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The molecular mechanisms of conidial germination

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Abstract

The asexual spore, or conidium, is critical in the life cycle of many fungi because it is the primary means for dispersion and serves as a 'safe house' for the fungal genome in adverse environmental conditions. This review discusses the physiological process of germination, conidial adhesion and initiation of protein synthesis and also the regulatory pathways used to activate conidial germination. These include Ca^{2+}/calmodulin-mediated signaling, the cyclic AMP/protein kinase A and the ras/mitogen-activated protein kinase pathways. Insights into the process of conidial germination will increase our understanding of the mechanisms of dormancy and sensing of environmental stimuli, and permit identification of novel therapeutic targets for the treatment of spore-borne fungal infections in plants and animals.

Keywords: Fungus; Spore germination; Signal transduction

1. Introduction

The selective pressure to survive and propagate during times of adverse environmental conditions has led many species, plant, animal and microbial, to develop strategies that incorporate dormant desiccated capsules, or spores, as genomic 'safe houses' that can survive during these periods. A defining characteristic of spores is their capability of developing into a new individual without fusion with another reproductive cell. Spores thus differ from gametes, which are reproductive cells that must fuse in pairs to give rise to a new individual.

Most lower vascular plants, such as ferns and mosses, produce dormant spores. Animals, because of their mobility, are less likely to use this strategy, although notable examples include various species of parasitic annelids and nematodes [1]. Many species of microbes, both eukaryotic and prokaryotic, produce spores during their life cycle. Among the former are the fungi and unicellular soil amoebae commonly referred to as 'slime molds' [2]. Well-studied examples of bacterial spores are found in species of bacillus and clostridium [3].

Fungi from the divisions Zygomycota (e.g. *Rhizopus*, the black bread mold), Ascomycota (e.g. *Penicillium, Neurospora* and some species of *Aspergillus*) and Basidiomycota (mushrooms and puffballs) produce both sexual spores called zygospores, ascospores and basidiospores, respectively, and asexual spores called conidia. Deuteromycota is a division of fungi for which a sexual cycle has not been observed, and its members (e.g. *Aspergillus fumigatus*) produce only conidia.

In view of our advanced ability to perform molecular manipulations in several species of fungi that serve as model organisms, it is somewhat surprising that the basic molecular steps of conidial germination have not already been defined. One plausible explanation for the difficulty in dissecting conidial germination at the molecular level is that it may be controlled by multiple sensors and pathways, each one sensitive to a particular environmental stimulus, working in a particular combination. This would greatly complicate genetic dissection. In addition, without clear molecular-output signals to define early germination, it is difficult to differentiate between the true early signaling events and the explosion of essential metabolic and housekeeping activities that occur almost at the very beginning of the process. It is also difficult to differentiate

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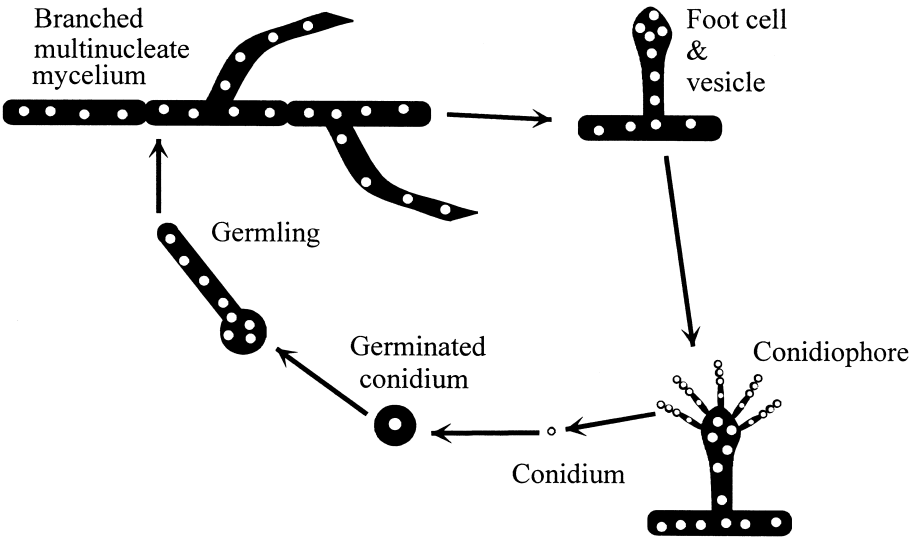

