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ENDOGENOUS SUBSTRATES FOR PROTEIN KINASES IN *COPRINUS MACRORHIZUS*

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Summary

The phosphorylation of cellular proteins in mycelia of strain Fis^c in *Coprinus macrorhizus* was examined. The phosphorylation of two proteins, Protein A and B, was stimulated by cyclic AMP in the presence of Mg^{2+}, and that of one protein, Protein C, was inhibited by cyclic AMP. The molecular weight of these proteins was determined, by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), to be 64 000 (Protein A), 46 000 (Protein B), and 18 000 (Protein C), respectively. These proteins were quickly phosphorylated and the phosphorylation reached to the maximal levels in 5 min. The concentration of cyclic AMP required for the half-maximal stimulation of phosphorylation of Protein A and B, and for the half-maximal inhibition of phosphorylation of Protein C was approx. $1.0 \cdot 10^{-7}$ M. Cyclic GMP and cyclic IMP were slightly effective for stimulation and inhibition of these proteins.

Introduction

As previously reported [1], high activities of adenylate cyclase and phosphodiesterase and accumulation of cyclic AMP were detected in monokaryotic mycelia and dikaryotic mycelia of a basidiomycete, *Coprinus macrorhizus*, which are able to form fruiting bodies, but not in monokaryotic mycelia which are unable to form fruiting bodies. From these data we suggested that cyclic AMP plays an important role in fruiting body formation of *C. macrorhizus* [1—5]. Mycelial cells of this fungus contain cyclic AMP-dependent protein kinases which phosphorylate histone, albumin and casein [6]. In mammalian cells cyclic AMP-receptor proteins were associated with cyclic AMP-dependent

Abbreviations: SDS, sodium dodecyl sulfate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

protein kinases, and these enzymes are known to be the major mediator for the metabolic and physiological effects of cyclic AMP [7—10]. Endogenous substrates for the cyclic AMP-dependent protein kinases were observed and characterized in many animal tissues [11]. Glycogen phosphorylase kinase and tyrosine hydroxylase were activated by phosphorylation via cyclic AMP and cyclic AMP-dependent protein kinase [11], but glycogen synthetase, acetyl-CoA carboxylase [12], and pyruvate kinase [13] were inactivated. Thus the activities of these key enzymes in metabolic pathways of animal tissues are regulated by cyclic AMP-dependent protein phosphorylation.

The present paper reports the detection and partial characterization of endogenous substrates for protein kinases in *C. macrorhizus*.

Materials and Methods

Materials. The following strains of *C. macrorhizus* Rea f. *microsporus* Hongo were used; Fis^{c1} A8B7, Fis^- A8B7, Fis^- A7B8, and dikaryon (Fis^- A7B8 + Fis^- A8B7). Origin and characterization of strains Fis^c and Fis^- (formerly designated *fis*^{c} and *fis*$^-$) have been described elsewhere [2].

Medium and cultivation. Mycelia were grown in a liquid malt-yeast medium [2]. Mycelial suspensions were inoculated and incubated in 100-ml Erlenmeyer flasks containing 20 ml of malt-yeast medium at 30° C for 7 days under continuous illumination (1000—6000 ergs/cm^2 per s).

Preparation of crude extract. Mycelia grown for 7 days were harvested on a filter paper, washed well with distilled water, and macerated in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM mercaptoethanol, and 1 mM EDTA with a Waring Blendor for 1 min. The suspension obtained was further homogenized with an Aminco French Pressure Cell Press (J5-598A) at 20 000 lbs/inch^2. The resulting homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant fluid was used as crude extract.

