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Stage-Specific Changes and Regulation of Endogenous Protein Phosphorylation in *Allomyces macrogynus*

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In the aquatic fungus *Allomyces macrogynus* the effects of Ca^{2+} and cAMP on the intracellular signal transduction of zoospore germination were studied using *in vitro* protein phosphorylation assay system. An endogenously phosphorylated protein (p50) having molecular weight of 50 kDa on SDS-PAGE was found in soluble fractions of both zoospore and mycelium. In zoospore extract, the endogenous phosphorylation of p50 was weak without any effectors, but was enhanced by Ca^{2+} and even more by cAMP. Phosphorylation of the same protein in mycelial extract was high only in the absence of cAMP. Irrespective of the presence of Ca^{2+} and cAMP, its phosphorylation was antagonistically suppressed in assay of combined zoospore and mycelial extracts. These results suggest that p50 is interconvertible in phosphorylation/dephosphorylation as a novel protein involved in germination of *A. macrogynus*. The antagonistic effect of cAMP to the phosphorylation of p50s from different developmental stages may be important in the regulation of cellular differentiation.

Key words: ***Allomyces macrogynus*, cellular differentiation, endogenous protein phosphorylation, cAMP, calcium ion**

The aquatic fungus, *Allomyces*, is known to be an excellent model for the study of various aspects of development. It has a well-defined gametophytic and sporophytic phase in its life cycle. During its diploid mycelial phases, diploid zoospores are produced. Upon reaching a suitable substratum, they immediately encyst and germinate. The synthesis of cell wall during encystment is known not to require protein synthesis (18). Therefore, programmed intracellular events are thought to be initiated by signal transduction, resulting in activation of suppressed proteins that are required for germination.

To investigate the regulatory mechanism of germination, extensive experimental approaches have been applied in many fungi, such as *Blastocladiella emersonii* (14, 15), *Dictyostelium discoideum* (12), *Candida albicans* (17), and *Saccharomyces cerevisiae* (16). Among many specific proteins phosphorylated in germination (1, 5, 21, 23), two have been well characterized. One is trehalase, identified in several organisms (3, 4, 16), and the other is glutamine fructose-6 phosphate amidotransferase in *B. emersonii* (6, 13). They exist in two forms, which are interconvertible by phosphorylation or dephosphorylation during cell growth. Trehalase is an enzyme catalyzing the hydrolysis of trehalose, a common reserve material in many fungi. It is activated in germination through phosphorylation by cAMP-dependent protein kinase (PKA) (22). The amidotransferase studied in *B. emersonii* catalyzes the first step in the hexosamine biosynthesis of cell wall. In zoospore, amidotransferase exists in a phosphorylated form that is inhibited by intracellular UDP-GlcNAc. Early during encystment, it is converted to a noninhibitable form by dephosphorylation and is present throughout the growth phase (8, 19). It is known to be phosphorylated by PKA and dephosphorylated by intracellular protein phosphatase 2A and 2C (7). However, it is not known how they are controlled to provoke phosphorylation/dephosphorylation of amidotransferase. Furthermore, in spite of many reports that show important roles of Ca^{2+} and cAMP in

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spore germination (9, 12, 17), it has not been demonstrated how their regulatory roles are connected to the activation of the suppressed proteins.

The aim of the present study was to examine the effects of Ca^{2+} and cAMP in endogenous protein phosphorylation of *A. macrogynus* as a strategy for identifying and further characterizing potentially novel proteins involved in germination. By using *in vitro* assay system, we have discovered on SDS-PAGE a phosphorylated 50-kDa protein that showed some unique phosphorylation features in the presence or absence of cAMP.

