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Snf1 Protein Kinase and the Repressors Nrg1 and Nrg2 Regulate *FLO11*, Haploid Invasive Growth, and Diploid Pseudohyphal Differentiation

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The Snf1 protein kinase of *Saccharomyces cerevisiae* is important for many cellular responses to glucose limitation, including haploid invasive growth. We show here that Snf1 regulates transcription of *FLO11*, which encodes a cell surface glycoprotein required for invasive growth. We further show that Nrg1 and Nrg2, two repressor proteins that interact with Snf1, function as negative regulators of invasive growth and as repressors of *FLO11*. We also examined the role of Snf1, Nrg1, and Nrg2 in two other Flo11-dependent processes. Mutations affected the initiation of biofilm formation, which is glucose sensitive, but also affected diploid pseudohyphal differentiation, thereby unexpectedly implicating Snf1 in a response to nitrogen limitation. Deletion of the *NRG1* and *NRG2* genes suppressed the defects of a *snf1* mutant in all of these processes. These findings suggest a model in which the Snf1 kinase positively regulates Flo11-dependent developmental events by antagonizing Nrg-mediated repression of the *FLO11* gene.

The budding yeast *Saccharomyces cerevisiae* responds to a variety of different environmental signals by altering its gene expression, metabolism, and morphology. Diploid pseudohyphal differentiation and haploid invasive growth are related but distinct cell type-specific developmental processes that occur in response to distinct nutrient limitation signals (6, 16, 22).

Pseudohyphal differentiation is a response of diploid cells to nitrogen limitation (8). Cells assume an elongated morphology, change their budding pattern, and generate chains of filamentous-form cells projecting from the main colony of yeast-form cells. At the molecular level, one of the key events is transcriptional activation of the *FLO11* (*MUC1*) gene, which encodes a cell-surface glycoprotein with roles in cell-cell adhesion and adherence to surfaces (15, 17, 18). *FLO11* has a large and complex promoter, and its transcription is regulated by at least two pathways: the cyclic AMP-dependent protein kinase A (PKA) pathway, which acts through the transcriptional activator Flo8 and the repressor Sfl1, and the Kss1 mitogen-activated protein kinase (MAPK) cascade, which regulates the activator Ste12-Tec1 (17, 21, 26, 31, 33).

Haploid invasive growth is a related process that similarly requires the Flo11 flocculin (15, 17, 30). Haploid cells elongate, alter their budding pattern, and invade the agar during growth on rich medium. Agar invasion does not initiate immediately but rather occurs after several days, suggesting that limitation for a nutrient triggers this process. Haploid invasive growth requires the same regulatory pathways as pseudohyphal differentiation, namely the PKA and MAPK pathways (30, 31); however, invasive growth occurs in response to limitation for glucose, not nitrogen (3). As is the case with many glucose limitation-induced processes, invasive growth also depends on the Snf1 protein kinase (3).

Finally, a distinct type of pseudohyphal and invasive growth occurs in haploids and diploids that lack the forkhead transcription factors Fkh1 and Fkh2, which control the CLB2 cluster of cell cycle-regulated genes (10, 39). This pseudohyphal growth is constitutive, rather than induced by nutrient starvation, and does not require *FLO11* or *STE12*.

The Snf1 kinase is conserved in fungi, plants, and animals (AMP-activated kinase in mammals) and has broad roles in transcriptional and metabolic responses to cellular stress (9, 13). In *S. cerevisiae*, the Snf1 kinase is primarily required for adaptation to glucose limitation but has also been implicated in other stress responses (2, 7). The dependence of haploid invasive growth on both the Snf1 kinase and the *FLO11* gene suggested that Snf1 regulates *FLO11* in response to glucose limitation. In support of this idea, *FLO11* transcription increases during the postdiauxic phase (33) and during growth on poor carbon sources (5), when the Snf1 kinase is active. In addition, the *FLO11* promoter is nearly identical over 3.9 kb to that of a Snf1-dependent gene, the glucoamylase gene *STA2* (5, 14).

The similarity of *FLO11* to *STA2* further suggested a role for the zinc finger repressor proteins Nrg1 and Nrg2, which interact physically with Snf1 (37). Nrg1 and Nrg2 contribute to glucose repression of several Snf1-dependent genes, including *SUC2*, *GAL*, and *DOG2* (37, 38). Most importantly, Nrg1 plays a major role in glucose repression of *STA* genes (27) and the Nrg1 binding site is conserved in *FLO11*.

