Conidial Germination in *Aspergillus nidulans* Requires RAS Signaling and Protein Synthesis

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ABSTRACT

The dormant spores of *Aspergillus nidulans* become competent for growth and nuclear division in a process called conidial germination. To analyze the molecular details of conidial germination, we developed a genetic screen in which we identified spore germination-deficient mutants that are blocked in this process at the restrictive temperature. These mutants defined eight genes, of which we identified five. Four of the five were directly involved in translation and protein folding, and the fifth showed a high degree of homology to a malonyl CoA synthetase. These results suggest that out of a wide array of processes occurring during conidial germination, translation is essential if germination is to proceed. We also show that conidia containing a mutant-activated form of *rasA*, the *ras* homologue in *A. nidulans*, germinate in the absence of an inducing carbon source, suggesting an important role for *rasA* signaling in conidial germination. Together these data suggest a model by which a carbon source activates a *ras*-dependent sensory mechanism, inducing translation and leading to conidial germination. This study shows that conidial germination in *A. nidulans* requires protein synthesis and that the initiation of translation is linked, through an as yet to be determined signaling cascade that includes *rasA*, to a carbon-source-sensing apparatus.

ASEXUAL sporulation is a common mode of reproduction for a variety of filamentous fungi. The asexual spore, or conidium, is formed from the tips of specialized spore-forming cells in a precisely regulated developmental plan (for a review see Adams *et al.* 1998). The conidia are a primary means of dispersion and a genomic safe house for the filamentous fungi during adverse environmental conditions. Their germination is typically triggered by nutrients in a process whose molecular details have not yet been determined. A detailed understanding of the molecular events occurring during conidial germination could well lead to novel ways to control fungal disease at this critical phase of their life cycle. The process has been best studied in the filamentous fungus *Neurospora crassa*, but progress has been hampered by the tendency of their conidia or macrospores to germinate sporadically in the absence of a specific growth stimulus and the inability to isolate relevant mutants (reviewed in Schmit and Brody 1976). The germination of sexual spores, or ascospores, has been fairly well characterized in the yeast *Saccharomyces cerevisiae*, but this fungus does not produce asexual conidia. The physical and biochemical differences between sexual and asexual germination make extrapolations from one to the other tenuous (Herman and Rine 1997).

In our studies of conidial germination, we have relied on *Aspergillus nidulans*, which begins its asexual life cycle as a dormant, metabolically inactive conidium containing a single, highly condensed nucleus (Bainbridge 1971). It has several advantages. Conidia can be stably stored for years in a desiccated state. Unlike *N. crassa*, they can also be stored for extended periods in distilled water with no detectable metabolic activity. When given a nutrient source they swell rapidly, the nucleus reorganizes, and hyphal growth begins several hours later (Bainbridge 1971). Early studies indicate that the first measurable effects (<20 min) are trehalose breakdown and translation (reviewed in D'enfert 1997). While biochemical studies have identified various enzymatic changes in germinating spores, such as transcription, translation, and later DNA replication, the initial signaling events controlling germination remain obscure. The few genetic screens of *N. crassa* have isolated a few germination-deficient mutants but even these have not led to any new insights into the process (reviewed in Schmit and Brody 1976). No one has reported isolating any germination-deficient mutants in *A. nidulans* or closely related pathogenic species, although a few specific mutants have been described as being impaired in germination. These include strains lacking mitogen-activated protein kinase (Bussink and Osmani 1998), Ras (Som and Kolaparthi 1994), and calmodulin (Dayton *et al.* 1996). Nevertheless, these strains do undergo partial germination characterized by spore swelling and nuclear reorganization, and therefore these proteins do not strictly define the early signaling events leading to conidial germination.

Nevertheless, we believed a study of this process in *A.*

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nidulans could lead to a better understanding of conidial germination. We devised an enrichment procedure based on the fungicide nystatin to increase the number of heat-sensitive germination-deficient conidia by ~20-fold. We isolated mutants whose conidia were completely unresponsive to germination-inducing media at the restrictive temperature. Genetic analysis showed that these mutants defined eight genes, of which we have cloned, sequenced, and identified five. Interestingly, four of the five were directly involved in protein synthesis and folding. The fifth showed a high degree of similarity to a malonyl CoA synthetase. These results led us to undertake a more detailed analysis in this article of the requirements for conidial germination. In addition to further highlighting the importance of translation as an obligatory step in germination, we show that a mutant-activated form of *A. nidulans rasA* induces precocious germination in the absence of a suitable carbon source.

On the basis of our results, we propose that a carbon source serves as an extracellular signal to activate translation, thereby initiating germination. These results serve as a starting point for further studies that will identify regulatory proteins that control translational initiation during conidial germination.

MATERIALS AND METHODS

***A. nidulans* strains and growth methods:** The genotypes of all strains used in this study are given in Table 1. MAG [2% malt extract, 0.2% peptone, 1% dextrose, trace elements (Cove 1977), 1 μg/ml pyroxidine, 8.8 μg/ml riboflavin, and 2% agar for solid medium] was used for routine propagation of strains. This medium was supplemented with 5 mm uridine, 10 mm uracil for *pyrG89* mutant strains. We germinated conidia in conidial germination studies in liquid minimal medium containing 70 mm $NaNO_3$, 7 mm KCl, 4 mm $MgSO_4$, 12 mm KPO_4, pH 6.8, trace elements, and a carbon source and/or inhibitor as indicated in the tables. Unless stated otherwise, chemicals were obtained from Sigma (St. Louis). H89 was purchased from Calbiochem (San Diego). Germination experiments involving mutant-activated *rasA*^{G17V}, strain A986, and wild-type *rasA*, strain A988 (Som and Kolaparthi 1994), were conducted on minimal medium agar plates containing 0.1% glycerol. After 48 hr of growth at 37° spores were harvested and promptly used in the germination experiments. These were performed in liquid minimal medium with or without the addition of 10 mm glucose as a sole carbon source. Genetic methods and other media employed were those described previously (Pontecorvo *et al.* 1953; Käfer 1977).