Fig. 1. The asexual life cycle of *A. nidulans*. Conidia are formed in chains on specialized structures called conidiophores. After dispersal and contact with a suitable substrate, conidia germinate and initiate hyphal growth (the germling stage). Germlings grow to form a branched multinucleate mycelium that subsequently develops specialized aereal hyphae that then form conidiophores.

genetically between early signal-transduction events and early essential housekeeping activities. Genetic damage in either one will yield a 'germination-defective' mutant, but only the former will be instructive. This review elaborates on these problems. We describe the process of conidial germination in detail, emphasizing well-characterized model fungi, such as *Aspergillus nidulans*, *Neurospora crassa* and recent studies of fungal plant pathogens. We examine the contributions of different signaling pathways to the process and attempt to incorporate them into a tentative model. This review focuses principally on our current understanding of the molecular details of asexual spore or conidial germination (Fig. 1). Inherent to our approach is the assumption that conidial germination is a regulated process that responds to environmental stimuli by a signaling cascade that is amenable to genetic and biochemical inquiry. Our definition of conidial germination takes into account only those very early events required to sense and transmit the signal to germinate. Later responses, such as the initiation of nuclear division and hyphal growth, are not included within our definition.

The study of conidial germination, in addition to being a scientific puzzle of great interest, has far-reaching practical implications. Intensive monoculture and inbreeding have greatly increased the incidence and severity of fungal infections in crops [4]. Often fatal fungal infections in immunodeficient patients have also increased markedly during the last decade [5]. In almost all cases in both plants and animals, fungal infection is initiated by contact of the host with airborne conidia, which begin the infective process by undergoing conidial germination. By achieving a molecular understanding of this process, it may be possible to develop novel therapeutic approaches that block infection at its outset.

2. Choice of a suitable model system for the study of conidial germination

Because the well-characterized fungi *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* do not produce conidia, *A. nidulans* and *N. crassa* remain the organisms of choice for the analysis of conidial germination. Both are genetically tractable, easily manipulated at the molecular level and are currently undergoing full-genome sequencing. *A. nidulans* has some additional advantages over *N. crassa*: it is closely related to several species of pathogenic fungi that infect humans, including *A. fumigatus* and *Aspergillus terreus*. Unlike *N. crassa*, whose conidia have a tendency to germinate sporadically in the absence of a specific growth stimulus, *A. nidulans* has a tightly controlled conidial germination pathway. Its conidia can be stored stably for years in a desiccated state or for extended periods in distilled water with no detectable metabolic activity. Studies of conidial germination have been conducted among the major plant pathogenic fungi in *Magnaporthe grisea*, *Ustilago maydis*, *Fusarium solani*, *Colletotrichum trifolii* and *Pythium ultimum*, to name a few. However, most research to date has been devoted to analyzing the molecular basis of the formation of an appressorium (a structure which enables the pathogen to penetrate the plant cuticle or pores), rather than early conidial germination. Another notable problem is the dilution of resources caused by pursuing a multitude of possible pathogens. Ideally, research should concentrate on those plant pathogens for which genetics and molecular techniques are available and a large body of research has accumulated, such as *M. grisea* [6] and *U. maydis* [7].