Here, we have explored the role of the Snf1 kinase pathway in regulating invasive growth. We present evidence that Snf1 regulates transcription of the *FLO11* gene in response to glucose depletion. We further show that Nrg1 and Nrg2 negatively regulate *FLO11* and invasive growth. Finally, we present genetic evidence that Snf1, Nrg1, and Nrg2 also affect two other

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
MY1401	*MAT*α *ura3*Δ *leu2*Δ *his3*Δ	Microbia
MY1402	*MAT***a** *ura3*Δ *leu2*Δ *trp1*Δ	Microbia
MCY4460	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *snf1*::*LEU2*	This study
MCY4461	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *snf1*::*LEU2 reg1*Δ::*URA3*	This study
MCY4462	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *reg1*Δ::*URA3*	This study
MCY4463	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *snf1*::*LEU2 nrg1*Δ::*His3MX6*	This study
MCY4464	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *nrg1*Δ::*His3MX6*	This study
MCY4465	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *nrg2*Δ::*His3MX6*	This study
MCY4466	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *snf1*::*LEU2 nrg2*Δ::*His3MX6*	This study
MCY4467	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *snf1*::*LEU2 nrg1*Δ::*His3MX6 nrg2*Δ::*His3MX6*	This study
MCY4468	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *nrg1*Δ::*His3MX6 nrg2*Δ::*His3MX6*	This study
MCY4469	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *reg1*Δ::*URA3 flo11*Δ::*His3MX6*	This study
MCY4470	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ *flo11*Δ::*His3MX6*	This study
MCY4471	*MAT***a** *ura3*Δ *leu2*Δ *his3*Δ	This study
MCY4472	MY1401 × MY1402	This study
MCY4473	*MAT***a**/*MAT*α *ura3*Δ/*ura3*Δ *leu2*Δ/*leu2*Δ *HIS3*/*his3*Δ *TRP1*/*trp1*Δ *snf1*::*LEU2*/*snf1*::*LEU2*	This study
MCY4474	*MAT***a**/*MAT*α *ura3*Δ/*ura3*Δ *leu2*Δ/*leu2*Δ *his3*Δ/*his3*Δ *nrg1*Δ::*His3MX6*/*nrg1*Δ::*His3MX6* *nrg2*Δ::*His3MX6*/*nrg2*Δ::*His3MX6*	This study
MCY4475	*MAT***a**/*MAT*α *ura3*Δ/*ura3*Δ *leu2*Δ/*leu2*Δ *his3*Δ/*his3*Δ *TRP1*/*trp1*Δ *snf1*::*LEU2*/*snf1*::*LEU2* *nrg1*Δ::*His3MX6*/*nrg1*Δ::*His3MX6 nrg2*Δ::*His3MX6*/*nrg2*Δ::*His3MX6*	This study

Flo11-dependent processes, the initiation of biofilm formation and diploid pseudohyphal differentiation. The genetic and physical relationships between Snf1 and Nrg proteins lead us to propose a model in which Snf1 positively regulates Flo11-dependent developmental processes by antagonism of Nrg-mediated repression of *FLO11*.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains are listed in Table 1. All strains were in the Σ1278b genetic background and were derived from the isogenic strains MY1401 and MY1402 of the Sigma2000 series (Microbia, Cambridge, Mass.). The alleles *snf1*::*LEU2* (11) and *reg1*Δ::*URA3* (35) were introduced into the diploid MY1401 × MY1402 by transformation using standard methods (32). To construct *nrg1*Δ::*His3MX6*, the *His3MX6* sequence (19) was amplified by PCR with oligonucleotide primers flanking the *NRG1* open reading frame, and the PCR product was used to transform MY1401. The *nrg2*Δ::*His3MX6* allele (37) was also introduced into MY1401. Combinations of these alleles were then obtained by genetic crossing, and genotypes were established by mutant phenotypes and by using the PCR for analysis of genomic DNA. A PCR product containing the *flo11*Δ::*His3MX6* sequence, amplified as above, was used to transform a *reg1*Δ::*URA3*/*REG1* diploid, and segregants were recovered by tetrad analysis.

Rich medium was yeast extract-peptone (YEP), and synthetic complete (SC) medium lacking appropriate supplements was used to maintain selection for plasmids (32).

Invasive growth assay. The plate washing assay described by Roberts and Fink (30) was modified as follows. Cells were spotted or replica plated onto YEP–2% glucose plates containing 2.5% agar and incubated at 26°C for 3 to 4 days. Plates were photographed, washed under a stream of distilled water by rubbing either with a gloved finger or with a cell spreader made from a smooth glass rod, and then photographed again.

Northern blot analysis. Preparation of RNAs and Northern blot analysis were performed as described previously (14), with minor modifications. ^{32}P-labeled probes were prepared from PCR products containing the *ACT1* gene or the first 208 codons of *FLO11* by using the Oligolabelling kit (Pharmacia LKB).

Assay for plastic adherence. Assays for adherence to the wells of a polystyrene 96-well microtiter plate (Falcon Microtest flat bottom plate, catalog no. 35-1172; Becton-Dickinson Labware) were carried out as described previously (29). Cells were grown in SC–2% glucose to an optical density at 600 nm (OD_{600}) of 0.5 to 1.5, collected, washed, and resuspended to OD_{600} of 1 in SC with 2% or 0.1% glucose. Cells (0.1 ml) were transferred to the wells of a microtiter plate and incubated at 30°C for 1 to 6 h. The cells were then stained with crystal violet, and the wells were washed repeatedly with water.