Materials and Methods

Organism and cultural condition

A. macrogynus(Strain Burma 3-35) was provided by Prof. C. W. Cho in the Department of Microbiology, Inje University and was maintained on PYG agar medium (peptone 1.25 g, yeast extract 1.25 g, glucose 3.0 g, agar 1.5 g in 1 liter) at 33°C. Zoospores liberated from a young sporophyte culture were inoculated on the PYG medium in petri dishes. After 3 days of incubation at 33°C, approximately 15 ml of dilute salt solution (KH_2PO_4, K_2HPO_4, $(NH_4)_2HPO_4$, $CaCl_2$, $MgCl_2$, 50 uM each) was poured onto plates and incubated at 25°C for 90 min. The zoospore suspension was collected, harvested by centrifugation at 2000 rpm, and stored in aliquots at -70°C until used. To culture mycelia, 7 ml of dense zoospore suspension was inoculated into 500 ml of 2X PYG medium in 1 liter flasks. After 24 hours of incubation at 33°C with gentle shaking, the mycelia were harvested by filtration, squeeze dried, and stored at -70°C.

Preparation of zoospore and mycelial extracts

To extract zoospore proteins, the frozen spores were suspended in four fold volumes (4 ml/gram of wet weight) of H buffer (Homogenization buffer, 10 mM Pipes, pH 7.5, 1 mM PMSF) in an Eppendorf tube and stored on ice to induce osmotic lysis. After an hour, the spore suspension was centrifuged at 13000 rpm for 3 min to discard precipitates. A small amount of mycelia was homogenized in four fold volumes (4 ml/gram of wet weight) of H buffer by using Polytron homogenizer. The mycelial suspension was centrifuged at 13000 rpm for 30 min at 4°C and the supernatant saved.

In vitro endogenous protein phosphorylation assay

Protein phosphorylation of zoospore or mycelial extract was performed for 10 minutes in a reaction mixture of 10 ul containing 10 mM Pipes, pH 7.5, 10 mM $MgCl_2$, 25~40 uM [γ-^{32}P]ATP (3000 Ci/mmol), and the extract (3.2~5.8 ug). The reaction was stopped by adding 4X SDS-PAGE sample buffer and the mixture was boiled for 2 min. The zoospore and mycelial extracts used in the assays were previously passed through Sephadex G-25 spin columns to remove endogenous small molecular weight substances. When an effector was used in the assay, it was preincubated with protein extracts for more than 30 min on ice and reaction was started by adding [γ-^{32}P]ATP. The effectors were routinely used at the following concentrations: Ca^{2+}, 1 mM; cAMP, 5 uM; EGTA, 5 mM; TFP, 200 uM. Phosphorylated proteins were analysed by SDS-PAGE (10) and autoradiography. SDS-PAGE was run on 12.5% polyacrylamide in minigel (7.3 cm×10.2 cm×0.75 mm, Biorad). The gels were stained for 15 min in Coomassie staining solution (50% methanol, 12.5% acetic acid, and 0.1% Coomassie Brilliant Blue R250), destained in 30% methanol and 10% acetic acid for 15 minutes, and equilibrated in distilled water for 5 minutes. The gels were vacuum dried and exposed for 6~12 hours to X-ray films at -70°C between intensifying screens.

Miscellaneous methods

Protein quantitation was performed with Bradford reagent (2) by using bovine serum albumin as standard protein.

Results and Discussion

Endogeneous phosphorylations of p50 in zoospore and mycelial extract

To observe endogenous protein phosphorylation in soluble fractions of both zoospore and mycelium from *A. macrogynus*, we have used an *in vitro* assay system. Optimal assay conditions were established and described in Materials and Methods. Although several phosphorylated proteins were observed during the time course of 2 min, incubation period of 10 min was used to focus our attention on a phosphorylated protein (p50) that showed a molecular weight of 50,000 on SDS-PAGE, which is different from the weights of trehalase and amidotransferase (6, 22).

The effects of Ca^{2+} and cAMP on the endogenous protein phosphorylation in zoospore extract is shown in Fig. 1A. Among several phosphorylated proteins, p50 was the most eminent. It was the only one that varied much in its phosphorylation depending on the presence of exogenous effectors in our assay condition. Phosphorylation was weak without any exogenous effectors, but was enhanced in the presence of Ca^{2+} (lane 2) and even more by cAMP (lane 5). However, the stimulatory effect of Ca^{2+} seems to be controversial, because the expected inhibitory activities of EGTA and TFP were not shown.