3. Approaches used to identify genes and proteins involved in conidial germination

Various approaches have been used to analyze the molecular events that occur during early germination. A genetic approach has been used in several fungi, including *A. nidulans* [8]. After random mutagenesis and one cycle of growth at the permissive temperature, so that defective genes can be translated into proteins that are stored in the dormant spores, temperature-sensitive mutants blocked in early conidial germination are isolated and characterized. Those arising from a single gene mutation can then be cloned by complementation. Demonstrating that the genetic defect specifically affects conidial germination and no other phase of the fungal life cycle is important. It is surprising that no phase-specific mutants have been isolated so far, indicating perhaps that the molecular components used in conidial germination are also used in other essential processes. A noteworthy finding is that most of the genes isolated from these screens are involved in protein translation. An analysis of existing *N. crassa* mutants also revealed that mutants defective in protein synthesis are completely blocked in conidial germination [9]. Restriction-enzyme-mediated insertional mutagenesis has been used to isolate *Colletotrichum* mutants defective in conidial pigmentation or cell wall thickness [10]. Subtractive hybridization has been used to isolate transcripts stored at elevated levels in dormant conidia in *A. nidulans, N. crassa* and *M. grisea* (Berlin, 1985 #6; Zimmermann, 1980 #10 [11]). A 'candidate-gene' approach has been very successful in isolating mutants defective in late germination and appressorium formation in *M. grisea* [12], *U. maydis* [13] and *Colletotrichum lagenarium* [14]. Inhibitors were widely used in many early studies (for example, see [15]), but interpretation of those results is complicated, because most inhibitors are not truly specific.

Monoclonal antibodies against cell wall determinants have been used on expression-clone outer cell wall glycoproteins [16], but their role in conidial germination remains unclear. Biochemical assays, such as protein, RNA, DNA synthesis rates [9,17], and specific activity of enzymes such as trehalose (reviewed in [18]) have also been used. However, although they may provide convenient 'read-out' points for further analyses, these methods cannot reveal the molecular mechanisms of germination.

4. A short description of conidial germination

The mechanics of conidial germination are as diverse and varied as the number of species that produce conidia. Nevertheless, several events generally occur in most instances: when supplied with the appropriate nutrients in the presence of water and air, conidia swell (hydrate) rapidly and undergo a change in surface properties, as evi-