RESULTS

Snf1 regulates the *FLO11* gene in response to glucose limitation. To explore the role of the Snf1 kinase pathway in the regulation of *FLO11* by glucose, we first examined the effect of a *snf1* mutation on *FLO11* expression. Isogenic wild-type and *snf1* mutant strains of the Σ1278b background were grown to mid-log phase in rich medium containing 2% glucose and then shifted to 0.05% glucose. In high glucose, the *FLO11* mRNA level was slightly lower (typically twofold) in the *snf1* mutant relative to that in the wild type (Fig. 1A and data not shown). In response to glucose limitation, the *FLO11* mRNA level increased dramatically in the wild type but showed only a modest increase in the *snf1* mutant. Thus, Snf1 is required for normal *FLO11* expression. Consistent with the partial derepression of *FLO11*, a *snf1* mutant exhibits a leaky invasive growth defect, as agar invasion can be observed after prolonged growth (data not shown).

To assess the effect of constitutively elevated Snf1 activity on *FLO11* expression and agar invasion, we examined a *reg1* mutant. Reg1 is a targeting subunit of protein phosphatase 1 (35), and evidence indicates that it directs the phosphatase to inhibit Snf1 (20, 23, 34). In the absence of Reg1, the Snf1 kinase is constitutively activated, leading to glucose-insensitive expression of many glucose-repressible, Snf1-dependent genes.

Deletion of *REG1* resulted in greatly increased expression of *FLO11* in glucose-grown cells (Fig. 1B). This effect was abolished in a *reg1 snf1* double mutant, confirming the involvement of Snf1. The *reg1* mutants also invaded agar much more efficiently than the wild type, and this phenotype was dependent on *SNF1* (Fig. 1B). To rule out the possibility that the *reg1* hyperinvasive phenotype is due to the unmasking of a *FLO11*-independent mechanism, we constructed *reg1 flo11* double mutants. The *reg1 flo11* mutants did not invade agar (Fig. 1B), even after prolonged growth (3 weeks; data not shown), strongly supporting a primary role for *FLO11*. These findings indicate that upregulation of the

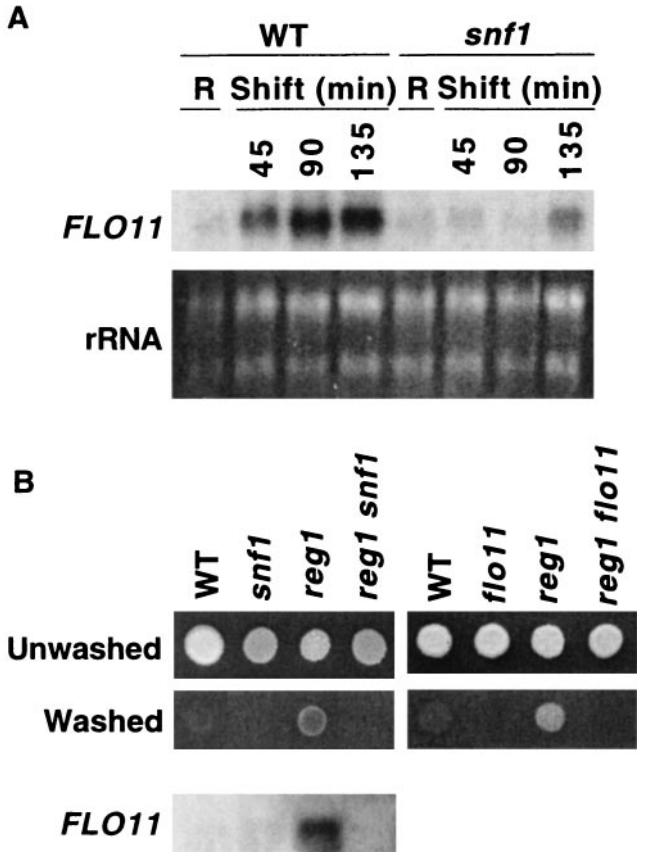

FIG. 1. Snf1 kinase regulates *FLO11* expression. (A) Wild-type (WT) and *snf1* mutant strains (MCY4460 and MCY4471) were grown to mid-log phase at 25°C in YEP–2% glucose (glucose repressed, R) and then shifted to YEP–0.05% glucose for the indicated times. Total RNAs were prepared and fractionated on a 0.8% agarose–formaldehyde gel, and the *FLO11* mRNA was detected by Northern blot analysis. Prior to membrane transfer, the gel was stained with ethidium bromide to visualize the rRNA, which served as a loading control. (B) Deletion of *REG1* increases invasion and *FLO11* expression. Cells were assayed for invasive growth as described in Materials and Methods. After 3 to 4 days of incubation at 26°C, plates were photographed, washed, and photographed again; at this temperature, wild-type cells require 5 to 6 days for substantial invasion. Strains were also grown in YEP–2% glucose to mid-log phase, and *FLO11* mRNA levels were assessed by Northern blot analysis. Visualization of the rRNA confirmed uniform sample loading (data not shown).

