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NRG1, a repressor of filamentous growth in *C.albicans*, is down-regulated during filament induction

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In response to a variety of external signals, the fungal pathogen *Candida albicans* undergoes a transition between ellipsoidal single cells (blastospores) and filaments composed of elongated cells attached end-to-end. Here we identify a DNA-binding protein, Nrg1, that represses filamentous growth in *Candida* probably by acting through the co-repressor Tup1. *nrg1* mutant cells are predominantly filamentous under non-filament-inducing conditions and their colony morphology resembles that of *tup1* mutants. We also identify two filament-specific genes, *ECE1* and *HWP1*, whose transcription is repressed by Nrg1 under non-inducing conditions. These genes constitute a subset of those under Tup1 control, providing further evidence that Nrg1 acts by recruiting Tup1 to target genes. We show that growth in serum at 37°C, a potent inducer of filamentous growth, causes a reduction of *NRG1* mRNA, suggesting that filamentous growth is induced by the down-regulation of *NRG1*. Consistent with this idea, expression of *NRG1* from a non-regulated promoter partially blocks the induction of filamentous growth.

Keywords: *Candida albicans*/filaments/hyphae/Nrg1/Tup1

Introduction

The yeast *Candida albicans* has been recognized as a human pathogen for over a century. Although *C.albicans* is found as a commensal organism present in the human digestive tract, this yeast is also the most common human fungal pathogen, causing oral and vaginal thrush, as well as more serious mucosal and systemic infections in immunocompromised individuals (Odds, 1988, 1994a,b; Dupont, 1995; Weig *et al*., 1998). *Candida albicans* can thrive in the bloodstream and on mucosal surfaces; during systemic disease it can infect all the major internal organs. *Candida albicans* infections are especially serious in AIDS patients, patients undergoing chemotherapy, transplantation patients undergoing immunosuppression therapy and recipients of artificial joints and other prosthetic devices (for reviews see Shepherd *et al*., 1985; Dupont, 1995; Weig *et al*., 1998). Despite its importance as a pathogen, relatively little is known about *C.albicans* when compared with 'model' yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

What features of *C.albicans* make it a human pathogen? It seems likely that many of the several thousand *C.albicans* genes that are not present in *S.cerevisiae* will turn out to be involved with the interaction between *C.albicans* and its mammalian hosts (Stanford DNA Sequencing and Technology Center, http://www-sequence.stanford.edu/group/candida). Even many of the gene products that are shared between *S.cerevisiae* and *C.albicans* appear to be put to somewhat different uses; for example signaling pathways and receptors in one organism may respond to different environmental signals and produce different responses in the other organism. Because of the many differences between *S.cerevisiae* and *C.albicans* and because *C.albicans* has been adapted to grow exclusively in warm-blooded animal hosts, the term virulence factor is difficult to define rigorously for *C.albicans*. Nonetheless, work of the past 10 years has identified several features of *C.albicans* that are required for its virulence (as measured in a simple mouse tail-vein injection model), but which are not essential for growth in laboratory media. These include the capacity of *C.albicans* to grow both in a single-celled yeast form (the blastospore) and in a spectrum of filamentous forms, adhesiveness to host cells, and secretion of degradative enzymes such as proteases and phospholipases (for reviews see Odds, 1994a; Corner and Magee, 1997; Kobayashi and Cutler, 1998; Madhani and Fink, 1998; Mitchell, 1998; Brown and Gow, 1999).

In this study, we identify and characterize a gene that controls one of these virulence factors, the transition between the blastospore and filamentous forms of *C.albicans*. This transition is regulated by many different environmental stimuli including serum, nitrogen levels, pH and temperature (for recent reviews see Mitchell, 1998; Brown and Gow, 1999; Ernst, 2000). Some of the evidence that this transition is important for virulence derives from experiments showing that a mutant of *C.albicans* defective in filament formation (a *Δcph1/Δcph1 Δefg1/Δefg1* double mutant) is avirulent (Lo *et al*., 1997). Likewise, a mutant that is locked into the filamentous forms (a *Δtup1/Δtup1* strain) is also avirulent (Braun and Johnson, 1997; Braun *et al*., 2000). Moreover, a mixture of these two strains is also avirulent (our unpublished observations). In the simplest view, these experiments, taken together, suggest that the transition between the blastospore and filamentous forms is required for virulence. However, the mutations used in these experiments produce multiple phenotypes, and it is not possible at this point definitively to ascribe the lack of virulence of these strains solely to a defect in the blastospore–filament transition.

The transcriptional repressor Tup1 is a key regulator of filamentous growth in *C.albicans*, as deletion of this gene

causes constitutive filamentous growth, in addition to other, more subtle phenotypes (Braun and Johnson, 1997). Deletion of Tup1 from *S.cerevisiae* results in viable cells that display a remarkable variety of phenotypes, including flocculence, loss of cell type control in α cells, inability to sporulate, enhanced dTMP uptake, temperature sensitivity and loss of glucose repression (Lemontt *et al*., 1980; Williams and Trumbly, 1990; Keleher *et al*., 1992; Tzamarias and Struhl, 1994; Treitel and Carlson, 1995; Mizuno *et al*., 1998; reviewed by Smith and Johnson, 2000). As far as is known, each specific phenotype is based on the loss of Tup1-mediated repression of a set of genes that normally is activated only under specific environmental or internal conditions. (One exception has been reported, however, where the Tup1–Ssn6 complex may cause activation rather than repression; Conlan *et al*., 1999.) Tup1 does not bind directly to the promoters of these target genes, but rather is brought to DNA by specific DNA-binding proteins each of which controls a set of co-regulated genes. In *S.cerevisiae*, 10 DNA-binding proteins that repress through Tup1 are known: Mig1, Mig2, Rox1, α2/Mcm1, Rfx1, a1/α2, Sko1 and Nrg1 (Keleher *et al*., 1992; Deckert *et al*., 1995b; Treitel and Carlson, 1995; Tzamarias and Struhl, 1995; Huang *et al*., 1998; Lutfiyya *et al*., 1998; Park *et al*., 1999; Proft and Serrano, 1999; reviewed by Smith and Johnson, 2000). Others, such as those responsible for flocculation, have not yet been described. The pleiotropic phenotypes of the *tup1* mutant result from derepression of all of these sets of genes, and subsets of the *tup1* phenotype can be created by the deletion of one or more of these DNA-binding proteins.