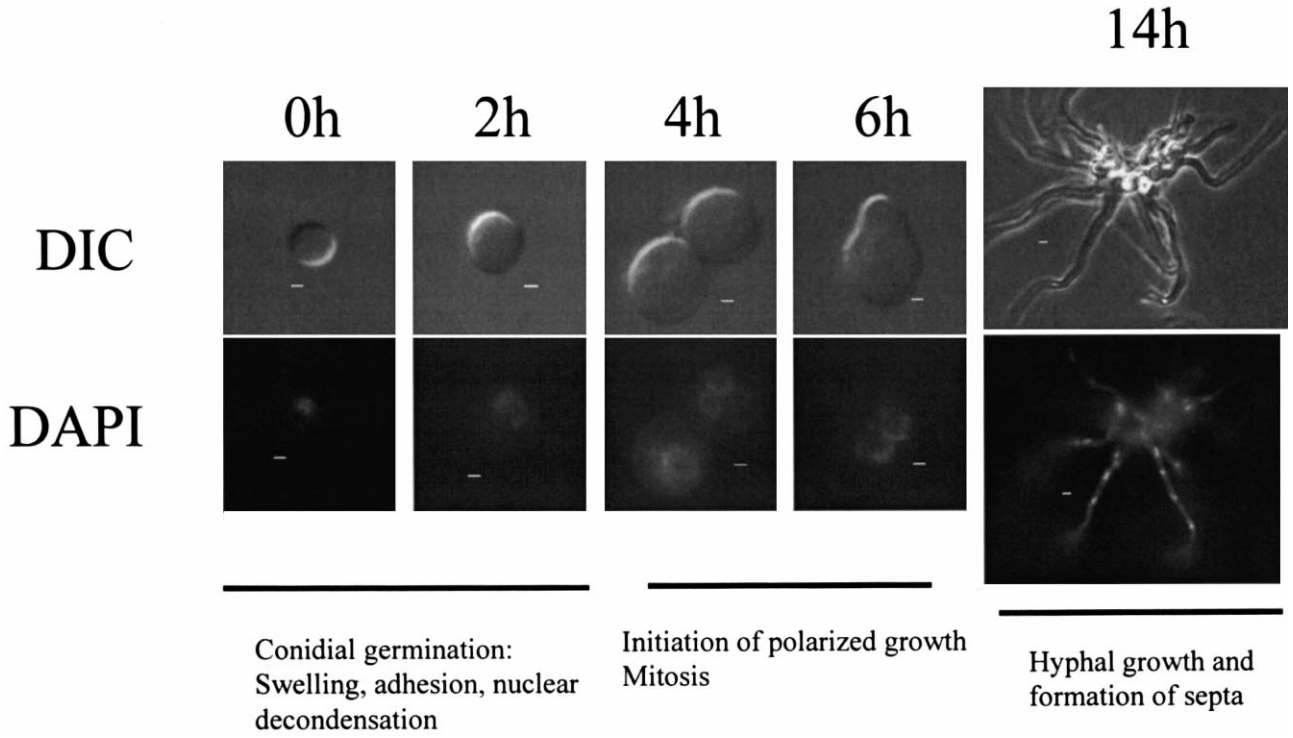

Fig. 2. Germination of *A. nidulans* conidia. Cells were fixed on glass coverslips and visualized by differential interference contrast (DIC) microscopy (top) and after DAPI nuclear staining (bottom). The morphological and biochemical changes that occur during the first 2 h (underlined on left) are the subject of this review.

denced by increased adhesion to one another and to the substrate. The nucleus reorganizes, and hyphal growth begins several hours later (Fig. 2). In many genera of plant pathogenic fungi (e.g. *Magnaporthe, Colletotrichum, Ustilago*), the hyphal tip swells to form the appressorium. At the same time, numerous metabolic activities, including respiration, RNA and protein synthesis, trehalose breakdown as well as differences in the composition of the cell wall can be detected [15,18].

5. Triggers of conidial germination

Conidial germination in most filamentous fungi requires the presence of low-molecular-mass nutrients such as sugars, amino acids and inorganic salts [19]. In *N. crassa*, germination occurs in the presence of salt and a carbon source alone [15], whereas in *A. nidulans*, the presence of glucose alone is sufficient to induce germination [8]. Considerable research has been carried out on the molecular basis of the glucose-sensing pathways in *S. cerevisiae* [20], and this, although not dealing directly with conidial germination, may provide interesting possible parallels between the two processes. Several signaling pathways allow *S. cerevisiae* cells to perceive the levels of glucose in their surroundings and initiate a transcriptional response. These pathways include the target of rapamycin (TOR) pathway which controls translation, and the hexose transporter induction (*snf3 rgt2*), cyclic AMP/protein kinase A (cAMP/PKA) activation and glucose repression (*Snf1* kinase/*Mig1*) pathways which control transcription [20]. These findings could be used to perform a 'candidate-gene approach' search for genes involved in the conidial response to glucose in filamentous fungi. For example, *cre-1* and *creA*, the homologs of *mig1*. have been cloned in *N. crassa* and *A. nidulans*, respectively. It is however important to note that homologs of *S. cerevisiae* genes are not always equivalent in function in filamentous fungi. For example, in *M. grisea*, loss of the MAP kinase *pmk1* does not affect sexual and asexual reproduction, whereas in *S. cerevisiae* deletion of the homologous MAP kinase *fus3* results in sterility [21].

