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Regulation of cAMP-dependent protein kinase during appressorium formation in *Magnaporthe grisea*

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Abstract

The rice blast fungus, *Magnaporthe grisea*, requires formation of an appressorium, a dome-shaped and highly melanized infection structure, to infect its host. cAMP was identified as an important second messenger in signaling systems for appressorium formation in this fungus. To understand further the role of cAMP in infection-related morphogenesis, cAMP-dependent protein kinase activity was measured during appressorium formation and germination of conidia. Much higher activity of protein kinase was detected in germlings forming appressoria on the hydrophobic surface of GelBond than germlings growing vegetatively on a hydrophilic surface. In liquid culture, protein kinase activity increased during conidial germination, and peaked at 8 h after incubation before declining. Two transformants which develop small non-functional appressoria, and which lack *cpkA*, a gene encoding the catalytic subunit of protein kinase, did not show protein kinase activity. These data suggest that protein kinase has significant roles in the regulation of functional appressorium formation in *M. grisea*.

Keywords: *Magnaporthe grisea*; Rice blast; Appressorium formation; Signaling system; Protein kinase

1. Introduction

Magnaporthe grisea (Hebert) Barr (anamorph: *Pyricularia grisea*) is a typical heterothallic Ascomycete and the causal agent of rice blast, one of the most destructive diseases on rice (*Oryza sativa* L.) worldwide. Successful infection by this fungus requires the formation of an appressorium, a dome-shaped and melanized infection structure. Differentiation of appressorium in *M. grisea* is induced by environmental stimuli, including hydrophobicity and hardness of the contact surface, and chemicals from the plant surface [4,7,13]. Recently, *MPG1*, a gene responsible for hydrophobin, has also been implicated to have the role of surface recognition for appressorium formation in *M. grisea* [2,10].

Much has been learned about the endogenous signaling systems involved in appressorium formation of *M. grisea* over the past few years. Lee and Dean found that addition of exogenous cAMP to germinating conidia of *M. grisea* induced appressorium formation on non-inductive surfaces [6]. Genes encoding adenylate cyclase (*MAC1*) and catalytic subunit of cAMP-dependent protein kinase (*CPKA*) have been cloned and characterized in relation to

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appressorium formation by assessing the phenotypes of gene knockout mutants [3,8,14]. Transformants carrying disrupted *mac1*, which are unable to differentiate into appressoria, formed appressoria when exogenous cAMP was added [3]. Beckerman and Ebbole also reported that addition of cAMP can bypass the inhibitory effect of both yeast extract and absence of Mpg1p on appressorium formation of *M. grisea* [2]. These series of experiments suggest that intracellular cAMP regulates appressorium formation in *M. grisea*.

Although much research has been conducted on the role of cAMP for appressorium formation of *M. grisea*, no attempt has been made to directly measure the level of intracellular cAMP and/or activity of cAMP-dependent protein kinase (PKA) during appressorium formation. We report here that much higher levels of PKA activity were detected during appressorium formation on hydrophobic surfaces than during vegetative growth on hydrophilic surfaces. These data further suggest that cAMP mediates its effect on appressorium formation through PKA in *M. grisea*.

2. Materials and methods

2.1. Fungal strain and cultural condition

Magnaporthe grisea 70-15 was kindly provided by Dr. A. Ellingboe at University of Wisconsin, Madison, and used throughout the experiments. Fungal culture was grown on oatmeal agar (50 g oatmeal per liter) at 22°C under constant fluorescent light to promote conidiation. Conidia were collected from 10-day-old cultures and washed with distilled water twice.

2.2. Protein extraction from germlings

For PKA activity measurement during conidial germination, conidial suspensions were inoculated into potato dextrose broth (PDB) and incubated at 25°C with constant shaking. Germlings were harvested at 2, 4, 8, and 16 h after incubation, washed, and macerated in liquid nitrogen. For *cpka* null transformants (I-27 and II-8), germlings were harvested at 8 h after incubation. For PKA activity during appressorium formation, conidial suspension was poured onto hydrophobic and hydrophilic surfaces of Gelbond (FMC Product, Rockland, ME) and incubated at 25°C. When 20% of germinating conidia had formed appressoria on hydrophobic surface, germlings from both hydrophobic and hydrophilic surfaces were harvested by scrapping with a razor blade. Fungal protein was extracted in ice-cold buffer including protease inhibitor (20 mM sodium phosphate, pH 7.6, 10 mM 2-mercaptoethanol, 4 mM EGTA, 0.5 mM PMSF). Protein concentration was measured by the Bradford method.

