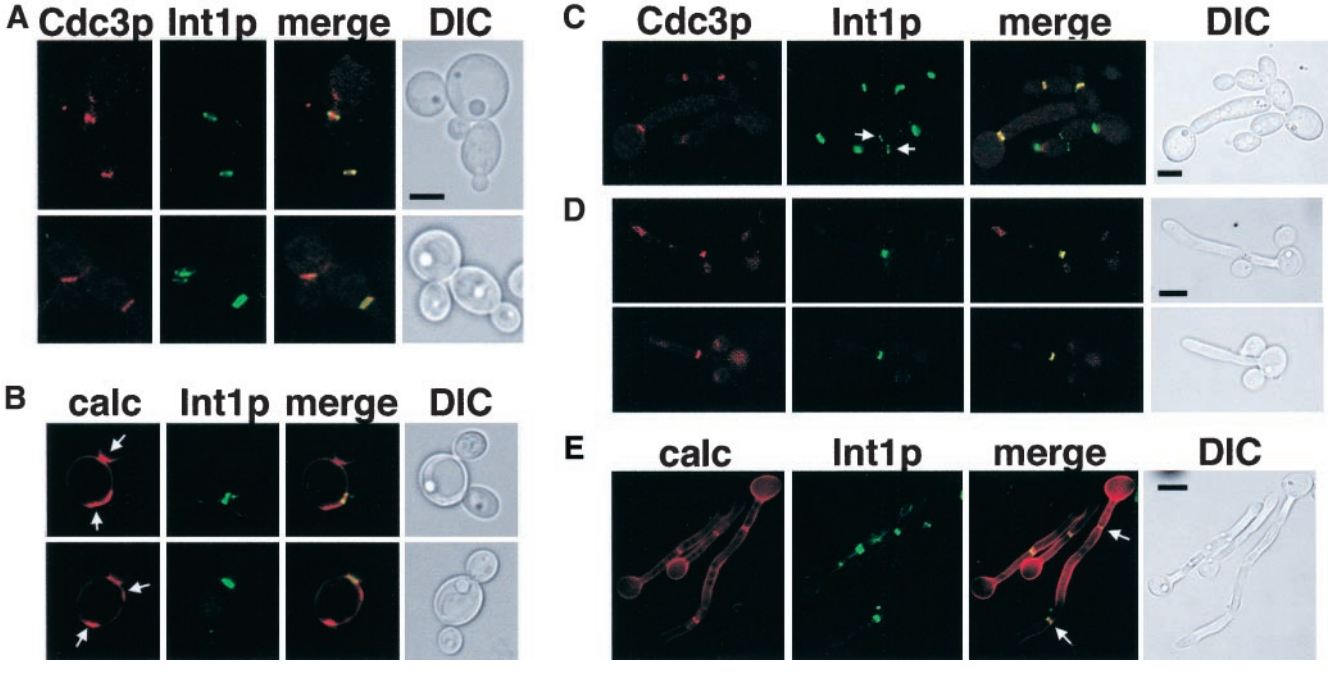

Figure 7. Colocalization of Int1p, Cdc3p septin, and septa in *C. albicans*. Bars (A–C), 5 μm; (D–E), 10 μm. YJB5850 grown at 30°C in minimal medium (A), 30°C in minimal medium with Calcofluor white (B), 37°C in YPAD to induce pseudohyphae (C), 37°C in YPAD, without shaking, to induce hyphae (D), or 37°C in YPAD, without shaking, and stained with Calcofluor white (E). Arrows, see description in RESULTS. The CFP and Calcofluor images were changed to red to facilitate merging.