Plant-pathogenic fungi have developed additional controls for regulating conidial germination. By sensing host-specific characteristics and substances these pathogens germinate only in the presence of a suitable host plant. For example, conidial germination of *M. grisea* is triggered by the hydrophobicity and hardness of the contact surface and by chemicals from the host plant (reviewed in [6]). Breakdown of conidial-contained germination inhibitors, called self-inhibitors, occurs upon contact with the waxy plant cuticle. In *Colletotrichum* species, conidial germination is triggered by waxes and ethylene produced by the host plant; contact with a hard surface is a prerequisite for the chemical signal to be effective in inducing germination [22]. Spores of *F. solani* are induced to germinate by both nutrients and pisatin, an isoflavonoid exuded by plant roots [23]. The sensory equipment that conidia presumably contain, which receives the various germination-inducing or -inhibiting signals, remains unidentified.

6. Conidial adhesion

Germinating conidia undergo a marked increase in their adhesive properties. This is usually a two-step process: initial adhesion that results from a pre-existing glycoprotein layer or from a component of the conidial cell wall, and later, tighter adhesion that results from metabolic activation and protein synthesis. Conidia of *Blumeria graminis* respond immediately, within seconds of contact with the plant host, by releasing extracellular matrix and taking up anionic materials of low molecular mass [24].

In *Colletotrichum* species, conidia are embedded in a pre-formed, water-soluble extracellular matrix composed of glycoproteins [25]. Additional secretion of mucilage occurs during germination and depends on protein synthesis [26]. Conidia of *M. grisea* stick to the repellant surface of rice plants by releasing an adhesive from the apex of the spore. This release is triggered by wetting of the conidium [27]. The surface of conidia from the powdery mildew fungus *Uncinuliella australiana* is coated with a thin network of extracellular mucilage which on contact with a wet surface, spreads instantly to form sticky adhesion pads [28]. So far, no adhesive glycoproteins have been cloned from any *Colletotrichum, Magnaporthe* or *Uncinuliella* species.

We have shown previously that adhesion of *A. nidulans* conidia to one another and to the polystyrene petri dishes in which they are germinated requires protein synthesis. Conidia from temperature-sensitive mutants blocked in protein synthesis were unable to adhere or germinate [8]. Using subtractive hybridization, we have isolated several mucin-like transcripts that are stored exclusively in dormant conidia and are potentially attachment molecules (unpublished data).

The adhesion of conidia from fungal pathogens of humans has been characterized in *A. fumigatus*. Conidia adhere to extracellular matrix proteins, such as fibronectin, collagen and laminins, that are found in host tissue. A 72-kDa cell wall laminin-binding protein has been isolated from *A. fumigatus* conidia [29]. Deletion of the *rodA* gene, which encodes a hydrophobin involved in the structure of the outer rodlet layer of conidia, decreased adherence to collagen but not to laminin and fibrinogen [30].

The most extensive studies of fungal adhesion have been carried out in the pathogenic dimorphic yeast *C. albicans*, and these may be instructive in the study of conidial adhesion. Multiple surface proteins called adhesins (Ala1p, Als1p, INT1) contribute to adherence of the yeast form of *C. albicans* (reviewed in [31]). During the transition to invasive hyphal growth, additional stable attachments

form through covalent cross-linking of Hwp1, a surface protein of *C. albicans*, to unidentified host cell membrane proteins. This cross-linking is mediated by host transglutaminases [32]. The molecular approach used to identify adhesin genes in *C. albicans* could be applied in the study of conidial adhesion. This approach uses heterologous gain-of-function experiments performed by transforming nonadherent strains of *S. cerevisiae* with a plasmid-based library of *C. albicans* DNA and screening for adherent transformants. The transformed plasmid presumably causing the increased adherence can then be isolated. Sequencing of the plasmid insert *C. albicans* DNA may reveal the genes responsible for the increase in adherence.