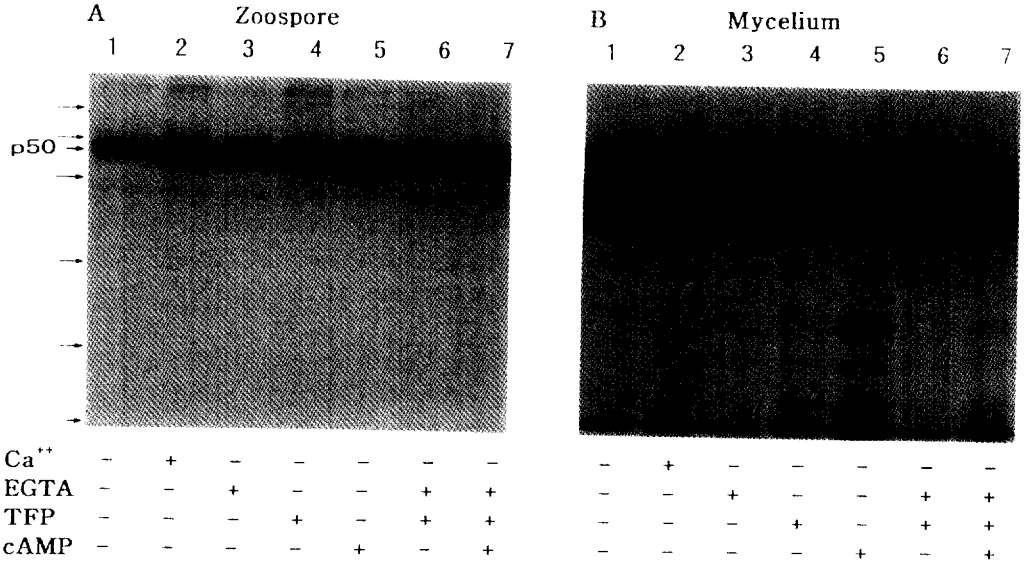

Fig. 1. Endogenous protein phosphorylations of zoospore and mycelial extracts. Desalted zoospore extract (3.5 μg) and mycelial extract (3.2 μg) were preincubated on ice for 1 h in the presence of 1 mM Ca^{2+}, 5 mM EGTA, 0.2 mM TFP, and 5 μM cAMP, respectively, as described below the figure. *In vitro* phosphorylation was performed for 10 min at 37.5℃ under standard reaction condition described in Materials and Methods. Phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Protein standard markers are indicated by arrows; phosphorylase B(97.4 kDa), serum albumin(66.2 kDa), ovalbumin(45 kDa), carbonic anhydrase(31 kDa), trypsin inhibitor(21.5 kDa), and lysozyme(14.4 kDa).

EGTA is known as a specific chelator of calcium ion and TFP is a calmodulin inhibitor. As excess amounts of EGTA and TFP were preincubated with the extracts, the effects of uneliminated Ca^{2+} or tivated form of calmodulin was thought to be negligible. It seems as if some basal level of phosphorylation activity was maintained independently of Ca^{2+} and calmodulin. It might have originated from the activity of Ca^{2+}/calmodulin-independent protein kinases, which have been identified in some fungi (11, 20).

The same endogenous phosphorylation assay was performed in the mycelial extract as shown in Fig. 1B. In contrast to the results obtained in the zoospore extract, two strong phosphorylated bands appeared only in the absence of cAMP (lane 5 and 7). The molecular weight of the higher one was also calculated as 50,000, which corresponds to the p50 found in zoospore extract. Another broad band just below the p50 was identified to consist of three bands, having similar molecular weights of 44 kDa, 42 kDa, and 40 kDa, respectively, when analysed on 10% acrylamide gel(data not shown). The phosphorylation of p50 was not responsive to the exogeneous effectors except cAMP; and the effect of cAMP was shown to be inhibitory. These characteristics are clearly the opposite of what was observed in the zoospore extract (Fig. 1A). It seems that the phosphorylation of p50 in mycelial extract is maintained in the opposite direction from that in zoospore