Assay for phosphorylation of cellular proteins. The standard reaction mixture (final volume 100 μl) contained 50 mM Pipes buffer (pH 7.0)/5 mM $MgCl_2$/4 μM [γ-^{32}P]ATP (12 Ci/mmol) and 70 μl of sample in the absence or presence of 1.0 μM cyclic AMP. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP and carried out at 30° C for 4 min with shaking. The reaction was terminated by the addition of 50 μl of a stop solution; 9% SDS/0.03 M Tris-HCl buffer (pH 7.0)/6% mercaptoethanol/3 mM EDTA and 15% glycerol, and the sample was heated at 100°C for 1 min; an aliquot (100 μl) of the sample was then subjected to SDS-polyacrylamide gel electrophoresis. The preparation gel contained 12% acrylamide and 0.34% *N,N'*-methylene bisacrylamide. The slab gels were stained for proteins with 0.025% Coomassie blue. The gels were destained and dried. Autoradiography was carried out as described previously [14]. Autoradiograph was scanned with a Toyo Digital Densitrol DMU-33C, and the absorbance tracing was used as a measure of the incorporation of ^{32}Pi into each protein. To obtain the scintillation spectrogram the gels were cut into pieces of 1-mm thickness and the radioactivity of each piece was counted in a liquid scintillation spectrometer. All values are expressed as pmols incorporated into each protein per mg protein.

Protein measurement. Protein concentration was measured by the method

of Lowry et al. [15] using bovine serum albumin as the standard.

Chemicals. [γ-^{32}P]ATP was purchased from The Radiochemical Centre (Amersham, U.K.); cyclic nucleotides from Sigma Chemical Co. (St. Louis, MO U.S.A.); molecular weight markers from BDH (Poole, U.K.); and Pipes from Calbiochem (La Jolla, CA, U.S.A.).

Results

Detection of endogenous substrates for protein kinases in mycelia of strain Fis^c

To find endogenous substrates for protein kinases of crude extracts of strain Fis^c were incubated with [γ-^{32}P]ATP in the presence or absence of cyclic AMP (1.0 μM), and subjected to SDS-polyacrylamide gel electrophoresis. The slab gels were stained for proteins and subjected to autoradiography. The densitograms of phosphorylated bands in the autoradiograms were prepared as shown in Fig. 1a. The same gel was dried and cut into small pieces and each piece was counted for the radioactivity (Fig. 1b). The examination of densitogram (Fig. 1a) and scintillation spectrogram (Fig. 1b) clearly indicated that the phosphorylation of two protein bands designated as Protein A and B was stimulated in the presence of cyclic AMP, and that of one other band, designated as Protein C, was inhibited.

Determination of molecular weight of Protein A, B and C

The molecular weight of Protein A, B and C was estimated by polyacrylamide gel electrophoresis in the presence of SDS, using marker proteins (Fig. 1). The minimal molecular weights estimated were approx. 64 000 for Protein A, 46 000 for Protein B and 18 000 for Protein C.

Characterization of phosphorylation reaction of Protein A, B and C

Under the standard assay conditions the levels of phosphorylation of Protein A, B and C attained to the maximum within 5 min and then decreased gradually (Fig. 2). The addition of cyclic AMP (1.0 μM) to the assay mixtures stimulated the phosphorylation reaction of Protein A and B, and inhibited that of Protein C at all incubation times tested (Fig. 2). No significant alteration in the staining pattern of these proteins by Coomassie blue was observed during incubation (data not shown), but the decrease of phosphorylation of these proteins was observed after long incubation (Fig. 2). The results indicated that crude extracts contain endogenous phosphoprotein phosphatase which dephosphorylates the proteins phosphorylated by protein kinases. The phosphorylation of Protein A, B and C was nearly proportional to the amount of crude extract proteins added up to about 0.2 mg. The pH optimum for the phosphorylation of these proteins was about 7.0. The effect of cyclic AMP on the phosphorylation of Protein A, B and C as a function of the concentration of ATP is shown in Fig. 3. The concentration of ATP was saturated for the phosphorylation of these proteins at a concentration of 20 μM, and an apparent K_m value for ATP was 5—10 μM. However, this value may be a minimum estimate of K_m value, as crude extract contains ATP hydrolyzing enzymes. The result indicated that the inhibition of phosphorylation of Protein C is not due to the relative shortage of ATP in the presence of cyclic AMP.