Microscopy and nuclear staining: Conidia were inoculated at 10^6 conidia/ml into petri dishes containing liquid medium and sterile coverslips. After incubation, cells were fixed using 4% freshly prepared paraformaldehyde and 0.1% Triton X-100 in 10 mm KPO_4, pH 6.8, for 10 min. Cells were washed twice in distilled water and stained for 15 min in 4′,6-diamidino-2-phenylindole (DAPI; 100 ng/ml). Cells were then washed twice in phosphate-buffered saline (PBS) and mounted in 50% glycerol, 0.5% *n*-propyl gallate in PBS. Cells were viewed using a Nikon microscope fitted with a Hamamatsu model C2400 camera using a SCSI adapter and twain driver into Adobe Photoshop.

TABLE 1

***A. nidulans* strains in this study**

Strain	Genotype
FGSC4[a]	Wild type
GR5[a]	*pyrG89; wA3*; *pyroA4*
R153	*wA2*; *pyroA4*
A122[a]	*riboA1 yA2*; *nicB8*
A612[a]	*acr*A1; *ribo*B2; *chaA1*
A988[a]	*biA1*; *argB*+::*palcA*:*rasA*
A986[a]	*biA1*; *argB*+::*palcA*:*rasA*^{G1V7}
sgdA 15.8.10	*pyrG89 riboA1 yA2; pyroA4; nicB8; sgdA*
sgdA 16.9.10	*pyrG89 riboA1 yA2; sgdA*
sgdB 11.18.5	*pyrG89; wA3; pyroA4; sgdB*
sgdC 1.1.25	*pyrG89 riboA1 yA2; pyroA; sgdC*
sgdD 2.2.3	*pyrG89; wA3; nicB8; sgdD*
sgdE 3.3.9	*pyrG89; nicB8; sgdE*
sgdF 4.4.24	*pyrG89; wA3; sgdF*
sgdG 13.7.6	*pyrG89; pyroA; sgdG*
sgdH 17.10.1	*pyrG89; wA3; sgdH*

[a] Available from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS.

Spore germination-deficient (*sgd*) mutant isolation: We isolated *sgd* mutants using a nystatin-based enrichment procedure as outlined in Figure 1. Freshly harvested conidia of strains A122 and A612 were mutagenized to ~30% viability using 4-nitroquinoline 1-oxide (4-NQO) as previously described (Holt and May 1996). 4-NQO-mutagenized conidia were plated in pools of 10^5 viable conidia/plate of solid medium and grown for 3 days at 32°. We used this "expression cycle" step because we reasoned that those gene products unique to the germination process are already present as proteins in the spore before germination. By employing a growth cycle all preexisting conidial proteins are of the mutant form. Conidia were harvested separately from each pool and germinated at a concentration of 10^6 conidia/ml in 5-cm petri dishes containing 5 ml liquid MAG medium for 4 hr at 42°. Nystatin was added directly to the plates at a concentration of 150 U/ml and incubated for a further 3–4 hr at 42°. The conidia were spun down and suspended in 0.5 ml of sterile distilled water. Aliquots of ~100 μl were mixed in 3 ml/plate top agar (MAG, 0.5 agar, 0.1% deoxycholate) and plated on MAG with 0.1% deoxycholate agar plates. Plates were incubated for 3–4 days at 32° and then replicated, grown at both restrictive (42°) and permissive (32°) temperatures, and compared. Colonies that exhibited heat-sensitive growth were purified and further analyzed for the *sgd* phenotype.

Cloning and identification of *sgd* genes: We constructed a genomic plasmid library in a derivative of pRG3 that contains the AMA1 sequence from *A. nidulans* because it allows autonomous replication and highly efficient transformation (Gems *et al.* 1991; Efimov and Morris 1998; reviewed in Aleksenko and Clutterbuck 1997). Indeed, incorporation of the AMA1 sequence into the vector increases transformation efficiency at least 100-fold to 1–2 × 10^4 transformants/μg DNA. Autonomous replication facilitates recovery of the plasmid from transformants and subsequent cloning steps. The library was constructed using *Sau*3AI partially digested *A. nidulans* genomic DNA from strain R153 that was size fractionated (6–20 kb) and ligated into a *Bam*HI-cut and phosphatase-treated pRG3-AMA1 vector. This library consists of enough independent clones with an average insert size of 9 kb to contain 20 genome

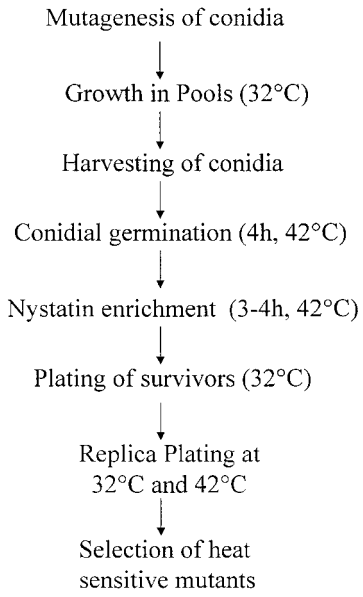

Figure 1.—Method for isolating *sgd* mutants. Conidia were mutagenized with 1 μg/ml NQO. Survivors were grown at permissive temperature in pools of 10^5 conidia to produce second generation conidia containing the mutated gene product. *sgd* mutants were enriched in the presence of the fungicide nystatin, which preferentially kills spores germinating at the restrictive temperature. Survivors were replicated at both permissive (32°) and restrictive (42°) temperatures, compared, and visually selected for complete lack of germination at 42°. Putative *sgd* were further identified by microscopy.

equivalents. Transformation-competent *sgd* conidia were prepared at the permissive temperature and transformed as described previously (May 1989). Transformants (~5000 Pyr^+ transformants/plate) were incubated at the restrictive temperature (42°) for 2–3 days, yielding ~1–5 rescued transformants per plate. Genomic DNA was prepared from these primary transformants and used to transform electrocompetent *Escherichia coli* cells. For each gene, 20 ampicillin-resistant colonies were analyzed for the recovery of the AMA1 library vector. Approximately 50% of the rescued plasmids contained identical or overlapping inserts that were then used to recapitulate the rescue of the *sgd* strain analyzed. The plasmids carrying the genes *sgdA*, *B*, *C*, *D*, and *E* complemented the heat-sensitive phenotype of the corresponding mutant at high frequency.