The fact that Tup1 is involved in filamentous growth and virulence in *C.albicans* does not provide specific information about the targets of repression or the signals that relieve it. It does, however, predict the existence of one or more DNA-binding proteins that bring Tup1 to DNA and thereby negatively regulate genes involved in filamentous growth and virulence. The work from *S.cerevisiae* also predicts that the regulation of these genes is brought about by modification of these DNA-binding proteins rather than by changes in Tup1 itself (Lutfiyya *et al*., 1998; Treitel *et al*., 1998; for reviews see Johnston, 1999; Smith and Johnson, 2000). Here, we report the identification and characterization of one such DNA-binding protein, *C.albicans* Nrg1, which appears to account for a large part of Tup1's role in filamentous growth. Several lines of evidence suggest a model whereby filamentous growth is induced through down-regulation of the Nrg1 repressor.

Results

Identification of C.albicans NRG1

To identify DNA-binding proteins that confer Tup1 repression in *C.albicans*, we searched the partially completed *C.albicans* genome sequence for sequences similar to those of known *TUP1*-dependent DNA-binding repressors in *S.cerevisiae*. Sequence data for *C.albicans* were obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida. Based on this information, we deleted from *C.albicans* close relatives of the *S.cerevisiae* genes *MIG1* (contig no. 4-2700), *SKO1* (contig no. 4-2301) and *NRG1* (contig no. 4-3083), and assessed their phenotypes with respect to filamentous growth. *Candida albicans* is diploid, and both copies of the indicated genes were deleted for this analysis. Deletion of the *C.albicans NRG1* relative produced dramatically increased filamentous growth compared with the parent strain (Figure 2), whereas deletion of *MIG1* or *SKO1* relatives did not produce any immediately obvious phenotypes (data not shown). The *C.albicans NRG1* gene is predicted to encode a 310 amino acid, 34 kDa sequence-specific DNA-binding protein of the zinc finger category (Figure 1). *Saccharomyces cerevisiae* has two closely related Nrg genes, Nrg1 and Nrg2. Nrg1 represses target genes in the presence of glucose, and the function of Nrg2 is not yet known (Park *et al*., 1999; see Discussion). The *C.albicans* Nrg1 DNA-binding domain (60 amino acids) is slightly more similar to that of Nrg2 (68% identity) than that of Nrg1 (61% identity). However, the *C.albicans* protein had no significant sequence similarity

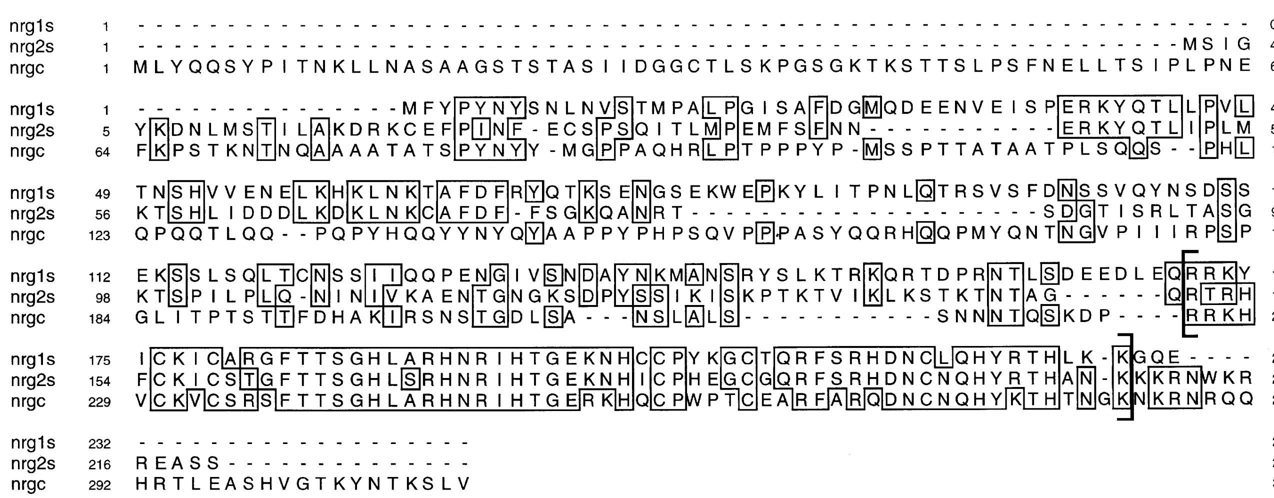

Fig. 1. Alignment of *C.albicans* Nrg1 (nrgc) with *S.cerevisiae* Nrg1 (nrg1s) and Nrg2 (nrg2s) (Park *et al*., 1999). Regions of identity are boxed. The zinc finger DNA-binding motif is shown in brackets and extends from amino acids 225 to 284 (of *C.albicans* Nrg1). Alignment was performed with Pileup in GCG 10 and prepared for display with SeqVu (Garvan Institute).