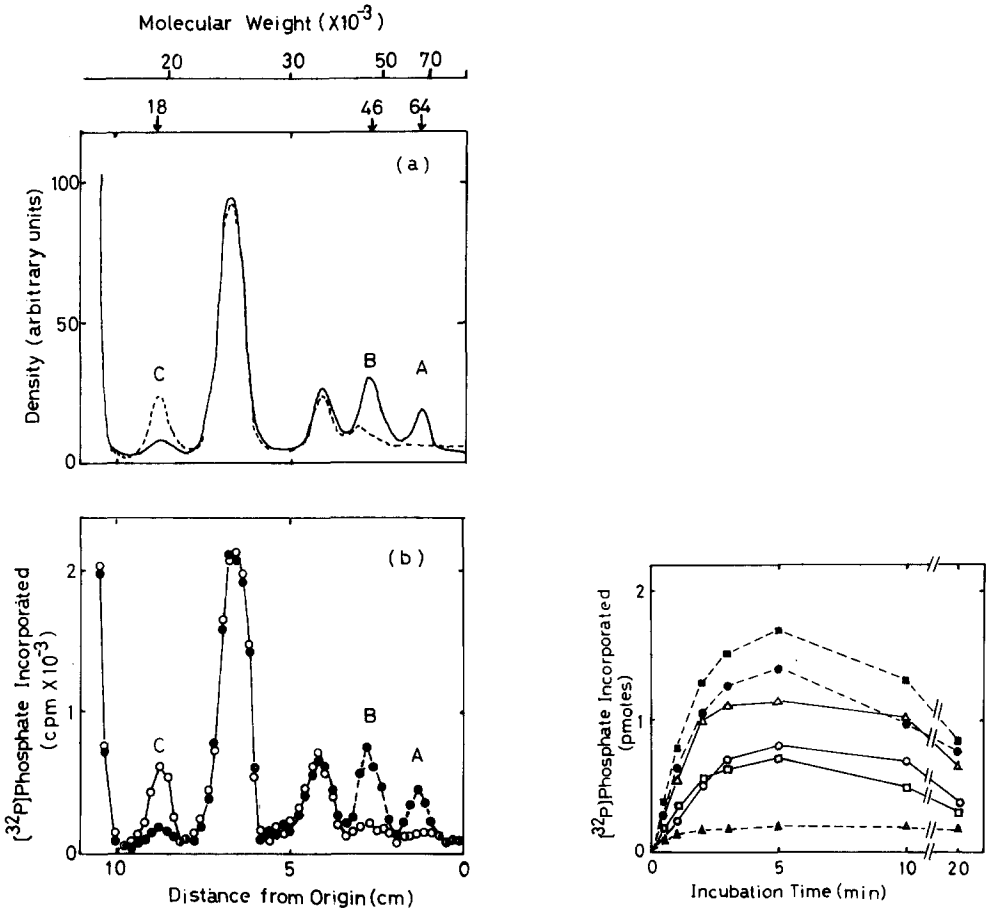

Fig. 1. Gel electrophoresis of crude extract from mycelia of strain Fis^{C} incubated with [γ-^{32}P]ATP in the presence or absence of cyclic AMP. The reaction mixtures were subjected to SDS-polyacrylamide gel electrophoresis. (a), The autoradiograms were scanned with a Toyo Digital Densitrol DMU-33C, and the density of bands was recorded. (b), The scintillation spectrograms of the gel were obtained. ———, ○, crude extract was incubated in the absence of cyclic AMP; — — —, ●, crude extract was incubated in the presence of cyclic AMP. A, B and C indicate the positions of Protein A, B and C. The BDH Molecular Weight Marker, M_r range 14 300—71 500 was used as molecular weight marker.

Fig. 2. The phosphorylation of Protein A, B and C in crude extract from mycelia of strain Fis^{C} as a function of incubation time. Incubation conditions were described in Materials and Methods, except for the variation in the incubation time. Amounts of ^{32}Pi incorporated from [γ-^{32}P]ATP into Protein A (○, ●), B (□, ■), and C (△, ▲) were measured. Open and closed symbols represent the incubation in the absence and presence of cyclic AMP (1.0 μM), respectively.