So we could rapidly identify the complementing gene within a cloned insert, we randomly mutagenized the rescuing plasmid with a transposon using an *in vitro* transposition kit (GPS-1 system, New England Biolabs, Beverly, MA). Individual transposon-tagged clones were transformed into the *sgd* mutant of interest. Plasmids that failed to rescue a *sgd* mutant at the restrictive temperature were assumed to have insertions disrupting the complementing gene. Typically, 30–40 transposon-tagged plasmids were tested, resulting in 3–5 that failed to complement. These were sequenced using primers unique for the transposon ends. In all cases tested, transpositions occurred within the same overlapping region defining a gene. Genes for *sgdA*, *B*, *C*, *D*, and *E* were identified and partially sequenced using the AMA1-based complementation/GPS transposon identification technique outlined above. In each case, 600–1500 bp were sequenced and the results were run through a Blast database search in order to identify the *sgd* genes' possible function by identifying homologous genes with known functions.

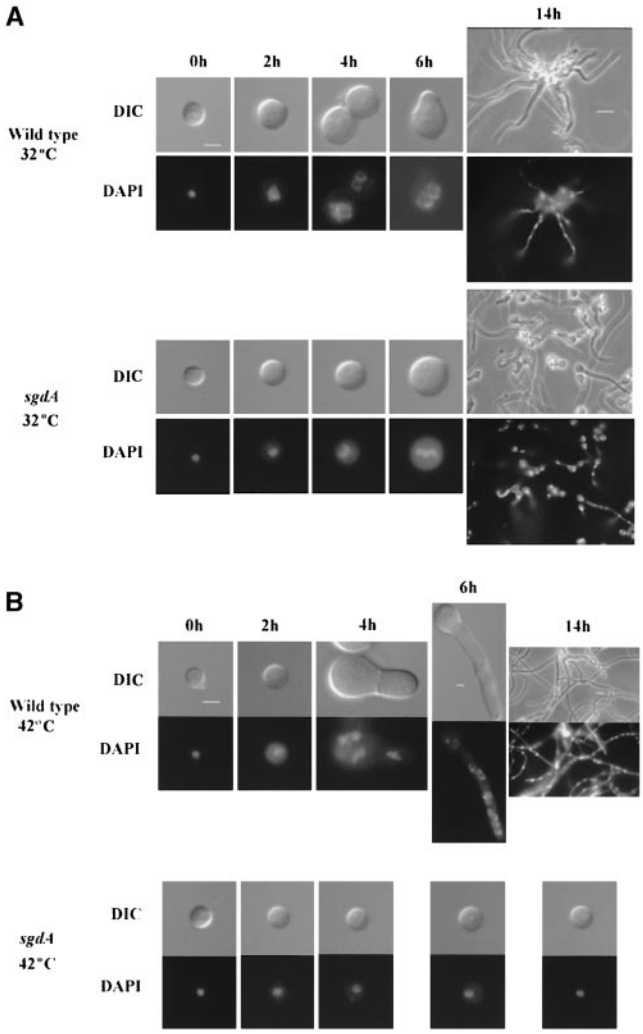

Figure 2.—Germination of wild-type and *sgdA* conidia at permissive 32° (A) and restrictive 42° (B) temperatures. Cells were fixed on glass coverslips and visualized by differential interference contrast (DIC) microscopy (top) and after DAPI nuclear staining (bottom). Scale bars denote length in micrometers.

RESULTS

Isolation of heat-sensitive mutants defective for spore germination: Addition of media to dormant *A. nidulans* conidia initiated a complex cascade of physiological and biochemical events (Figure 2, A and B, top). At 42° the earliest visible changes that took place were spore swelling (<1 hr), nuclear reorganization (as spores entered interphase; <1 hr), and cell wall changes by which conidia adhered to one another and to the substrate (1–2 hr). Polarity and the establishment of a hyphal germ tube proceeded at ~4 hr at 42° and 6 hr at 32°. At the same time the nucleus underwent mitosis, with nuclei migrating into the germ tube.

To parse the molecular events of conidial germination, we isolated heat-sensitive germination-deficient mutants and analyzed their functional deficits. Details

TABLE 2

Results of nystatin enrichment protocol

	Number	% of total
Colonies screened	~12,000	100
Heat-sensitive mutants recovered	252	2.1
Growth mutants	201	1.7
Polarity defective	31	0.26
Spore germination defective	20	0.17

of the screening protocol are described in materials and methods (Figure 1). We developed a fungicide-based enrichment protocol to isolate *sgd* mutants as they make up only a small fraction of the total range of heat-sensitive mutants derived by mutagenesis. Nystatin is a polyene fungicide that interferes with fungal membrane integrity, selectively killing germinating, metabolically active conidia. It has been used to select auxotrophic mutants of *A. nidulans* (Ditchburn and Macdonald 1971). In this study, we used nystatin to selectively kill germinating conidia incubated at the restrictive temperature, enriching for spores that did not undergo germination or whose nystatin-sensitive phase of growth was delayed.