7. Calcium signaling during conidial germination

Although calcium signaling plays a clear role in fungal morphogenesis and development, its involvement in early conidial germination seems to vary between species. Calcium signaling appears to be nonessential in the early stages of conidial germination in *A. nidulans*. Deletion or delayed expression of the Ca^{2+}/calmodulin-dependent kinases slows entry into the cell cycle but does not completely block germination [33]. In *M. grisea* and *C. trifolii*, appressorium formation, but not germination, is completely blocked in the presence of calcium chelators, ionophores and calcium regulators [34,35]. In contrast, in *Phyllosticta ampelicida*, conidial germination is completely blocked in the presence of several specific channel blockers [36].

Clearly, the differing results could be species-specific, but they could also arise from the use of different methods. Differences also exist in how different researchers define and characterize germination. A more rigorous molecular analysis, involving the construction of specific null and conditional mutants defective in Ca^{2+}-sensing components would go a long way toward clearing up this uncertainty.

8. The ras/mitogen-activated protein kinase (ras/MAPK) pathway

Evidence that ras signaling plays an important role in conidial germination is conflicting. In *A. nidulans*, overexpression of dominant negative or activated forms of ras affects germination by delaying or precociously activating germination respectively [8,37], which suggests a role for ras in controlling conidial germination. In contrast, expression of constitutively active CT-Ras^{G17V} in *C. trifolii* does not influence germination rates but does cause defects in polarized growth and differentiation [38]. In *N. crassa*, deletion of the ras homolog *smco7*, which is upregulated during germination, does not inhibit germination, although hyphal growth is severely impaired [39]. The evidence for MAPK involvement in conidial germination is equally diverse between species. Deletion of the *C. lagenarium* MAPK gene *CMK1* blocks germination, whereas deletion of the *M. grisea* MAPK gene, *pmk1*, inhibits appressorium formation but not germination [21,40].

9. The PKA pathway

Conflicting evidence exists about the involvement of the PKA pathway in conidial germination. In *F. solani*, *C. trifolii* and *M. grisea*, the PKA pathway is essential for germination. *F. solani* and *C. trifolii* PKA-specific inhibitors block conidial germination, and activators induce it [23,41]. In *M. grisea*, the PKA pathway plays a role in both appressorium formation [42] and germination [43]. Deletion of MAC1, the gene encoding adenylate cyclase in this fungus, results in delayed germination and an inability to form appressoria [43]. In contrast, PKA-specific inhibitors or activators have no effect on germination in *A. nidulans* [8]. Deletion of the regulatory subunit of PKA in *N. crassa* results in defects in growth polarity and in the formation of septa and conidia, but the effects on conidial germination have not been reported [44].

Some problems arise in the analysis of these experimental results. First, different species of fungi may possess different permeabilities and target susceptibilities for the various PKA pathway inhibitors and activators. Second, since the PKA pathway has a central role throughout the life cycle of most fungi, it is difficult to dissect its specific role during conidial germination. Gene deletion will often be lethal. However, ways to circumvent this problem exist. One way is to replace existing components of the PKA pathway (e.g. PKA) with temperature-sensitive versions of the gene that are inactivated *at the protein level* by a temperature shift. One could then germinate mutant conidia at the restrictive temperature, causing inactivation of any pre-stored or translated PKA protein, and measure the effect of its absence on germination. Such mutants can also be used subsequently to identify additional signaling components involved in conidial germination by performing suppressor screens. In this approach, one looks for secondary induced mutations that enable the temperature-sensitive germination-deficient mutant to undergo germination at the restrictive temperature.