Snf1 pathway results in increased *FLO11* expression and Flo11-dependent agar invasion.

Together, these analyses of *snf1* and *reg1* mutants strongly suggest that Snf1-dependent regulation of *FLO11* is a major mechanism by which glucose levels control invasive growth in haploid yeast.

Genetic evidence that Nrg1 and Nrg2 function downstream of Snf1. What are the downstream effectors that convey the regulatory input from Snf1 to the *FLO11* promoter? The Nrg1 and Nrg2 repressor proteins seemed to be likely candidates for this role. Both proteins interact physically with Snf1 and contribute to repression of various Snf1-dependent genes (37, 38). Most significantly, Nrg1 plays a major role in glucose repression of the *STA* genes, and the promoter of *FLO11* is nearly identical to that of the *STA* genes with the Nrg1 binding site conserved (5, 15, 27). Another possible candidate, the repressor Mig1, has already been shown to be dispensable for repression of *STA2* (12).

TABLE 2. Deletion of *NRG1* and *NRG2* partially suppresses the *snf1* mutant defect in *STA2-lacZ* expression[a]

Relevant genotype	Units of β-galactosidase activity in:	
	2% glucose	0.05% glucose
Wild type	0.1	37
nrg1	8.1	120
nrg2	0.7	62
nrg1 nrg2	22	190
snf1	0.1	0.1
snf1 nrg1	4.0	3.1
snf1 nrg2	0.4	0.5
snf1 nrg1 nrg2	7.0	8.0

[a] Strains with the indicated genotypes were transformed with pLCLG-Staf, a centromeric plasmid carrying *STA2-lacZ* (12). Transformants were grown to mid-log phase in selective SC–2% glucose and shifted to SC–0.05% glucose for 4 hr. β-Galactosidase activity levels were assayed in permeabilized cells and are expressed in Miller units, as described previously (37). Values are averages of activity levels for three transformants, and standard errors were <15%.

To investigate the epistatic relationship of the *nrg1* and *nrg2* mutations to *snf1*, we assayed expression of a *STA2-lacZ* promoter fusion on a centromeric plasmid (12) (Table 2). In the wild type, *STA2-lacZ* expression was strongly derepressed in response to glucose limitation (0.1 U of β-galactosidase activity in high glucose and 37 U in low glucose) whereas a *snf1* mutant was defective in derepression (0.1 U). The *nrg1* and *nrg2* mutations together not only relieved glucose repression in the wild type but also substantially restored *STA2-lacZ* expression in the *snf1* mutant (7 U in high glucose and 8 U in low glucose). These data indicate that Snf1 antagonizes repression of *STA2* by Nrg1 and Nrg2 and support the view that these repressors are targets of regulation by Snf1 at the *FLO11* promoter.

Nrg1 and Nrg2 are negative regulators of invasive growth and *FLO11*. We examined the effects of Nrg1 and Nrg2 on invasive growth. The *nrg1 nrg2* double mutant was hyperinvasive, but the single mutations did not noticeably affect invasive growth, implying that each repressor alone is sufficient (Fig. 2A). Moreover, overexpression of Gal4 activation domain (GAD) fusions to Nrg1 and Nrg2 also caused increased invasiveness (Fig. 2B); we previously showed that fusion to an activation domain converts these repressors into activators of a target gene (37). The hyperinvasive phenotype caused by the GAD-Nrg proteins was dependent on *FLO11* (Fig. 2B), and no invasiveness was detected for the *flo11* mutant even after prolonged growth (data not shown).

Analysis of *FLO11* mRNA levels showed that the *nrg1* and *nrg2* single mutations caused no significant change, but *FLO11* expression was elevated in the *nrg1 nrg2* double mutant compared to that in the wild type (Fig. 2A). The effect was modest, but small increments in *FLO11* expression have been shown to increase invasive growth (25). Collectively, these results provide evidence that the Nrg1 and Nrg2 proteins repress invasive growth by repressing *FLO11*.