When mutagenized conidia were directly plated without incorporating the nystatin enrichment procedure, only 0.1% of the colonies were heat sensitive, and none were spore germination deficient. Nystatin treatment increased the recovery of heat-sensitive growth mutants ~20-fold to 2% of the total (Table 2). Conidia from these strains were plated on rich medium at the restrictive temperature and were microscopically examined. Of the strains, 201 or 80% were categorized as heat-sensitive growth-deficient mutants, *i.e.*, at the restrictive temperature conidia germinated and formed short hyphae, in some cases forming small fuzzy colonies on agar. An additional 31 or 12% of the mutant strains were categorized as polarity defective, undergoing spore swelling, nuclear reorganization, and in some cases nuclear division. Only 20 mutants or 8% of the total number of heat-sensitive mutants were defined as putative *sgd*. We defined the *sgd* phenotype narrowly, as complete blockade of spore germination on rich medium at the restrictive temperature for up to 14 hr. Conidia could not undergo swelling or nuclear reorganization as assessed by DAPI staining, but after shift to permissive temperature they were viable. We selected the mutants that met these criteria for further study.

Genetic characterization of the *sgd* mutants: The 20 putative *sgd* mutant strains were outcrossed to strain GR5 and scored for heat sensitivity and the *sgd* phenotype. Because *A. nidulans* is haploid, single-gene mutations are expected to segregate in a 1:1 ratio in crosses to wild type. Of the 20 putative *sgd* strains, 12 showed 1:1 segregation and linkage of heat sensitivity to the *sgd* phenotype. To determine the number of genes defined by the 12 *sgd* mutant strains, pairwise crosses were made between all mutants using the different auxotrophic and color markers. Crosses were verified by using different color markers on each crossed pair. If we could not identify any viable progeny at the restrictive temperature, we assumed each parent strain contained a mutation in the same *sgd* gene. Based on the genetic analysis, the 12 *sgd* mutants defined eight genes. Four independent mutations were isolated in the *sgdA* gene, two for *sgdB*, and one each for the *sgdC-H* genes. The fact that the *sgd* screen produced several independent mutations in the *sgdA* and *sgdB* genes indicates that mutants for only a limited number of genes can be isolated in this selective regime, and it is possible that the action of very few genes is required in the earliest steps of conidial germination in *A. nidulans*.

Phenotypic characterization: The phenotypes of the *sgd* mutants were characterized in both liquid and solid medium and compared to a wild-type control. In liquid medium at the permissive temperature, *sgd* strains underwent swelling, nuclear reorganization, mitosis, and hyphal growth just as the control wild type did (Figure 2A). At the restrictive temperature there was no conidial swelling or nuclear reorganization for up to 14 hr, as defined by our parameters (Figure 2B). Because mutants containing genes that are specifically needed during germination should exhibit a defined period of thermal sensitivity early in germination, we allowed the *sgd* mutants to undergo early germination for 6 hr at the permissive temperature. During this time, the *sgd* gene would be expressed and conidia would germinate. We then shifted them to the restrictive temperature and tested for continued growth. An inability to grow after shift was interpreted to mean that the *sgd* gene product was needed during both germination and vegetative growth. The mutant strains were unable to grow after the shift, indicating that the mutated genes were also necessary for vegetative growth.

***sgd* genes encode factors that are required for protein synthesis or folding:** The genes *sgdA–E* were cloned by complementation using the AMA1-based genomic library. We then used transposon-mediated insertional inactivation of the rescued plasmid to map the complementing gene (see materials and methods). Sequences derived from transposon-inactivated genes were used to identify the gene's function through homology with known genes in the data banks. All the sequences scored a significant homology to well-characterized genes (Figure 3). Three of the five genes encode proteins involved in translational initiation (*sgdA*) and elongation (*sgdB, C*) and a fourth (*sgdE*) encodes a protein involved in protein stabilization and folding.

sgdA showed 40% identity over ~0.8 kb to yeast *PRT*1 (Evans *et al.* 1995), a homologue of the mammalian p116 subunit of *eif-3*. This protein is essential for translational initiation, and in yeast a temperature-sensitive