to either of the *S.cerevisiae* proteins outside its DNA-binding domain. The *C.albicans* gene had no other relatives in the *C.albicans* sequence database, so it seems likely that there is only one representative of the Nrg proteins in this organism. The near completion of the *Candida* sequencing project has so far confirmed this conclusion. Based on this information, we refer to the *C.albicans* gene as *NRG1*. The fact that the two *S.cerevisiae* proteins are more related to each other than either is to the *C.albicans* protein is consistent with the idea that duplication and divergence of these proteins probably occurred in *S.cerevisiae* after the last common *Candida–Saccharomyces* ancestor, estimated to have existed ~300 million years ago (Pesole *et al*., 1995).

nrg1 mutants exhibit a high degree of filamentous growth

Deletion of both copies of *NRG1* in *C.albicans* led to highly wrinkled, convoluted colonies of cells on YPD and on SD minimal media (Figure 2); this appearance is caused typically by a high proportion of filamentous cells (see below). These colonies also show unusual aerial projections. In contrast, the wild-type colonies were smooth under these same conditions. Microscopic examination of

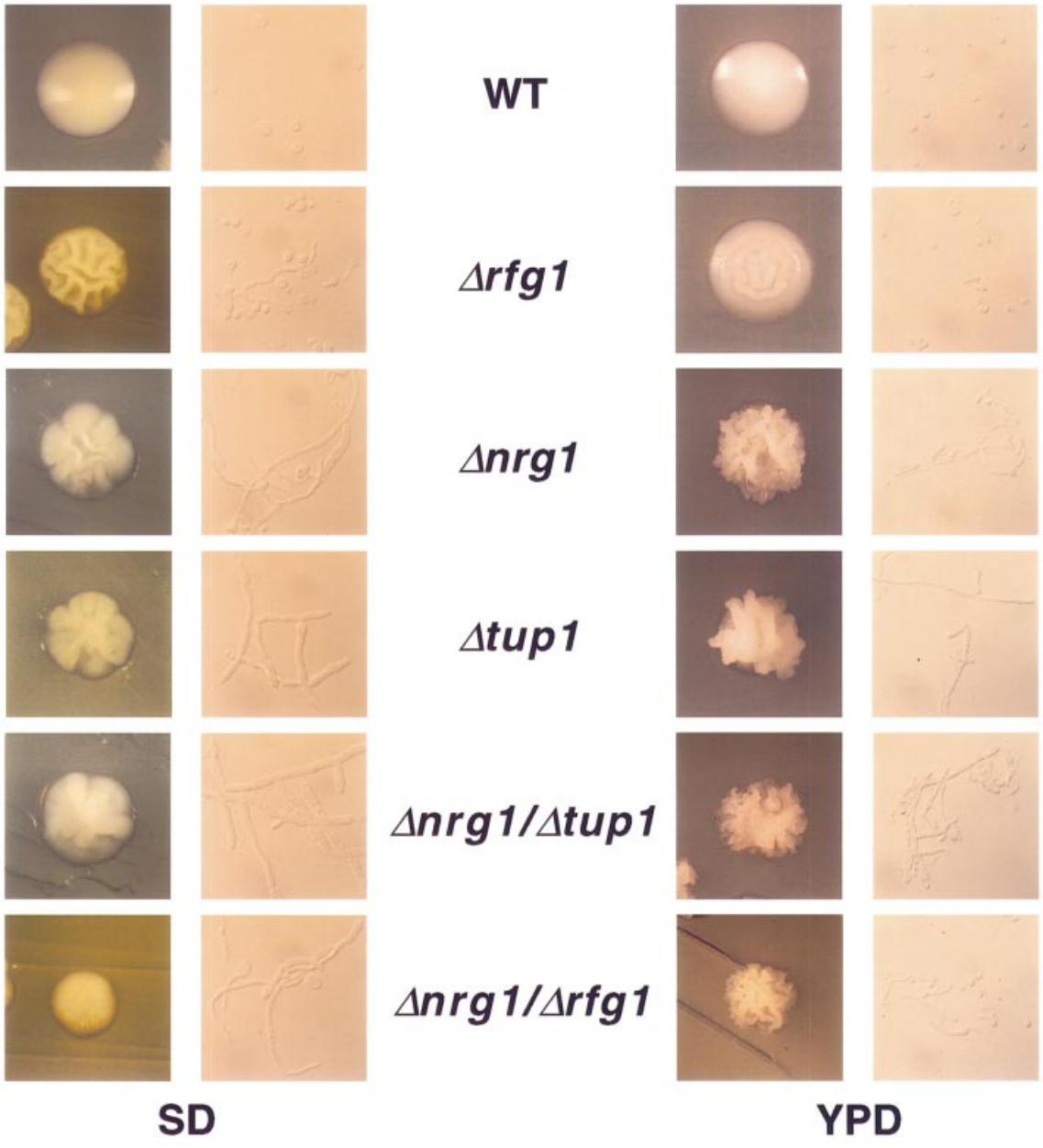

Fig. 2. Cell and colony morphology of *nrg1*, *rfg1* and *tup1 C.albicans* mutants. Strains deleted for both copies of the indicated genes were streaked on YPD and SD plates, incubated for 4 days (SD) or 3 days (YPD) at 30°C and the resulting colonies were photographed at approximately ×3 magnification (left panels). Cells corresponding to these colonies were then visualized by Nomarski optics and photographed under ×100 magnification (right panels).

colonies of the *nrg1* disruptant grown on SD and YPD showed them to contain a large number of filamentous cells, with the remainder appearing as normal blastospores (Figure 2). In contrast, wild-type cells grown on those same media for the same time periods were 100% blastospores. Growth on Spider medium, which partially induces filamentous growth in wild-type cells (50% of the cells were filamentous in this experiment), caused a complete conversion of the *nrg1* strain to filamentous growth (data not shown). We conclude from these observations that *Δnrg1* cells are predisposed to filamentous growth. Although the *Δnrg1* mutant colonies appeared similar to *Δtup1* colonies on YPD medium, the proportion of filamentous cells in the *Δtup1* strain was higher, approaching 100%.