2.3. Measurement of cAMP-dependent protein kinase activity

PKA activity was measured by using a non-radioactive cAMP-dependent protein kinase assay system (Promega, Madison, WI) following the manufacturer's instructions. This assay system uses colored and fluorescent A1 peptide (L-R-R-A-S-L-G, kemptide) as a substrate. Phosphorylation of A1 peptide by PKA alters the peptide's net charge from +1 to −1. This change allows the phosphorylated and non-phosphorylated peptides to be separated by agarose gel electrophoresis at neutral pH. Phosphorylated peptides (migrate toward the positive electrode) were cut from agarose gel and kinase activity was quantified spectrophotometrically at 570 nm following the manufacturer's instructions. One unit of kinase activity was defined as the number of nanomoles of phosphate transferred to a substrate per minute per milligram. Preliminary experiments indicated that about 0.5 μg of total protein is enough to obtain reproducible data for PKA activity visually or quantitatively with spectrophotometer in *M. grisea*.

3. Results and discussion

To understand the role of PKA on appressorium formation in *M. grisea*, PKA activity was measured in germlings incubated on hydrophobic and hydrophilic surfaces of GelBond. Germlings were harvested from both sides of GelBond at a time when 20% of germlings were forming appressoria on the hydrophobic surface. Germlings incubated on hydro-

philic surfaces did not form appressoria. Germlings on hydrophobic surface showed high activity of PKA, but only detectable levels of activity were observed in germlings on the hydrophilic surface of GelBond (Fig. 1). This suggests that the PKA activity is elevated during appressorium formation on the hydrophobic surface compared to vegetative growth on the hydrophilic surface of GelBond in *M. grisea*. Since the PKA activity was proportional to the concentration of intracellular cAMP, the higher activity of PKA implies higher concentrations of cAMP in the fungal cells. PKA activity was also measured in *Colletotrichum trifolii* from conidia, germinating conidia, appressoria, and mycelia. High levels of PKA activity were detected from germinating conidia, but only basal levels of activity were detected from appressoria [15]. This might be due to the fact that appressoria were collected after maturity at which

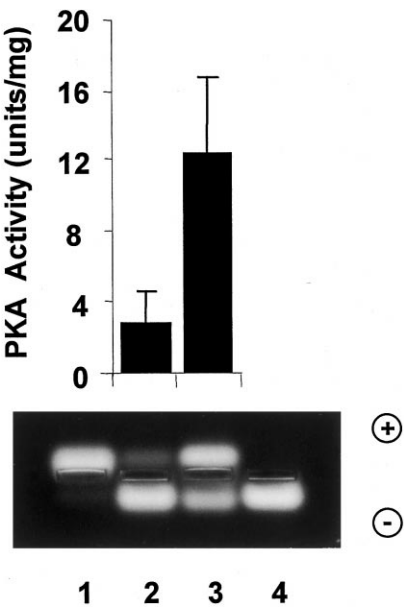

Fig. 1. Activities of cAMP-dependent protein kinase during appressorium formation in *Magnaporthe grisea*. Total protein was extracted from germlings incubated on hydrophilic (lane 2) and hydrophobic (lane 3) surfaces of the GelBond. Lanes 1 and 4 indicate positive and negative controls, respectively. Bottom: electrophoretic separation of phosphorylated and non-phosphorylated peptides. Phosphorylated (negatively charged) and non-phosphorylated (positively charged) peptides migrate toward the positive and negative electrodes, respectively. Top: quantification of PKA activity.