Table 4. Effect of *INT1* expression on the bud site selection pattern of *C. albicans* strains

Strain (n)	Relevant genotype	% Axial	% Bipolar	% Random
CAF2 (490)	*INT1/INT1*	58	42	0
CAG3 (531)	*int1/int1*	26	73	1
CAG5 (317)	*int1/int1::INT1*	47	51	2

Strains were grown overnight to saturation in rich (YPAD) medium at 28°C and stained with Calcofluor white to detect bud scars. χ^2 analysis showed that CAF2, CAG3, and CAG5 were significantly different from each other at the $p < 0.005$ level.

tin structures in polarized *swe1* cells expressing *INT1*, indicating that *SWE1* is not required for the disruption of the septin ring by Int1p.

Int1p and Septins in C. albicans

Int1p contributes to morphogenesis in *C. albicans* (Gale *et al.*, 1998) and colocalizes with septins at the mother-bud neck of yeast and pseudohyphal cells as well as at the septa of hyphal cells. Classic studies of *C. albicans* morphogenesis suggested that the commitment to yeast versus hyphal growth is determined by the timing and position of cytokinetic structures (Mitchell and Soll, 1979a,b; Soll and Mitchell, 1983) and that the ultrastructure of septa differ in hyphal and yeast cells (Gow *et al.*, 1980). During hyphal induction, we consistently observe septa, septins, and Int1p within the growing germ tube and at a distance of 10–20 μm from the mother–daughter cell junction. This is consistent with the recent observations of others (Sudbery, 2001; Warenda and Konopka, personal communication) with the use of anti-Cdc11p antibody and septin fluorescent protein fusions, respectively, and supports the model that yeast and pseudohyphae are fundamentally different from true hyphae. Our results are also consistent with the results of Mitchell and Soll (Mitchell and Soll, 1979a,b; Soll and Mitchell, 1983) except that in our study the septin ring appears at a greater distance from the mother–daughter cell junction. This disparity may be due to the difference in hyphal-inducing medium used or the growth temperature used, or both. Interestingly, Sudbery (2001) noted a transient Cdc11p signal at the mother–daughter junction that disappeared as germ tube growth progressed. In our experiments, we did not observe such a transient signal for Cdc3p. The discrepancy between the observations may be due to a difference in visualization between the fluorescent-tagged antibody used by Sudbery (2001) and our fluorescent protein fusions or may indicate a difference in the actual localization of Cdc11p versus Cdc3p in *C. albicans* hyphae.

In *C. albicans*, *int1/int1* cells are defective in hyphal growth under some, but not all, environmental conditions (Gale *et al.*, 1998). Thus, Int1p may play an important role in a subset of the environmental sensing and signal transduction pathways that trigger hyphal growth in *C. albicans*. Because several *S. cerevisiae* proteins (e.g., Swe1p, Hsl1p, Hsl7p, Gin4p, Bud3p, Bud4p, Myo1p, and Cdc5p) that influence morphogenesis also localize to the septin scaffold (Sanders and Herskowitz, 1996; Bi *et al.*, 1998; Longtine *et al.*, 1998a, 2000; Barral *et al.*, 1999; Lord *et al.*, 2000; Song *et al.*, 2000), the colocalization of Int1p and septins in *C. albicans* suggests that Int1p influences the response to some hyphal induction conditions through its interactions with proteins in the septin ring.

Int1p affects axial bud site selection in *C. albicans*, as does Bud4p in *S. cerevisiae*, but *INT1* does not complement a *bud4*Δ strain for bud-site selection (Gale *et al.*, 1998), and high levels of Bud4p do not produce the aberrant morphology seen with Int1p overexpression. Thus, Int1p and Bud4p are not complete functional homologues. These proteins may have evolved different related functions that both occur at the cell membrane or the mother-bud neck, or both. It is notable that the only known role for Bud4p is in haploid bud-site selection, yet *BUD4* is expressed in both haploid and diploid *S. cerevisiae* cells (Sanders and Herskowitz, 1996). This suggests that Bud4p may have additional, uncharacterized functions independent of its known role in bud-site selection. Just as studies of Int1p in *S. cerevisiae* have provided insights into Int1p function in *C. albicans*, possible roles for Bud4p in diploid *S. cerevisiae* cells may be revealed by studying Int1p functions in *C. albicans*.

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