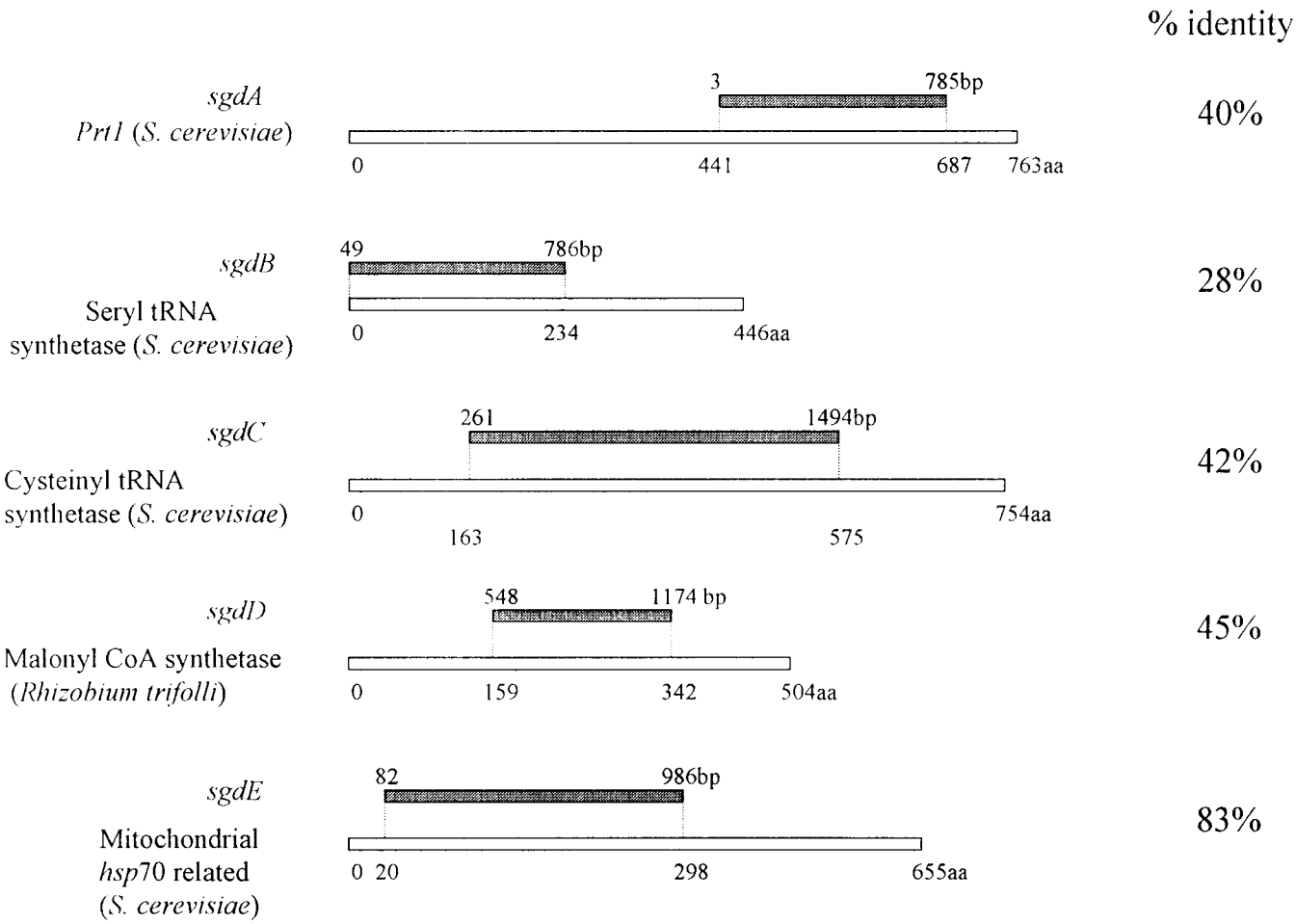

Figure 3.—Comparison between *sgdA–E* and known genes in the database. *sgdA–E* were cloned by complementation and sequenced utilizing the AMA1-based complementation/GPS transposon identification technique outlined in materials and methods. In each case, between 600 and 1500 bp were sequenced, and the results were run through a Blast database search in order to identify the *sgd* genes' possible function through homology with known genes in the data banks. Accession numbers for the *sgdA, B, C, D,* and *E* gene sequences are AF180560, AF180324, AF179866, AF180325, and AF180323, respectively.

mutant, *cdc63-1*, causes arrest at G_1 when shifted to the restrictive temperature (see discussion). *sgdB* and *C* showed 28 and 42% identity over ~0.7 and ~1.2 kb, respectively, to seryl and cysteinyl tRNA synthases. These enzymes esterify the appropriate amino acid to their corresponding tRNA prior to their attachment to the ribosome. *sgdD* showed 45% identity over ~0.6 kb to a malonyl CoA synthetase from *Rhizobium trifolii* (An and Kim 1998). This class of enzyme is involved in the synthesis of malonyl CoA from malonate. *sgdE* showed 83% identity over ~0.9 kb to the mitochondrial hsp70 protein *SSC1/ENS1* from yeast (Morishima *et al.* 1990). The *SSC1* protein supports the assembly of some protein complexes inside the mitochondria, probably aiding in their refolding in the mitochondrial matrix. These results show that our genetic approach has begun to illuminate translation and protein folding as key biochemical events in conidial germination in *A. nidulans.* To gain further insight into these results, we asked what effects would various well-characterized inhibitors of macromolecular synthesis, or cytoskeletal function, have on spore germination.

The *sgd* phenotype can be duplicated by an inhibitor of translation but not by inhibitors of other key cellular systems: The requirement for translation during conidial germination was tested using cycloheximide, an inhibitor of translation. Addition of cycloheximide resulted in complete inhibition of germination (Table 3). Conidia and nuclei remained condensed and were identical in phenotype to the *sgd* mutants, as described above. Surprisingly, inhibition of conidial germination could not be achieved using hydroxyurea, an inhibitor of DNA synthesis, or actinomycin D, an inhibitor of RNA synthesis. Similarly, benomyl, an inhibitor of microtubule function, and cytochalasin D, an inhibitor of actin polymerization, did not block spore germination, although growth rates and cell shapes were severely affected.

TABLE 3

Effects of various inhibitors on conidial germination

Addition of	Conidial swelling, nuclear reorganization	Hyphal growth
No treatment	+++	+++
Cycloheximide (2 mg/ml)	−	−
Cytochalasin D (25 μg/ml)	++	+ (swollen)
Benomyl (25 μg/ml)	++	+ (swollen)
Hydroxyurea (50 mm)	++	+ (swollen)
Actinomycin D (25 μg/ml)	++	+

Inhibitors were added to fresh FGSC4 or GR5 conidia in rich medium and incubated for 14 hr at 37°. Cells were fixed, DAPI stained, and analyzed microscopically. +++, ++, and + indicate that >90, ~60, or 30% of spores, respectively, underwent this stage in germination. − indicates that no spores underwent this stage.

Nutritional requirements for conidial germination in *A. nidulans*: To gain further insight into the molecular events occurring upstream of protein synthesis in conidial germination we initially determined which components of the medium were required for germination by systematically omitting them from defined minimal medium. Freshly harvested GR5 and FGSC4 wild-type conidia were incubated for 2–14 hr in the different media. Omission of nitrogen or phosphate had no effect on spore germination, whereas omission of the carbon source blocked germination. Even 2% glucose in distilled water was sufficient for conidial germination (Table 4), as were sucrose, galactose, raffinose, maltose, and fructose and other carbon sources such as acetate, glycerol, and starch. In contrast, such carbon sources as malate, propionate, and citrate did not activate germination (Table 4). The addition of 2-deoxyglucose or 6-deoxyglucose, which are nonmetabolizable analogues of glucose, did not support conidial germination, indicating that not only glucose but also metabolites of glucose were needed for germination. Both compounds were nontoxic, as their removal and replacement with glucose resulted in normal germination (data not shown).