The *nrg* mutations were next tested for their ability to suppress the *snf1* mutation with respect to its defects in invasive growth and *FLO11* expression. The significant increase in invasive growth caused by the double deletion of *NRG1* and

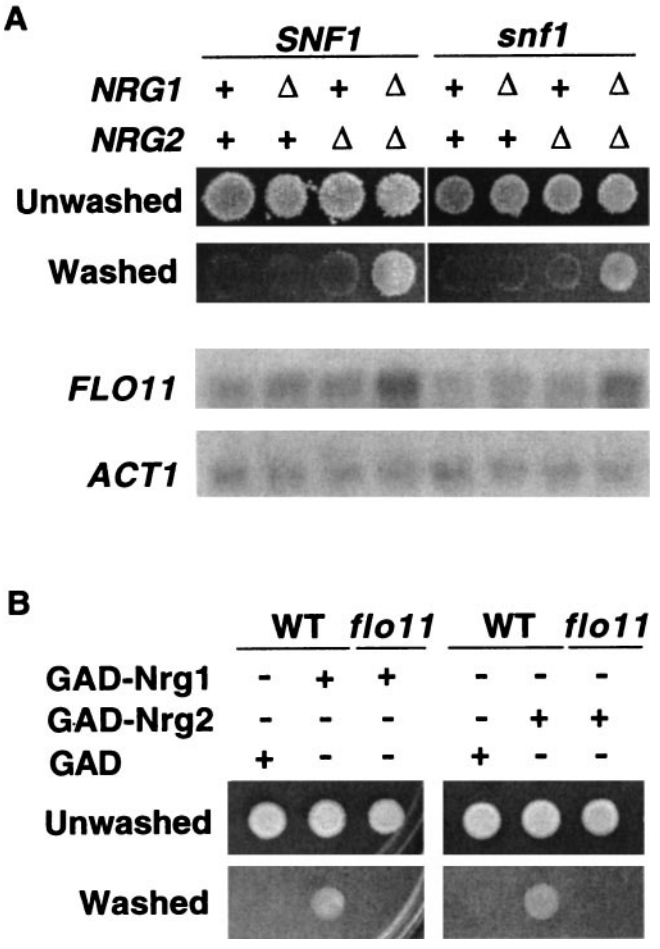

FIG. 2. Nrg1 and Nrg2 affect agar invasion and *FLO11* expression. (A) Strains with the indicated genotypes were assayed for invasive growth. Plates were photographed before wild-type cells had invaded the agar to any significant extent so that the increased invasiveness caused by the double *nrg1*Δ *nrg2*Δ mutation would be apparent. The same strains were grown to mid-log phase in YEP–2% glucose and subjected to Northern blot analysis of *FLO11* mRNA and, as a control, *ACT1* mRNA. (B) Wild-type and *flo11* strains were transformed with plasmids expressing GAD-Nrg1, GAD-Nrg2, or GAD from the *ADH1* promoter (pV40, pV39, or pACTII, respectively) (37). After growth on SC–2% glucose plates lacking leucine (for plasmid selection), cells were resuspended in sterile 10 mM Tris-HCl (pH 7.5)–1 mM EDTA and spotted onto plates for invasive growth assays. We were also able to detect invasive growth on selective SC–2% glucose plates, with similar results (data not shown).