To test the idea that Nrg1 represses filamentous growth via the Tup1 pathway, we generated a *Δnrg1/Δnrg1 Δtup1/Δtup1* double mutant. On YPD plates, this mutant appeared nearly identical to the *Δtup1/Δtup1* mutant but possessed a more highly wrinkled crown and large numbers of tiny aerial projections not found on *Δtup1/Δtup1* colonies (Figure 2). Based on these results, we believe that Nrg1 functions as a Tup1-dependent repressor, and that Nrg1 may account for a large part of the regulation of filamentous growth by Tup1. However, these results also suggest that Nrg1 may have a Tup1-independent role, perhaps as a gene activator in combination with a different protein. It is also possible that Tup1's partner protein, Ssn6 (whose homolog exists in *C.albicans*, but has not been studied to date), may be able to provide some repression in a Tup1-independent manner. Both of these possibilities have precedents in *S.cerevisiae* (Treitel and Carlson, 1995).

The Δnrg1/Δnrg1 strain appears avirulent in a mouse model

As reviewed in the Introduction, most, if not all, *Candida* mutants that are altered in the blastospore to filament transition show defects in virulence as determined in the mouse tail-vein injection model (for reviews see Mitchell, 1998; Brown and Gow, 1999; Ernst, 2000). To determine whether the correlation holds for *NRG1*, we tested the effect of deleting *NRG1* on virulence in an experiment that utilized 14 mice. Two mice were injected with saline, four with 1×10^6 cells of a wild-type strain (CAF2-1), four with 1×10^6 cells of a *Δnrg1/Δnrg1* strain and four with 3.2×10^6 cells of a *Δnrg1/Δnrg1* strain. By the end of the second day following injection, only the four mice injected with CAF2-1 had died. At the end of 30 days, all the mice injected with saline or with the *Δnrg1/Δnrg1* strain were still alive and appeared healthy. These results indicate a strong requirement for *NRG1* in a murine model of systemic candidiasis.

Nrg1 directs transcriptional repression of a subset of filament-specific genes

To investigate the *nrg1* phenotype more fully, we studied the expression of three genes whose transcription is repressed in blastospores but activated during the transition to filamentous growth. *ECE1* and *HWP1* are both Tup1-controlled filament-specific genes (Birse *et al*., 1993; Staab *et al*., 1996; Braun and Johnson, 2000; Tsuchimori *et al*., 2000), and we found that both are

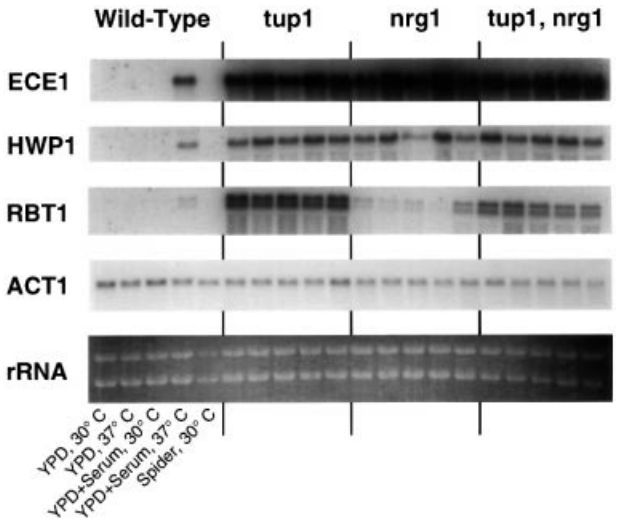

Fig. 3. Comparison of filament-specific gene expression in *nrg1*- and *tup1*-deleted cells. RNA was prepared from a wild-type strain and strains bearing homozygous deletions of *tup1*, *nrg1* and *tup1 nrg1* grown under the indicated conditions for 2 h (serum corresponds to 10% serum in YPD). Northern blots containing 5 μg of each RNA sample were then probed for expression of the indicated hyphal transcripts. An *ACT1* control as well as an ethidium-stained gel showing the large and small rRNA subunits of each sample are shown to confirm equal loading of RNA in all lanes.

constitutively expressed in the *nrg1* mutant, regardless of growth conditions (Figure 3). The level of *HWP1* and *ECE1* expression is the same in the *nrg1 tup1* double mutant as in either of the two single mutants, suggesting that Nrg1 directs transcriptional repression of these two genes via the Tup1 pathway. *RBT1*, another filament-specific gene also known to be under Tup1 control (Braun and Johnson, 2000; Braun *et al*., 2000), is only minimally, if at all, regulated by Nrg1. These results, as well as the colony morphologies (Figure 2), indicate that Nrg1 represses only a subset of the Tup1-repressed genes involved in filamentous growth, and suggest that filamentous growth in *C.albicans* is regulated by more than one DNA-binding protein that depends on Tup1 for repression activity. This situation is reminiscent of the multiple mechanisms of glucose repression in *S.cerevisiae* (Lutfiyya *et al*., 1998; Park *et al*., 1999).

Phenotypes of an nrg1 rfg1 double mutant

Two laboratories recently reported the identification of another Tup1-dependent DNA-binding protein from *C.albicans*, Rfg1, which is similar to the Rox1 protein of *S.cerevisiae* (Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001). Like Nrg1, Rfg1 represses filamentation in *C.albicans*, although the effects of an *RFG1* deletion are more subtle than those of an *NRG1* deletion. Deletion of both *RFG1* and *NRG1* from *C.albicans* led to more wrinkled and 'hairy' colony morphologies on SD medium than those seen for either deletion alone, indicating a higher proportion of cells in the filamentous form (Figure 2). Although similar, the phenotypes of the double mutant and the *tup1* mutant were not identical either in the morphology of colonies or in the complete conversion of cells from blastospore to filamentous forms. This result suggests that additional Tup1-dependent DNA-binding

repressors may be involved in the regulation of filamentous growth and that Rfg1 and Nrg1 may have additional roles.