***rasA*^{G17V}, an activated mutant form of *rasA*, induces conidial germination in the absence of a carbon source:** In *S. cerevisiae* the *ras* pathway appears to serve as a specific sensor for the detection of fermentable sugars (for review see Thevelein 1991). In *A. nidulans* overexpression of a dominant negative mutant form of *rasA*^{S22N} under the inducible *alcA* promoter delays germination (Som and Kolaparthi 1994). In contrast, overexpression of a dominant activated form, *rasA*^{G17V} that is locked in the active GTP-bound form, causes the formation of giant swollen multinucleate spores, indicating that RASA plays an important role in conidial germination (Som and Kolaparthi 1994). We hypothesized that the activated mutant form of *rasA* might induce conidial germination in the absence of a carbon source by bypassing the requirement for the activating signal. To test this hypothesis, conidia were collected from strain A986, which contains a wild-type copy of *rasA* and a constitutively activated mutant form, *rasA*^{G17V}, driven by the inducible *alcA* promoter inserted in tandem. Conidia were collected from A986 grown on minimal medium glycerol plates in which the *alcA* promoter expresses moderate levels of activated RASA^{G17V} and subsequently incubated in minimal medium for 14 hr

TABLE 4

Test of carbon sources that support conidial germination

	Minimal medium plus	
Supports germination (>95%)	Glucose (10 mm)	2% fructose
	2% sucrose	Acetate (10 mm)
	2% galactose	0.5% glycerol
	2% raffinose	2% soluble starch
	2% maltose	Glucose alone[a]
Does not support germination	No addition	Citrate (10 mm)
	6-Deoxyglucose	Malate (10 mm)
	2-Deoxyglucose	Proprionate (10 mm)

Conidia (10^6/ml) were incubated in the presence of medium for 14 hr at 37°, fixed, DAPI stained, and analyzed by microscopy.

[a] Conidia were incubated in 10 mm glucose in distilled water.

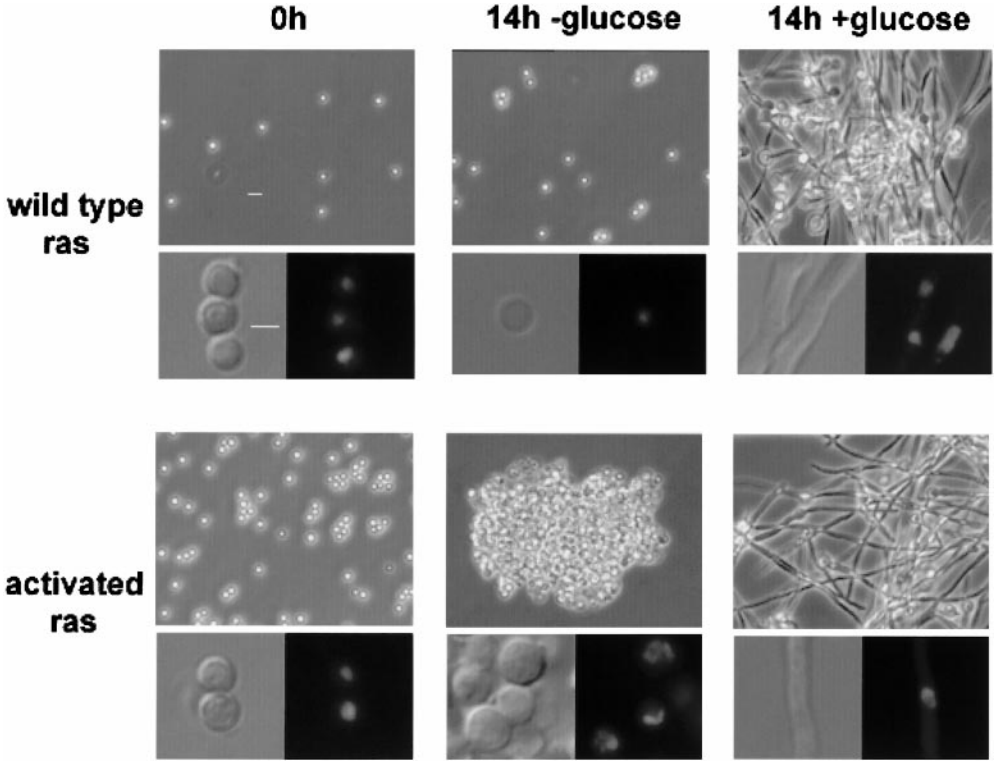

Figure 4.—Conidia expressing the mutant-activated *rasA*^{G17V} precociously germinate in the absence of glucose. Conidia expressing wild-type RASA (top) or mutant form of activated RASA^{G17V} (bottom) were incubated for 14 hr at 37° in the presence or absence of glucose. Conidia expressing a mutant form of activated RASA^{G17V} undergo swelling, nuclear decondensation, and aggregation in the absence of glucose (bottom, middle) in contrast to conidia expressing wild-type RASA (top, middle). For each time point three images are shown: at top a low-magnification phase-contrast image, at bottom left a high-magnification DIC image, and at right its corresponding DAPI-stained image. Scale bars denote length in micrometers.

at 37° in the presence or absence of glucose (see materials and methods). As a negative control, strain A988, which contains both a wild-type and an inducible *alcA*-driven wild-type copy of *rasA* in tandem, was treated in an identical fashion and assayed in parallel. Microscopic analysis revealed that in minimal medium lacking glucose, conidia containing the activated mutant form of *rasA*^{G17V} underwent spore swelling (45% diameter increase, $n = 100$), nuclear reorganization, and adhesion (Figure 4, bottom). In contrast, conidia containing wild-type *rasA* did not undergo these changes under these conditions (Figure 4, top). The inclusion of glucose resulted in normal growth in both strains (Figure 4, right). These results indicate that Ras signaling plays a role in inducing conidial germination in *A. nidulans*.