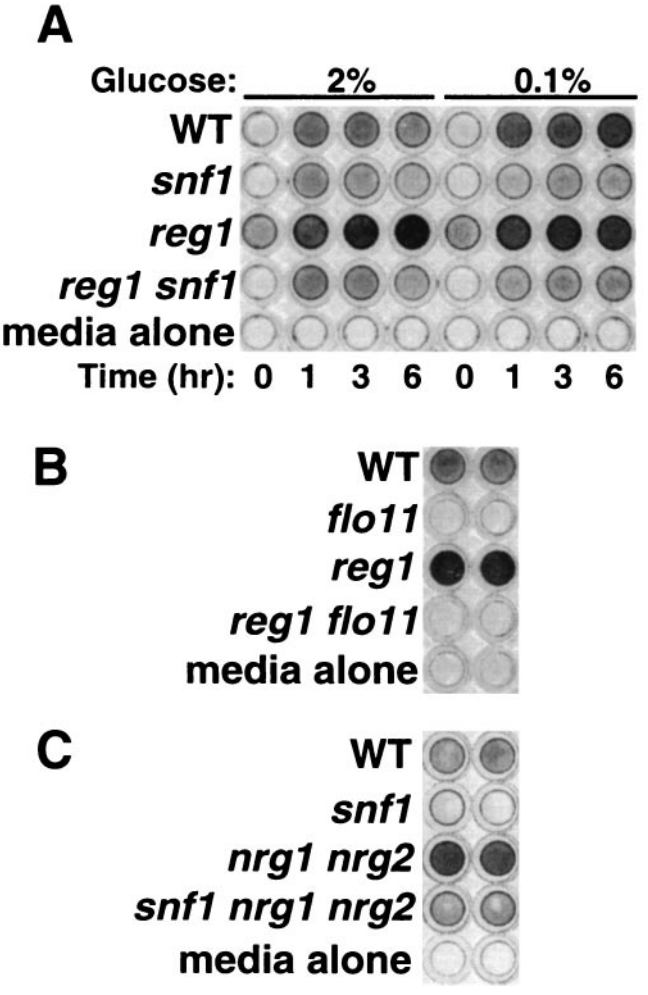

FIG. 3. The Snf1-Nrg pathway affects adherence to plastic surfaces. Cells were assayed for adherence to polystyrene (29) as described in Materials and Methods. All strains tested were *MAT***a**. (A) Cells were resuspended in SC with 2% or 0.1% glucose, transferred to the wells of a microtiter plate, and incubated for the indicated times. (B) Cells were resuspended in SC–2% glucose, and incubation was for 6 h. Duplicate samples are shown. (C) Cells were grown in SC–2% glucose to an OD_{600} of 2 and resuspended in SC–0.1% glucose. Incubation was for 2.5 h. Duplicate samples are shown. We also monitored the growth of samples of the same cultures for 2.5 h after resuspension; differences in growth rate did not correlate with differences in adherence (not shown). The *nrg1 nrg2* cells also adhered better than wild-type cells when resuspended in SC–2% glucose, and when assayed together, *nrg1 nrg2* and *reg1* cells adhered similarly (data not shown).

NRG2 was epistatic to *snf1*; in fact, the triple mutant was more invasive than the wild type (Fig. 2A). This phenotype correlated with an increase in *FLO11* mRNA (Fig. 2A). These findings lend further support to a model in which the Nrg1 and Nrg2 proteins function downstream of Snf1 to repress *FLO11*.

Snf1, Nrg1, and Nrg2 affect biofilm formation. The potential of *S. cerevisiae* cells to form biofilms has been demonstrated using an assay based on ability to adhere to plastic (29). Adherence required *FLO11* and improved when glucose levels were reduced (29), suggesting that Snf1 and the Nrg repressors could have a role in regulating biofilm formation. To test this idea, we grew *snf1* and *reg1* mutant cultures in SC–2% glucose, collected the cells, and resuspended them in SC with 2% or 0.1% glucose. Cells were inoculated into the wells of a polystyrene microtiter plate, incubated for 1 to 6 h, and stained (Fig. 3A). We found that *snf1* mutant cells adhered to the plastic somewhat less well than the wild type, particularly in 0.1% glucose, whereas the *reg1* mutant adhered extremely well; moreover, the *reg1* phenotype depended on *SNF1*, as a *reg1 snf1* double mutant adhered as poorly as the *snf1* mutant. In a control experiment, a *reg1 flo11* mutant showed no adherence, confirming that the *reg1* mutation does not bypass the requirement for *FLO11* (Fig. 3B). Finally, the *nrg1 nrg2* double mutation significantly improved adherence in both wild-type and *snf1* mutant cells (Fig. 3C). Thus, the regulatory mutations

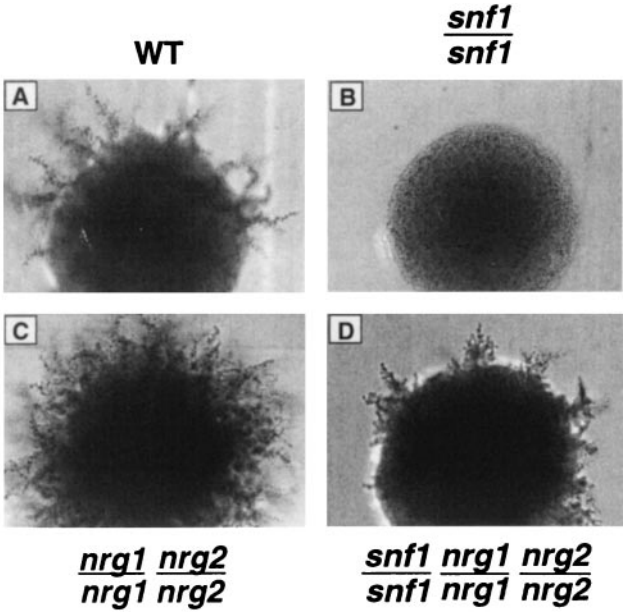

FIG. 4. Snf1, Nrg1, and Nrg2 affect diploid pseudohyphal growth. Diploid cells were streaked on solid low ammonia (SLAD) medium (8) and incubated at 30°C for 5 days. Colonies were viewed using a Nikon Eclipse E800 fluorescent microscope. Images were taken with an Orca100 (Hamamatsu) camera using Open Lab (Improvision) software and processed using Adobe Photoshop 5.5 software. Diploid strains were MCY4472, MCY4473, MCY4474, and MCY4475, which were transformed with pLCLG-Staf, a centromeric plasmid with *URA3* and *LEU2* (12) to confer prototrophy.