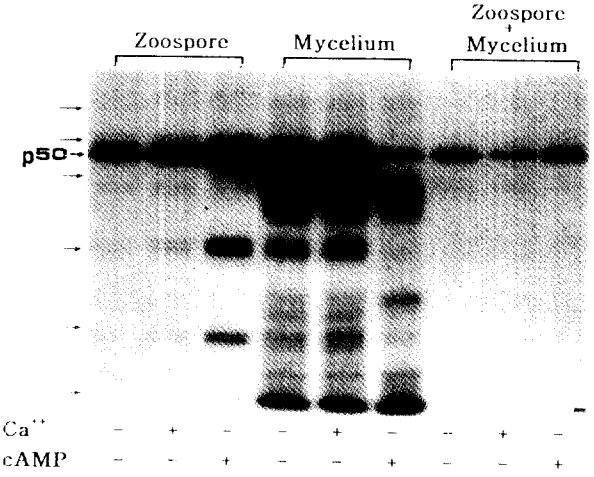

Fig. 2. Endogenous protein phosphorylation of mixed extracts. Zoospore extract (3.4 μg) and mycelial extract (3.2 μg) were assayed in the presence of Ca^{2+} (1 mM), cAMP (50 μM), or neither, as indicated below the figure. Protein extracts were desalted and preincubated with the effectors. Assay conditions were as described in Fig. 1. A half amount of each extract (zoospore, 1.7 μg; mycelial, 1.6 μg) used in individual assays was combined and assayed in the same reaction condition. Protein standard markers are indicated by arrows; 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.55 kDa, and 14.4 kDa.

extract and its reversible regulation is controlled mainly by cAMP. cAMP is implicated in the mediation of cellular events during zoospore germination in another

aquatic fungus, *B. emersonii* (9). However, the antagonistic effect of cAMP on the phosphorylation of a common substrate from different developmental stages has not been reported in any fungi. These results strongly suggest that p50 is interconvertible by phosphorylation/dephosphorylation during the life cycle, and is a novel protein involved in cellular differentiation of *A. macrogynus*.

Suppressed endogenous phosphorylation of p50 in mixed assay

To confirm whether the p50s phosphorylated in zoospore and mycelial extracts were identical and to analyze how their phosphorylation/dephosphorylation were maintained antagonistically, we have attempted an indirect approach of mixing the zoospore extract with the mycelial extract in the endogenous phosphorylation assay. A half amount of the proteins used in individual reactions of both extracts were mixed in the presence of Ca^{2+}, cAMP, or neither (Fig. 2). Compared with those in individual assays of both extracts, total protein phosphorylation was suppressed in every lane of the mixed extracts. The bands corresponding to the mobility of p50 were less intensive than predicted in the case of the simple additive. These results supports that the p50s found in both zoospore and mycelial extract are identical. Suppressed phosphorylation in mixed extracts is thought to be caused by the simultaneous presence of both activated protein kinase(s) and activated protein phosphatase(s), which are opposite in their activation states in individual extract. Simply explained, in the presence of cAMP, the protein kinase(s) are activated and protein phosphatase (s) are inactivated in zoospore extract and *vice versa* in mycelial extract. In the absence of cAMP, the situation is reversed for each of them. Therefore, irrespective of the presence of cAMP, their activities on the common substrates are counterbalanced in the mixed assay and the net results are less than predicted for simple additive behaviour. On the other hand, nonspecific interactions could have been possible between more than one kind of protein kinases and protein phosphatases, which are under different developmental regulation during the life cycle. In *B. emersonii*, PKA and protein phosphatase type 1, type 2A, and type 2C showed developmental regulation, with the highest activity found in zoospore, followed by a decrease during germination (7, 15). Although the stimulatory effect of Ca^{2+} in zoospore extract is not definitely shown, similar interpretation could be possible for the results obtained in the mixed assay; or it may reflect the fact that signal transduction leading to the phosphorylation of p50 might be more complicated *in vivo* than our simple explanation.

To summarize, (i) p50 found in *A. macrogynus* is thought to be a novel protein involved in signal transduction of germination, and (ii) the antagonistic effect of cAMP observed in the phosphorylation of p50s from different developmental stages is unique in its regulation, which has not been described in any fungi. We believe that our results presented in this paper provide a model system for elucidating germination mechanisms in fungi. In order to verify the functional significance of our suggestions *in vivo*, we are currently doing purification of p50.

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