10. The role of protein synthesis in conidial germination

Conidial germination cannot take place when protein synthesis is blocked. In contrast, studies conducted with either specific inhibitors or mutants have indicated that DNA synthesis is apparently not necessary for early germination [8,9]. Studies using RNA synthesis inhibitors are conflicting. The results of one study have suggested that this process is essential for germination to proceed in

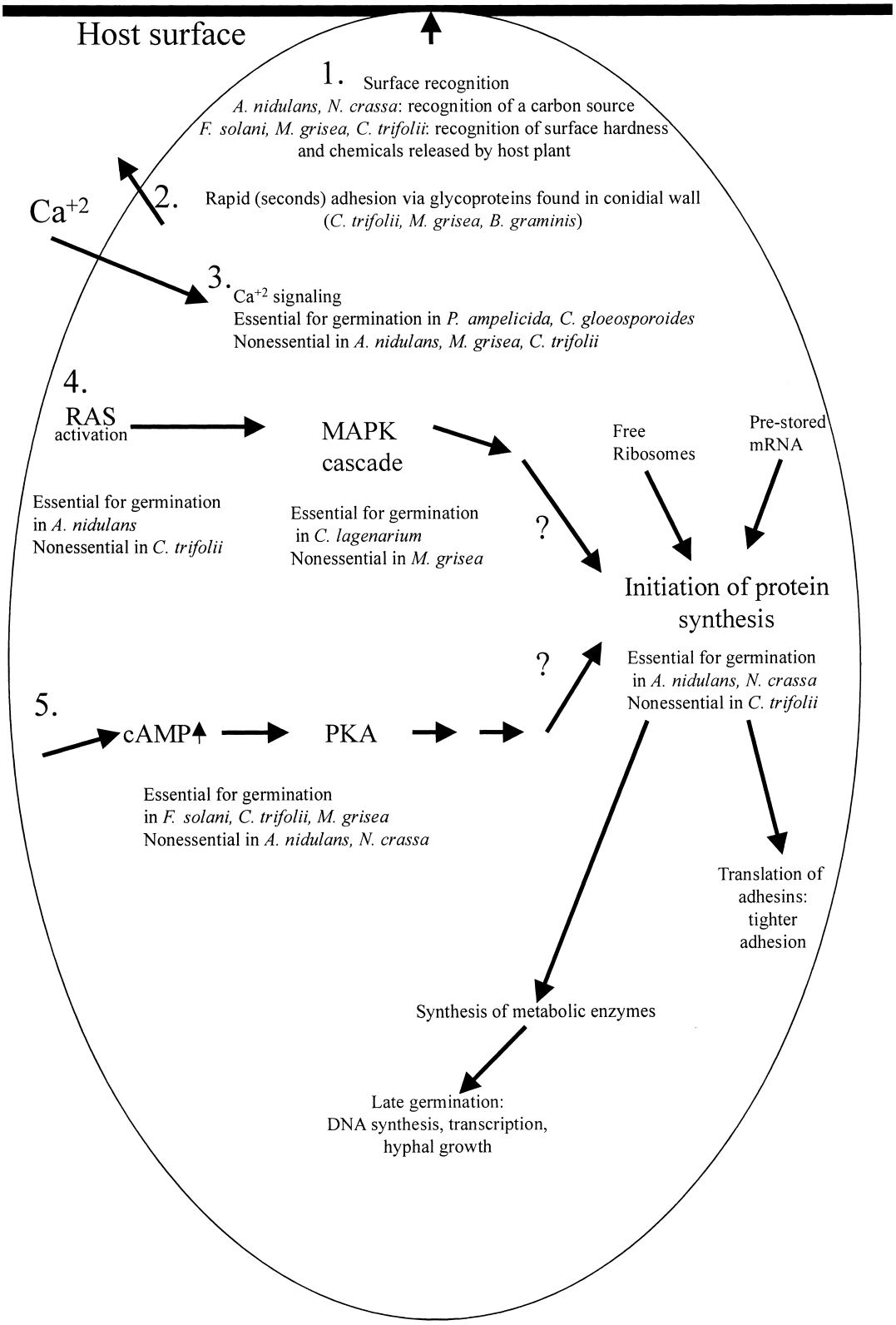

Fig. 3. In this tentative model of conidial germination we have tried to incorporate all the data from the different fungi described in this review. One of the first steps following substrate recognition is rapid adhesion that results from a pre-existing glycoprotein layer or a component of the conidial cell wall. We hypothesize that the RAS/MAPK, cAMP/PKA and possibly other unrecognized signaling pathways control the initiation of protein synthesis, which is the main bottleneck controlling conidial germination. Rapid assembly of polysomes onto pre-stored mRNA is followed by translation of key enzymes and proteins necessary for tighter adhesion, metabolic activation, DNA synthesis and hyphal tip growth.