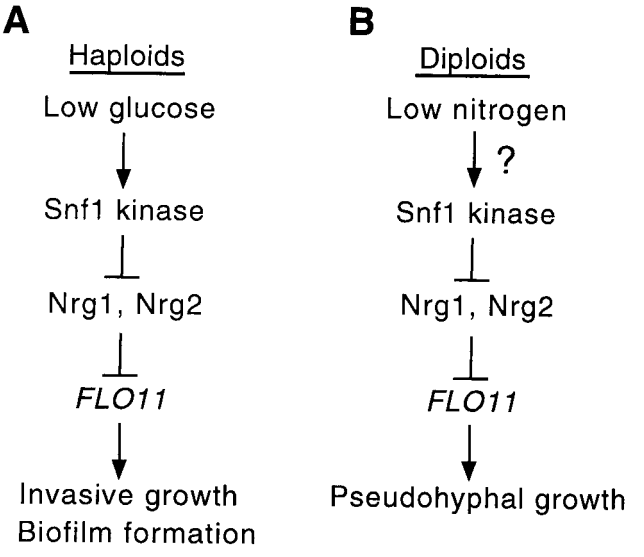

FIG. 5. Model for regulation of *FLO11* gene expression by the Snf1-Nrg pathway. (A) In haploid cells, the Snf1 kinase is activated in response to glucose limitation and relieves Nrg-mediated repression of *FLO11*. Expression of *FLO11* is critical for invasive growth and biofilm formation. It is also possible that Snf1 affects *FLO11* by other Nrg-independent mechanisms. (B) In diploid cells, the Snf1 kinase is activated under conditions of nitrogen limitation that lead to pseudohyphal differentiation. The simple model is that Snf1 responds to a low nitrogen signal, but there is no evidence to exclude other possibilities (see text).

tested here all affect plastic adherence in agreement with their effects on *FLO11*. These findings implicate the Snf1-Nrg pathway as one of the signal transduction pathways that regulate biofilm formation.

Snf1, Nrg1, and Nrg2 regulate diploid pseudohyphal growth. The involvement of the Snf1 kinase in agar invasion and biofilm formation by haploids is in accord with the well-established role of Snf1 in response to glucose limitation. Diploid pseudohyphal differentiation occurs in response to nitrogen limitation in the presence of abundant glucose; however, this process is similar to that of haploid invasive growth and requires *FLO11* (8, 15, 17). We therefore examined the roles of Snf1 and the Nrg proteins in pseudohyphal differentiation.

We first constructed a diploid homozygous for the *snf1* mutation and assessed its ability to form pseudohyphae during growth on low ammonia (SLAD) plates (8). While an isogenic wild-type diploid developed normal pseudohyphae in 5 days, the homozygous *snf1* mutant was predominantly in the yeast form, indicating that Snf1 is required (Fig. 4). In contrast, a homozygous *nrg1 nrg2* double mutant diploid showed increased pseudohyphal differentiation compared to that of the wild-type diploid (Fig. 4). Finally, to examine the relationships of Snf1, Nrg1, and Nrg2 in this process, we constructed a diploid homozygous for all three mutant alleles. The ability to form pseudohyphae was partially restored in the *snf1*/*snf1 nrg1*/*nrg1 nrg2*/*nrg2* diploid.

Thus, these results indicate that the Snf1 kinase plays a role in a response to nitrogen limitation and suggest that Snf1 regulates pseudohyphal differentiation, at least in part, by antagonizing Nrg1- and Nrg2-mediated repression.

DISCUSSION

We have here examined the roles of the Snf1 protein kinase and two repressors, Nrg1 and Nrg2, in the regulation of haploid invasive growth in response to glucose depletion. We present evidence that Snf1 regulates transcription of *FLO11* and that Nrg1 and Nrg2 function as negative regulators of *FLO11* and invasive growth. Evidence regarding the physical and genetic relationship of Snf1 to the Nrg proteins suggests that Snf1 kinase activity inhibits their repressive function. We propose that Snf1, Nrg1, and Nrg2 are components of a pathway for regulation of *FLO11* that, together with the PKA and MAPK pathways, contributes to the complex regulation of this key gene (Fig. 5A).

We first showed that the Snf1 kinase is required for derepression of *FLO11* expression in response to glucose limitation. A *snf1* mutant derepressed *FLO11* only weakly, consistent with its reduced ability to invade agar, whereas constitutive elevation of Snf1 activity in a *reg1* mutant led to elevated *FLO11* expression and improved invasion. Thus, manipulating Snf1 kinase activity either genetically or physiologically, by adjusting the glucose concentration (3), results in highly correlated responses in *FLO11* expression and agar invasion. We conclude that Snf1-dependent regulation of *FLO11* is a primary mechanism by which glucose levels control invasive growth; however, it remains possible that Snf1 also regulates other targets that contribute to invasion. We also note that

FLO11 expression is reduced but not abolished in *snf1* mutants, consistent with evidence that multiple regulatory pathways converge on *FLO11*.