The Nrg1 transcript is down-regulated in response to serum at 37°C

How is Nrg1-dependent repression relieved during the blastospore–filament transition? Many mechanisms have been found to regulate Tup1-dependent transcriptional repressors in *S.cerevisiae*, including phosphorylation, subcellular localization and transcriptional control (Deckert *et al*., 1995a; De Vit *et al*., 1997; Treitel *et al*., 1998; for a review see Smith and Johnson, 2000). We studied the expression of *NRG1* mRNA under various conditions that induce filamentous growth, and found that it was substantially reduced in response to growth in YPD medium with 10% fetal calf serum (FCS) at 37°C (Figure 4). The time course of this loss of *NRG1* expression (half-life of ~1 h) roughly approximates the induction of filamentation, which begins to be visible within ~45 min under the conditions used. In contrast, we found that when cells were grown in Spider medium at 37°C, levels of *NRG1* mRNA were not obviously reduced (Figure 4). Some caution is needed in interpreting this result, as Spider medium does not induce filamentous growth in 100% of the cells, and it is possible that *NRG1* mRNA levels drop in those cells that do undergo the blastospore–filament transition. Down-regulation of the *NRG1* transcript in response to serum at 37°C did not occur in strains deleted for *EFG1*, a major activator of filamentous growth. This observation suggests the possibility that one role of Efg1 during the induction of filamentous growth is the down-regulation of Nrg1. We note that Efg1 must have additional roles in controlling filamentous growth, as it can act independently of the Tup1 pathway (Braun and Johnson, 2000).

To test directly the idea that down-regulation of *NRG1* mRNA is involved in the induction of filament formation by serum at 37°C, we expressed *NRG1* constitutively from the *C.albicans ACT1* promoter. When transformed into a *Δnrg1/Δnrg1* strain, *ACT1::NRG1* fully suppressed the enhanced filamentous growth phenotype (data not shown). The *ACT1::NRG1* construct (as well as a vector control) was next integrated in a wild-type strain and the corresponding transformants were tested for their ability to undergo the transition to filamentous growth in response to serum at 37°C. As shown in Figure 5 (rows 1 and 2), a wild-type strain transformed with vector only and grown on YPD medium with 10% serum at 37°C exhibited a wrinkled colony morphology and contained a significant proportion of hyphal cells when compared with the same strain grown at 30°C in the absence of serum. In contrast, however, the *ACT1::NRG1* strain grew almost entirely in the blastospore form in both inducing (serum plus 37°C) and non-inducing (30°C) conditions (Figure 5, rows 3 and 4). These results demonstrate that unregulated (and possibly overexpressed) Nrg1 leads to a significant reduction in filamentous growth. Similar results were obtained when these strains were tested for induction by temperature alone and on Spider and cornmeal media (data not shown), indicating that the reduction of filamentous growth caused by unregulated Nrg1 is not restricted to the serum induction pathway. Northern analysis indicated that the *ACT1::NRG1 Δnrg1/Δnrg1* strain produces roughly the same level of *NRG1* mRNA as does the wild-type strain grown under non-filament-inducing conditions (data not shown).

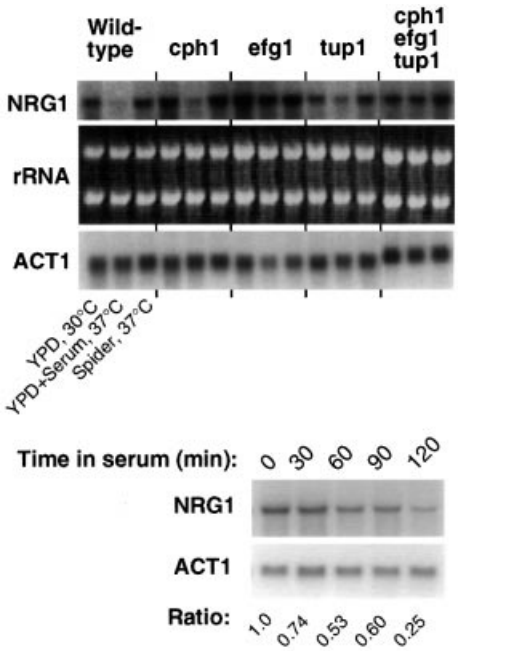

Fig. 4. Regulation of *NRG1* mRNA abundance. Wild-type or homozygous mutant cells (as marked) were grown in the media indicated and probed for *NRG1* mRNA via northern blot. Cells were grown in liquid for 1 h for YPD and YPD plus serum, and for 2 h for Spider. The only significant variation in mRNA abundance was seen after growth in 10% serum in YPD at 37°C. A time course investigating this decrease in expression is shown in the lower panel, for which cells were transferred from YPD at 30°C to YPD plus 10% FCS at 37°C and incubated with vigorous shaking for the time indicated. An *ACT1* control as well as an ethidium-stained gel showing the large and small rRNA subunits of each sample are shown to confirm equal loading of RNA in all lanes. Numbers in the lower panel indicate the ratio of *NRG1* to *ACT1* mRNA.