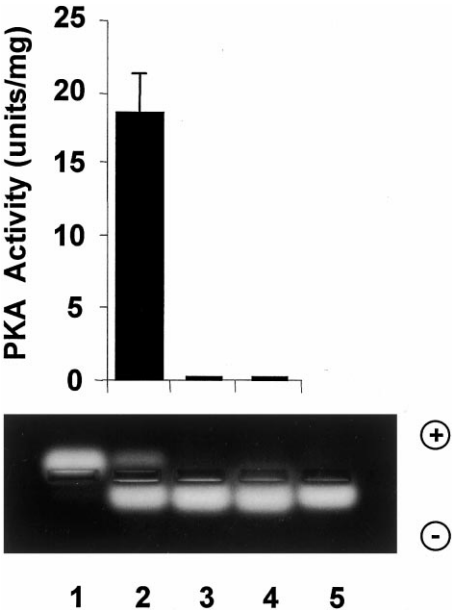

Fig. 2. Activities of cAMP-dependent protein kinase of wild type (70-15; lane 2), *cpkA* disrupted transformants (I-27 and II-8; lanes 3 and 4, respectively) of *Magnaporthe grisea*. Lanes 1 and 5 indicate positive and negative controls, respectively. Bottom: electrophoretic separation of phosphorylated and non-phosphorylated peptides. Top: quantification of PKA activity.

PKA may be unnecessary. Direct measurement of intracellular cAMP would be required to understand the precise roles of cAMP on fungal cell differentiation. However, technical difficulties exist to directly measure the concentration of cAMP in fungal cells. It has been reported that cAMP levels in fungi can change within 2 min during extraction [9].

To further understand the role of cAMP and PKA for appressorium formation, two transformants (I-27 and II-8) lacking *cpkA* through targeted disruption were tested for appressorium formation and PKA activity. These transformants delayed forming appressoria on the inductive surface when incubated over 24 h. Appressoria were small in size and not functional to penetrate host plants. No PKA activity was detected from germlings in these two transformants (Fig. 2). This finding implies that *M. grisea* contains a single copy of a catalytic subunit gene of PKA, and it further suggests that PKA is necessary to form functional appressoria in *M. grisea*. However, some other fungi have been shown to contain more than one gene encoding the catalytic sub-

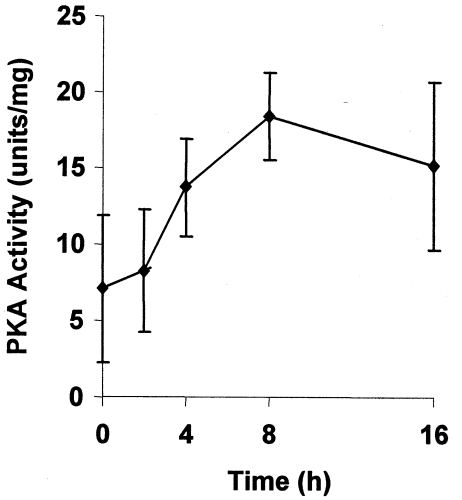

Fig. 3. Activities of cAMP-dependent protein kinase during germination of conidia in *Magnaporthe grisea*. Total protein was extracted from conidia, and 2, 4, 8, and 16 h after conidial germination in PDB.

unit of PKA [5,11]. In yeast, three catalytic subunit genes (*TPK*s) of PKA have been cloned and characterized. No two of the three genes are essential by themselves, but at least one *TPK* gene is required for a cell to grow normally [11]. However, no additional catalytic subunit gene was identified by extensive Southern blot analysis and polymerase chain reaction with degenerate primers in *M. grisea* [1,8]. Recently, divergent cAMP signaling pathways were also proposed for infection-related morphogenesis in *M. grisea* [1].

During conidial germination of *M. grisea* in PDB, PKA activity increased upon germination and peaked at 8 h after incubation before declining (Fig. 3). A similar phenomenon was also reported from *Colletotrichum trifolii* [15]. In *Mucor rouxii*, however, the level of intracellular cAMP rapidly increases within several minutes after germination, but then drops during the emergence of hyphal germ tubes and remains low during hyphal growth compared with budding growth [12].

In this paper, we demonstrated that high levels of PKA activity occurred during appressorium formation by *M. grisea* on inductive surfaces, but not during vegetative growth on non-inductive surfaces. These data further suggest that cAMP regulates appressorium formation in *M. grisea*.

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