We next present evidence that Nrg1 and Nrg2 are negative regulators of invasive growth and act, at least in part, by repressing *FLO11*. Mutation of *NRG1* and *NRG2* together strongly enhanced agar invasion and relieved glucose repression of *FLO11*. Overexpression of GAD-Nrg1 or GAD-Nrg2 caused a hyperinvasive phenotype, presumably by upregulating genes that are normally repressed by the native Nrg proteins, and this phenotype was strictly dependent on *FLO11*. Finally, *nrg1* and *nrg2* mutations relieved glucose repression of *STA2-lacZ*; the finding that *nrg1* alone caused a loss of repression may reflect the fact that the *STA2* promoter is stronger than that of *FLO11* due to two deletions of 20 and 64 bp (5), or the slight differences in DNA sequence may differentially affect the binding of Nrg1 and Nrg2. Further studies will be required to determine whether Nrg1 and Nrg2 also regulate other genes, besides *FLO11*, that are involved in invasive growth.

We provide genetic evidence that Snf1 kinase activity counteracts Nrg1- and Nrg2-mediated repression. The interactions of the double *nrg1 nrg2* mutation and *snf1* with respect to invasive growth, biofilm formation, pseudohyphal growth, derepression of *STA2-lacZ*, and *FLO11* expression all support the idea that Nrg1 and Nrg2 function downstream of the Snf1 kinase. Although we cannot rule out the possibility that Snf1 indirectly antagonizes repression by Nrg1 and Nrg2, their physical interaction with Snf1 (37) suggests a direct functional interaction. It is also possible that Snf1 exerts some control over these repressors at the transcriptional level; evidence that *NRG1* RNA levels are sixfold lower in glycerol-ethanol than in glucose (27) would be consistent with this idea, but it has also been reported that *NRG1* RNA is induced 2.7-fold during the diauxic shift (4).

Snf1 is known to have multiple regulatory targets, including both transcriptional activators and repressors, that mediate different responses to glucose limitation (2). It may therefore be worthwhile to consider the possibility that Snf1 also affects *FLO11* expression by other mechanisms that do not involve Nrg proteins. In support of this idea, *nrg1* and *nrg2* only partially suppressed the *snf1* defect in *STA2* expression, suggesting that other Snf1-dependent mechanisms act on *STA2* and, by extension, on *FLO11*.

The roles of Snf1, Nrg1, and Nrg2 in regulating *FLO11* expression raised the possibility that this signaling pathway affects the potential of cells to form biofilms. We found that a *snf1* mutation impaired adherence to a plastic surface, whereas *reg1* increased adherence (dependent on the presence of *SNF1*). Mutation of *NRG1* and *NRG2* strongly enhanced adherence and also suppressed the *snf1* defect. These results implicate the Snf1-Nrg pathway in the regulation of biofilm formation.

We also report that the Snf1 kinase is required for diploid pseudohyphal growth, in accord with unpublished observations of Cullen and Sprague (see reference 3). We further show that mutation of the *NRG* genes both enhances pseudohyphal growth in wild-type cells and restores pseudohyphal growth in a *snf1* mutant. These findings suggest that the regulatory relationships between Snf1, the Nrg repressors, and *FLO11* are the same for pseudohyphal growth as for invasive growth (Fig. 5B); however, pseudohyphal differentiation is regulated by nitrogen rather than glucose. Thus, these findings suggest a role for Snf1 in nitrogen signaling. Snf1 activity may be induced by low nitrogen; alternatively, the basal activity of Snf1 in the presence of glucose may be required during the response to nitrogen limitation, or Snf1 may relay a general nutritional stress signal. Two different pathways regulate the activity and localization of Snf1 in response to different carbon source signals (36), so it is easily conceivable that Snf1 receives yet other signaling inputs.

The finding that pseudohyphal growth depends on Snf1 expands the known repertoire of this kinase in yeast cell differentiation. It is now clear that the Snf1 kinase has roles in diverse, cell type-specific developmental processes that occur in response to distinct nutrient signals: invasive growth of haploid cells in response to glucose limitation, pseudohyphal differentiation of diploid cells in response to nitrogen limitation, and meiosis and sporulation of diploids in response to limitation for both glucose and nitrogen. Snf1 has multiple regulatory roles in the metabolic adaptation of vegetatively growing cells to changes in carbon availability and may similarly prove to have multiple roles in regulating developmental events.

The *SNF1* and *NRG* genes are conserved in the fungal pathogen *Candida albicans*, raising the possibility that this regulatory pathway is functionally conserved. In accord with this idea, the *C. albicans* Nrg1 represses filamentous growth and represses expression of hypha-specific genes, including the adhesin genes *HWP1*, *ALS3*, and *ALS8* (1, 24). The functions of the Snf1 kinase in *C. albicans* are not yet understood because the gene is essential for viability (28). Based on the present study, we suggest a role for the *C. albicans* Snf1 kinase in the morphological transition from yeast form to filamentous growth, a process that is essential for the pathogenicity of *C. albicans*.

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