N. crassa and *A. nidulans*, but not in *F. solani* and *Peronospora tabacina* [45]. In contrast, other studies performed in *N. crassa* and *A. nidulans* conclude that RNA synthesis is not essential for conidial germination [8,46].

The dependence of conidial germination on protein synthesis has been demonstrated in *N. crassa*, *A. nidulans* and *F. solani* using either protein-synthesis inhibitors or temperature-sensitive mutants defective in this process [8,9,47]. Dormant conidia contain high levels of free ribosomes, which in the presence of a carbon source, associate with mRNA within 15 min to form polysomes [46].

These results have suggested that dormant conidia contain a pre-existing pool of mRNA and ribosomes, primed for rapid activation and translation in the presence of nutrients. The identity of these mRNAs could prove instructive, possibly revealing which proteins need to be translated at the very beginning of germination. We have recently used suppressive subtraction hybridization [48] to identify and clone 12 transcripts that are stored at relatively high levels in dormant conidia of *A. nidulans*. Of these, according to sequence homology, three are metabolic enzymes, two show similarity to mucins, and the rest have no homology to any known genes (our unpublished results). These transcripts could serve as 'reporters' for dissecting earlier upstream signaling events.

The molecular mechanism of translational control has been studied intensively in both *S. cerevisiae* and mammalian systems. In yeast, the two main control points are the TOR-dependent activation of eIF-4E cap-binding protein by *TAP42* and *Sit4* phosphatase [49] and the phosphorylation of eIF-2a by *GCN2* ([50]. It would be interesting to clone the homologs of these genes in a species of fungus that produces conidia and assess their involvement in conidial germination.

11. The protein disulfide model in *N. crassa*

In *N. crassa*, early conidial germination is associated with the rapid reduction of oxidized glutathione and glutamic acid pools, the generation of NADH and NADPH and the concomitant reduction of protein disulfide bonds. This has led to the hypothesis that glutamic acid reduction generates NAD(P)H which is used to generate reduced glutathione. In turn, glutathione activates the proteins necessary for germination by breaking protein disulfide (Cys-S–S-Cys) bonds into Cys-SH-free residues, leading to their activation ([51] and reviewed in [15]). The validity of this model has not been pursued experimentally, nor does it explain the necessity for the other elements of signaling discussed in this review. Nevertheless, it highlights additional biochemical events that should not be left out of a final model.

12. Conclusions

Clearly, conidial germination is a complex process that varies somewhat between different species of fungi. Dissecting this process is difficult because its sensory and signal-transduction aspects are inextricably linked to the more general 'housekeeping' duties that are activated in the awakening conidium. Although the currently available data create a somewhat fractured mosaic, several generalizations can be made (Fig. 3). Adhesion occurs in all germinating conidia, and although it is still poorly understood at the molecular level, it is eminently amenable to future genetic and biochemical dissection. Several signal-transduction pathways have been implicated in conidial germination, including Ca^{2+}/calmodulin signaling and the ras/MAPK and cAMP/PKA pathways (Fig. 3). We hypothesize that the crucial bottleneck in conidial germination is the initiation of protein synthesis, which is probably influenced by the activation of the ras/MAPK cascade and the cAMP/PKA pathways. How these pathways interrelate, and how they influence the initiation of protein synthesis leading to the subsequent steps of conidial germination are some of the important questions that should be addressed in future studies.

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