The concentration of cyclic AMP required to give the half-maximal levels of phosphorylation of Protein A and B, and that of inhibition of phosphorylation of Protein C was estimated to be approx. $1.0 \cdot 10^{-7}$ M (Fig. 4). On the other hand, high concentrations of cyclic GMP (more than $1.0 \cdot 10^{-6}$ M) were required to stimulate the phosphorylation of Protein A and B, and to inhibit the phosphorylation of Protein C, but the effects of cyclic GMP were slight comparing those of cyclic AMP (Fig. 4). The effects of cyclic IMP on the phosphorylation of these proteins were similar to those of cyclic GMP, but the addition of cyclic

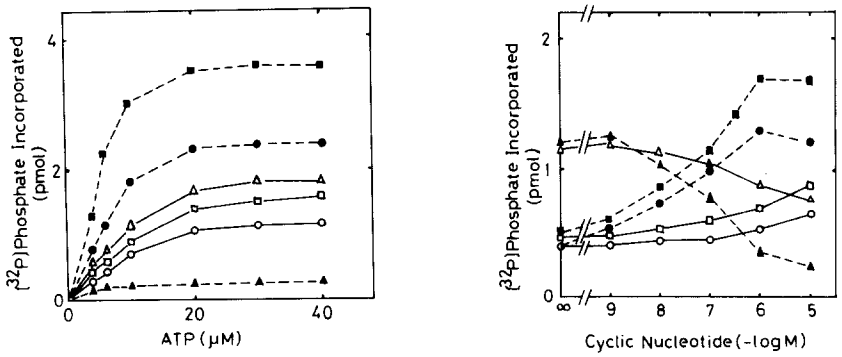

Fig. 3. Effect of varying the concentration of ATP on the phosphorylation of Protein A, B amd C. Incubation conditions were as described in Materials and Methods except for the variation in ATP concentration. See Fig. 2 for symbols.

Fig. 4. Effect of varying the concentration of cyclic AMP and cyclic GMP on the phosphorylation of Protein A, B and C. Incubation conditions were as described, except for the variation in the concentration of cyclic nucleotides. Closed and open symbols represent the addition of cyclic AMP and cyclic GMP, respectively. See Fig. 2 for symbols.

CMP and cyclic UMP showed no positive effects. The effects of these cyclic nucleotides on protein phosphorylation were essentially the same, when these experiments were carried out for 2 min.

Discussion

The occurrence of cyclic AMP-dependent and inhibited protein kinases was reported in the crude extract of *C. macrorhizus* [6]. The substrates used for the assay of these eznymes were calf thymus histone, bovine serum albumin, and casein. The phosphorylation of Protein A and B identified in the present study was stimulated by the addition of cyclic AMP, and therefore these proteins may be in vivo substrates of cyclic AMP-dependent protein kinases that are activated by cyclic AMP. As shown in Fig. 2, phosphorylation sites sensitive to cyclic AMP may be different from those phosphorylated in the absence of cyclic AMP, because cyclic AMP increased the extent and the rate of phosphorylation of Protein A and B. We have previously reported that a cyclic AMP-dependent protein kinase was involved for the activation of glycogen phosphorylase in this fungus [16]. It is suggested that the glycogen phosphorylase may be one of the in vivo substrates of cyclic AMP-dependent protein kinases in the mycelial cells of this fungus, but no direct evidence for this has been obtained.

The concentrations of cyclic AMP required to give the half-maximal levels of phosphorylation were higher than those previously reported [6]. Although crude extracts contains phosphodiesterase were used as enzyme source in this study, partially purified cyclic AMP-dependent protein kinase free from phosphodiesterase was used in the previous study. Therefore, this difference may be caused by hydrolysis of cyclic AMP by phosphodiesterase action.

On the other hand, the phosphorylation of Protein C was inhibited by cyclic AMP, and therefore this protein may be a substrate of cyclic AMP-inhib-

ited protein kinase. In a few organisms cyclic AMP-inhibited protein kinase was observed [17], but in many other organisms this kind of enzyme could not be detected. The present paper first indicates the presence of an endogenous substrate for cyclic AMP-inhibited protein kinase. In animals cyclic AMP-dependent phosphorylation of many proteins was observed [11]. However, cyclic AMP exceptionally inhibited autophosphorylation of the regulatory subunit of type II cyclic AMP-dependent protein kinase in the presence of $ZnCl_2$, but stimulated in the absence of $ZnCl_2$ [14]. In *C. macrorhizus* cyclic AMP inhibited Protein C phosphorylation in the presence of excess amount of EDTA (data not shown), and therefore this possibility could be excluded in the case of Protein C.

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