We next determined whether the reduction in filamentous growth caused by the unregulated *NRG1* construct was dependent on Tup1. As shown in Figure 5 (rows 5–8), *Δtup1/Δtup1* strains grown at 30°C in YPD or at 37°C in YPD plus 10% serum show constitutive hyphal growth regardless of whether they are expressing the *ACT1* or *ACT1::NRG1* constructs. These results indicate a clear requirement for Tup1 in the reduction of filamentous growth caused by unregulated Nrg1 and provide further support for the idea that in *C.albicans*, as in *S.cerevisiae*, Nrg1 carries out transcriptional repression via recruitment of the Tup1 co-repressor complex.

Discussion

The switch between the blastospore (single-celled) form and the filamentous (many cells attached end-to-end) forms of the fungal pathogen *C.albicans* has been strongly implicated in its virulence. In this study, we identify a regulator of this transition, the DNA-binding repressor Nrg1. We provide evidence that Nrg1 acts through the general co-repressor Tup1 and we identify two filament-specific genes that are repressed by Nrg1 under non-inducing conditions. Finally, we provide evidence that the

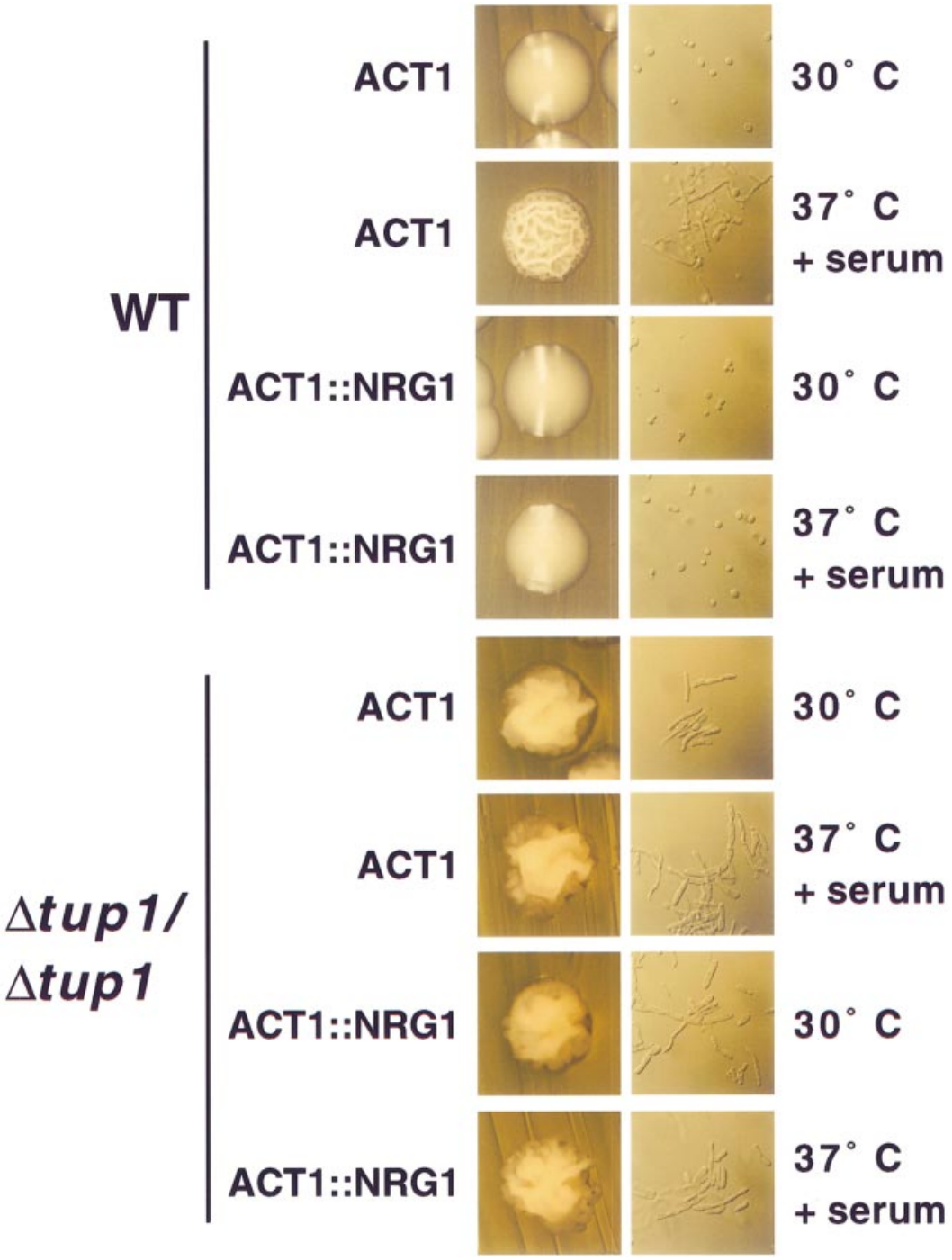

Fig. 5. Unregulated expression of Nrg1 in wild-type and Tup1 deletion strains. A construct expressing Nrg1 constitutively from a *C.albicans ACT1* promoter was integrated at the *ACT1* locus in wild-type and *Δtup1/Δtup1* strains. Strains bearing the vector only (*ACT1*) or the Nrg1 expression construct (*ACT1::NRG1*) were streaked on YPD plates and grown at 30°C for 3 days or on YPD + 10% FCS (serum) plates and grown at 37°C for 3 days (colony photographs at approximately ×3 magnification, left panel). Cells from these colonies were visualized at ×100 magnification by Nomarski optics (right panel).

down-regulation of Nrg1 synthesis is responsible, at least in part, for the induction of filamentous growth in response to environmental stimuli.