Conidial germination is not affected by 8-Br-cAMP, forskolin, and H89: In *Fusarium solani* 8-Br-cAMP, an activator of cAMP-dependent protein kinase (PKA), can induce spore germination in the absence of a carbon source, and H89, a highly specific inhibitor of PKA, blocks flavonoid-induced germination but does not interfere with germination on rich medium (Ruan *et al.* 1995). We therefore tested the ability of 8-Br-cAMP and forskolin, which activate PKA and adenylate cyclase, respectively, to activate conidial germination in *A. nidulans*. These compounds have been used in a variety of fungal systems, including *S. cerevisiae* (Rana *et al.* 1999) and *N. crassa* (Techel *et al.* 1990). The compounds, either in combination or separately, were added to conidia in either minimal medium lacking a carbon source, or acetate, a poor carbon source. Conidial germination was not induced or enhanced by the presence of 8-Br-cAMP or forskolin or both. The specific PKA inhibitor H89 did not inhibit conidial germination on rich medium at concentrations of up to 100 μm. Therefore it is unlikely that germination is triggered by a carbon source activating the PKA pathway, although the possibility that these compounds are unable to enter ungerminated conidia cannot be ruled out. Taken together our results indicate a role for *rasA* signaling but not cAMP signaling in conidial germination in *A. nidulans*.

DISCUSSION

In this study we describe a genetic analysis of the early steps of conidial germination in *A. nidulans* that revealed the importance of protein synthesis and metabolism in this process. In addition, we show the important role played by *rasA* in controlling germination.

Identification of *sgd* mutants: To isolate mutants blocked early in conidial germination, we set very rigorous criteria for the definition of the *sgd* phenotype, namely a complete lack of visible signs of germination in the presence of rich medium at the restrictive temperature. Since this class of mutants is relatively rare, we developed an enrichment scheme based on the ability of the fungicide nystatin to kill germinating spores. We isolated 12 *sgd* conditional heat-sensitive mutants that do not undergo spore swelling, nuclear reorganization, or adhesion at the restrictive temperature. Genetic characterization indicated that the 12 mutants defined eight

genes. Five of the genes were cloned by complementation and their gene products were identified. None of the mutants showed a defined window of early heat sensitivity, indicating that they were also necessary throughout vegetative growth. This parallels the results in *N. crassa*, where no phase-specific germination mutants have been described so far, and may indicate that conidial germination utilizes established regulatory and metabolic pathways that are necessary throughout the vegetative life cycle.

Genetic evidence that translation is required for conidial germination: A role for translation at an early stage of germination has been suggested previously, using biochemical measurements, for *N. crassa* conidia (Schmit and Brody 1976). The screen we used provided direct genetic evidence for the importance of this process in conidial germination in *A. nidulans*. Of the five *sgd* genes cloned, three (*sgdA*, *B*, and *C*) play a direct role in translation and a fourth (*sgdE*) is involved in protein folding in mitochondria. *PRT*1 and *SSC*1/*ENS*1, the budding yeast homologues of *sgdA* and *sgdE*, respectively, are essential for ascospore germination.

Many events have been measured biochemically and have been shown to become highly active during early *A. nidulans* and *N. crassa* conidial germination, including trehalose metabolism, respiration, RNA and protein synthesis, hydration, and the release of extracellular enzymes (reviewed in D'enfert 1997), but their relative contribution to germination was unknown. In this study, we show that out of a bewildering array of early events, an unbiased genetic approach specifically highlights a central role for translation in spore germination. Therefore, although a variety of events take place during the first hour of germination, a crucial process is the initiation of translation. Indeed, our demonstration that germination proceeds in the presence of inhibitors of RNA and DNA synthesis, but not in the presence of cycloheximide, an inhibitor of translation, strengthens this observation.

The initiation of translation in eukaryotes is a complex process with multiple control points (reviewed in Merrick 1992). In yeast the two main control points are the target of rapamycin (TOR)-dependent activation of eIF-4E cap binding protein by *TAP42* and *Sit4* phosphatase (reviewed in Dennis *et al.* 1999) and the phosphorylation of eIF-2a by *GCN2* (reviewed in Hinnebusch 1997). TOR is inactivated by the immune-suppressant drug rapamycin, leading to a rapid block in translation and arrest in Go in yeast. Surprisingly, addition of rapamycin did not inhibit *A. nidulans* germination and growth (data not shown). During the first 20 min of germination in both *N. crassa* and *A. oryzae* conidia, the percentage of ribosomes that sediment as polysomes rapidly increases (Horikoshi *et al.* 1965; Mirkes 1974). This shift from monosomes to polysomes is induced solely by the presence of a carbon source. The rate of RNA synthesis during this period is too low to be sufficient for the rate of increase of polysome formation, indicating that there is a preexisting pool of both mRNA and free ribosomes, whose coupling is induced by the presence of a carbon source.

***sgdD*, a putative malonyl CoA synthetase:** *sgdD* showed a 45% identity to a malonyl CoA synthetase (*MatB*) from *R. trifolii* (An and Kim 1998). This class of enzyme is involved in the formation of malonyl CoA from malonate, enabling its metabolic breakdown. Since we have also shown that conidial germination can proceed in the presence of acetate as sole carbon source, we speculate that a malonyl CoA synthetase might be involved in the generation of acetyl CoA through malonyl CoA and that this molecule may then be involved in activating translation, thereby linking the metabolic sensory pathways to those leading to protein synthesis.

Activators of conidial germination: Research in a variety of fungal species, including *F. solani* (Nelson 1991), *Pythium ultimum* (Nelson and Hsu 1994), *A. niger* (Abdel-Rahim and Arbab 1985), and *N. crassa* (Schmit and Brody 1976), has implicated sugars and amino acids as general essential activators of germination. We have extended these studies to *A. nidulans* and have shown that a variety of sugars and other carbon-containing compounds can induce germination in the absence of amino acids. Conidia also underwent nuclear division and hyphal growth in the presence of glucose alone, unlike yeast ascospores (Xu and West 1992; Herman and Rine 1997), and may indicate that perhaps conidia, unlike ascospores, contain internal stores of essential nutrients that sustain them through early stages of spore germination and growth. Since the nonmetabolizable glucose analogues 2-deoxyglucose and 6-deoxyglucose did not induce germination, we speculate that a metabolite of glucose is the trigger for this process, although the possibility that 2-deoxyglucose and 6-deoxyglucose are not recognized as glucose by the sensor cannot be ruled out.

***rasA* plays an important role in initiating spore germination:** The evidence that *ras* signaling is important for fungal spore germination is conflicting. Several lines of evidence point to a central role for *ras* in regulating spore germination in yeast and filamentous fungi. In *A. nidulans*, overexpression of a dominant negative mutant form of *rasA* delays germination (Som and Kolaparthi 1994), whereas in *N. crassa* deletion of the *ras* homologue *smco7*, which is upregulated during germination, did not inhibit germination (although hyphal growth was severely impaired; Kana-Uchi *et al.* 1997). Expression of a constitutively active CT-Ras^{G17V} in the fungus *Colletotrichum trifolli* did not influence germination rates but did cause defects in polarized growth and differentiation (Truesdell *et al.* 1999). In *S. cerevisiae*, ascospore germination is blocked in strains defective in the genes encoding the CDC25/*Ras*/CDC35 pathway (Herman and Rine 1997). These conflicting results prompted us to examine the role of *rasA* in conidial germination

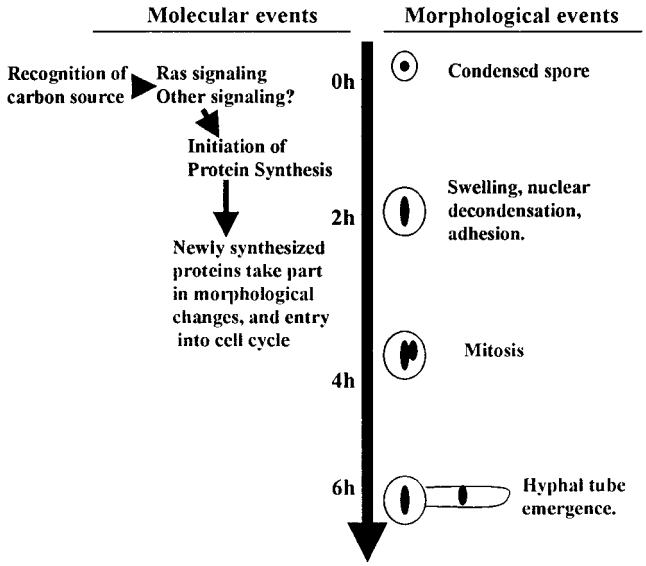

Figure 5.—Model for conidial germination. Spore germination as indicated by swelling and nuclear reorganization begins in the presence of an appropriate carbon source in the medium. Signaling is probably initiated by a metabolite of the carbon source through *ras*, leading to initiation of translation. Newly synthesized proteins enable the cell to enter the cell cycle and grow.

in *A. nidulans*. The conidia we collected from a strain overexpressing the mutant-activated form of *rasA* underwent spore swelling, adhesion, and nuclear reorganization in the absence of a carbon source. Thus the mutant-activated $rasA^{G17V}$ bypasses the need for a carbon-source-sensing apparatus that is crucial in wild-type organisms. The result agrees with the notion that *ras* has a function in controlling germination.

Is there a role for PKA or adenylate cyclase in conidial germination? There is likewise conflicting evidence regarding the role of PKA in fungal spore germination. In *S. cerevisiae* and *F. solani*, PKA plays an essential role in ascospore and conidial germination, respectively (Ruan *et al.* 1995; Herman and Rine 1997). In contrast, in the rice blast *Magnaporthe grisea*, disruption of PKA blocked appresorium formation but not conidial germination or development (Mitchell and Dean 1995). In this study we tested the ability of 8-Br-cAMP and forskolin, which activate PKA and adenylate cyclase, respectively, to activate conidial germination in *A. nidulans* in the absence of an inducing carbon source. Neither had any effect on germination, either in the absence of glucose or in the presence of acetate, a partial activator of germination. In contrast, in *F. solani*, conidia undergo germination in the presence of 8-Br-cAMP in the absence of a carbon source (Ruan *et al.* 1995). Addition of H89, a specific inhibitor of PKA, had no effect on conidial germination at concentrations as high as 100 μm. Together these results suggest that probably neither PKA nor adenylate cyclase performs essential roles in conidial germination in *A. nidulans*.

A model for conidial germination: On the basis of our results, we propose that the first essential step of conidial germination in *A. nidulans* is the uptake and breakdown of a carbon source, leading to the activation of the *rasA* signaling pathway. This and possibly other pathways activate the translation machinery to initiate loading of preexisting pools of ribosomal subunits onto mRNA, producing a rapidly growing pool of newly synthesized proteins. These proteins subsequently initiate a cascade of morphological changes leading to entry into the cell cycle, conidial swelling, and hyphal growth (Figure 5). Analysis of additional *sgd* mutants and the use of novel screens for genes that induce precocious germination in the absence of a carbon source should help us identify additional elements that connect the glucose sensory apparatus to the initiation of translation and subsequent conidial germination.

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