Nrg1, a DNA-binding protein that represses C.albicans filamentous growth via the Tup1 pathway

Nrg1 and Rfg1 (another DNA-binding repressor protein) each have roles in regulating filamentous growth in *C.albicans*. They are similar in sequence to well-characterized DNA-binding proteins in *S.cerevisiae* that work through the general co-repressor Tup1. Here, we present four lines of evidence that directly implicate Tup1 in repression by Nrg1 in *C.albicans*: (i) *nrg1* mutants are highly filamentous on non-inducing medium and appear similar (but not identical) to *tup1* mutants; (ii) several filament-specific genes repressed by Nrg1 were identified and shown to be a subset of those genes repressed by Tup1;

(iii) the transcripts of these genes are derepressed to the same extent in the *nrg1 tup1* double mutant as in either of the two single mutants; and (iv) an *ACT1::NRG1* construct inhibits *C.albicans* filamentous growth in a Tup1-dependent manner.

Comparisons between mutant strains (see Results) suggest that Nrg1 repression may account for a large part of the overall Tup1 phenotype. In contrast, *rfg1* mutants are less filamentous than *nrg1* strains and their colonies contain a smaller percentage of hyphal cells. While Rfg1 and Nrg1 may each regulate a subset of filament-specific genes, it seems likely that additional DNA-binding proteins (not yet identified) are also important for Tup1 repression of filamentous growth. Consistent with this idea, cells of the *rfg1 nrg1* double mutant are not 100% hyphal, as are *tup1* cells.

The observation that different Tup1-dependent DNA-binding proteins can regulate genes involved in the same process has precedents in *S.cerevisiae*. For example, Nrg1 of *S.cerevisiae* represses *STA1*, which encodes a starch-degrading enzyme, in the presence of glucose (Park *et al*., 1999). It has sequence similarity to several other *S.cerevisiae* proteins, including Mig1, Mig2 and Yer028, all of which are also glucose-dependent repressors that act by recruitment of Tup1 to promoters of target genes (Treitel and Carlson, 1995; Tzamarias and Struhl, 1995; Lutfiyya *et al*., 1998). Mig1 and Mig2 act on the same set of genes, although the way in which their repression is released is different. Mig1 is phosphorylated by Snf1 and excluded from the nucleus in the absence of glucose, while Mig2 is unaffected by Snf1, is constitutively nuclear and is turned into an active repressor in the presence of glucose by an unknown mechanism (Lutfiyya *et al*., 1998; Treitel *et al*., 1998; for a review see Johnston, 1999). Yer028 is known to be a glucose-dependent and Tup1-dependent repressor on the basis of LexA fusion protein experiments (Lutfiyya *et al*., 1998), but its target gene(s) and mechanism of glucose regulation are not known.

A model for induction of filamentous growth by serum at 37°C via the Nrg1–Tup1 pathway

The results presented in this paper provide insight into at least one mechanism by which growth in serum at 37°C induces filamentous growth in *C.albicans*. Under non-inducing conditions, Nrg1 functions as a strong Tup1-dependent repressor of a set of filament-specific, serum-inducible genes (Figure 3). In addition, the *NRG1* transcript is itself regulated by serum at 37°C. Growth of blastospores in the presence of serum at 37°C rapidly induces filamentous growth, and the level of *NRG1* mRNA decreases during this induction (Figure 4). When *NRG1* is ectopically expressed from the actin promoter, it inhibits the blastospore–filament transition (Figure 5), consistent with the idea that Nrg1 expression normally is down-regulated as a part of the filamentous growth induction process.

These results suggest a model where, in the absence of inducing conditions, *NRG1* is expressed and functions as a Tup1-dependent transcriptional repressor of a subset of filament-specific genes. In the presence of serum at 37°C, *NRG1* transcript levels are down-regulated and, as a consequence, a set of filament-specific genes are derepressed and cells undergo the transition from blastospores to filaments (Figure 6). At this point, we do not know whether transcription of the *NRG1* gene is repressed or whether *NRG1* mRNA is degraded during induction of filamentous growth, or both. Given that *Δnrg1* mutants are only partially filamentous, it seems likely that *NRG1* down-regulation is only one of several mechanisms by which filamentous growth is induced.

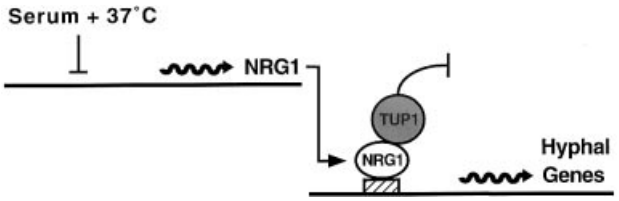

Fig. 6. A model for induction of hyphal-specific genes by Nrg1 in the presence of serum at 37°C. In the absence of serum and at 37°C, Nrg1 is expressed and binds to the promoters of a subset of hyphal-specific genes. Nrg1 functions as a major transcriptional repressor of these genes via recruitment of the Tup1 co-repressor complex. In the presence of serum and at a temperature of 37°C, the levels of Nrg1 transcript are reduced (by a mechanism that is not yet determined). As a consequence, Nrg1 protein levels fall and hyphal transcripts are derepressed. This model does not exclude the possibility that other serum- and temperature-dependent transcriptional regulators may regulate identical or overlapping sets of hyphal-specific target genes.

Regulation of filamentous growth in C.albicans by condition-specific DNA-binding proteins

Candida albicans undergoes the transition from blastospore to filament forms in response to a wide variety of environmental conditions including neutral pH, serum, high temperature (37°C), high CO_2/O_2 ratio and starvation (Gow and Gooday, 1984; Odds, 1985, 1988). The finding that Nrg1, a strong transcriptional repressor of filament-specific genes, is specifically down-regulated in response to growth in serum at 37°C supports recent observations that different DNA-binding proteins regulate transcription of hyphal genes in response to different environmental conditions. For example, the Prr2 regulatory protein induces alkaline-specific transcripts and represses acid-specific transcripts at neutral pH (Ramon *et al*., 1999; Davis *et al*., 2000). Efg1, an APSES domain basic helix–loop–helix (bHLH) DNA-binding protein, has been shown to regulate induction of *C.albicans* filamentous growth in response to serum, *N*-acetyl glucosamine (GlcNAc) and starvation; Efg1 is believed to be the downstream target of a cAMP/PKA signaling pathway (Lo *et al*., 1997; Stoldt *et al*., 1997; Ernst, 2000). Czf1, a zinc finger DNA-binding protein, is important for filamentous growth induction specifically in the presence of micro-aerophilic, or embedded, conditions (Brown *et al*., 1999).

These findings all suggest that induction of filamentous growth in *C.albicans* is a complex process involving multiple DNA-binding proteins that respond to a wide variety of environmental conditions. Moreover, it is becoming increasingly apparent that each different DNA-binding protein regulates a different subset of filament-specific genes (Murad *et al*., 2001). This observation may, in part, explain how a wide variety of filament types can be generated by *C.albicans* in response to different environmental conditions.

Materials and methods

Strains and plasmids

Deletion of *NRG1* was performed by the *URA* blaster technique described previously (Fonzi and Irwin, 1993; Braun *et al*., 2000), starting from the strain CAI4 (Fonzi and Irwin, 1993), creating heterozygous strain BCa23-1 and homozygous strain BCa23-3. Short DNA segments flanking the *NRG1* open reading frame (ORF) were synthesized using the following primers: 5′-GTATAAAGGCATGCAGATTTCCCTCT-3′ (*Sph*I), 5′-TGATTGTTGGATCCTTAATGAAACT-3′ (*Bam*HI), 5′-CAAGAG-CCTAGTATGCATGTGGTCAA-3′ (*Nsi*I) and 5′-TTTTTGGGGTAC-CCAAGAAATAATTGC-3′ (*Asp*718), and placed on either side of a *URA3* marker gene from pBB510 (Braun and Johnson, 2000) to create the disruption constructs pBB571A and pBB571B. The transforming DNA fragment was released by cleaving these constructs with *Sph*I and *Asp*718, followed by transformation into CAI4 or heterozygous BCa23-1 cells, respectively. PCR was used to verify each junction of the new locus and the removal of the native locus. These disruptions removed the entire coding region of *NRG1*, from 10 bp before the ATG, to the T of the TAG stop codon. Disruption of *RFG1* has been described previously (Kadosh and Johnson, 2001). Sequence data for *C.albicans* were obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida.

The *ACT1::NRG1* expression construct was generated as follows: a PCR fragment containing the entire *NRG1* ORF (flanked by an upstream *Xho*I site and a downstream *Bam*HI site) was cloned into the pDK1 *ACT1* expression vector cut with *Xho*I and *Bam*HI (pDK1 was generated by cloning a 3.4 kb *Sma*I–*Avr*II restriction fragment containing *rahB–URA3–rahB* repeats into *ACT1* expression vector pAU34; Uhl and Johnson, 2001). The resulting construct (p576) was then cut in the *ACT1* promoter DNA with *Bsr*GI to direct integration to the native *ACT1* locus. Positive transformants were verified by PCR.

Growth conditions and RNA preparation

Cells were grown routinely on YPD and SD minimal selective medium at 30°C (Guthrie and Fink, 1991). Cells were tested for filamentous growth on YPD, Spider agar, liquid Spider medium and liquid YPD + 10% FCS at both 30 and 37°C, as described previously (Braun and Johnson, 2000). The wild-type strain used was CAF2-1 (Fonzi and Irwin, 1993), which carries one copy of *URA3*, as do all of the disruptant strains.

For RNA analysis, cells were grown overnight to OD_{600} ~4.0, rediluted to OD_{600} ~0.5 in either inducing or non-inducing medium and grown at either 30 or 37°C for the times indicated. RNA was prepared by the hot phenol method (Guthrie and Fink, 1991; Ausubel *et al*., 1992) and 5 µg were electrophoresed on formaldehyde gels before capillary transfer to Gene Screen nylon membranes. Transfer and loading were monitored by ethidium bromide staining. Northern blots were hybridized as described previously (Church and Gilbert, 1984) at 67–70°C. The *NRG1* hybridization probe was made from a PCR fragment covering the entire gene (5′-CAATTTGGATCCCATCTATACTAGGC-3′ to 5′-GGCCTC-GAGCATTATGCTTTATCAACAATCA-3′). The *RBT1*, *HWP1* and *ECE1* probes, comprising unique portions of their ORFs, have been described previously (Braun and Johnson, 2000). Quantitation of *NRG1* mRNA was performed by phosphoimager.

Virulence experiments

General procedures were as described in Braun *et al*. (2000). *Δnrg1/Δnrg1* and wild-type cells were grown overnight to ~OD_{600} 1.0 in YPD (wild-type cells were back-diluted once from OD_{600} 1.0 to OD_{600} 0.2 to allow the slower growing *nrg1* cells to catch up). At this point, the larger (uninjectable) clumps were allowed to settle out briefly and the suspended cells were collected, rinsed twice in phosphate-buffered saline and counted on the hemocytometer. This sample consisted of small clumps of 1–50 pseudohyphal cells. Due to the filamentous nature of the *Δnrg1/Δnrg1* cells, their OD_{600} and cell counts were less reliable than those of wild-type cells. For this reason, we performed the *nrg1* injections with both 1×10^6 and 3.2×10^6 cells, for comparison with wild-type cells at 1×10^6 cells per mouse. Injections were performed on female Balb/c mice (8–10 weeks old; Charles River Co., Cambridge, MA) and survival was monitored over a period of 30 days.

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