Evidence for an arginine-specific mono(ADP-ribosyl)transferase in dormant spores of the fungus *Phycomyces blakesleeanus*

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A soluble mono(ADP-ribosyl)transferase was detected in dormant spores of *Phycomyces blakesleeanus*. Soluble proteins incubated with [³²P]NAD revealed, after two-dimensional electrophoresis, three major ADP-ribosylated substrates with molecular masses of 38, 37 and 36 kDa and pI values of 6.9, 8.1 and 4.6, respectively. When these endogenous substrates were first ADP-ribosylated with [³²P]NAD and then incubated for different times with either 3 M hydroxylamine (pH 7.0) or 1 mM HgCl₂, only hydroxylamine released the incorporated radioactivity after 30 min incubation. Additionally, agmatine was used as a substrate for this enzyme. These data suggest that the mono(ADP-ribosyl)transferase is an arginine-specific enzyme. This enzymic activity was stimulated by 10 mM MgCl₂ and by 250 µM of the nitric-oxide-releasing agent sodium nitroprusside, and inhibited by 8 mM benzamide, 0.4 mM *m*-iodobenzylguanidine and 0.5 mM novobiocin. The three ADP-ribosylation inhibitors affected the germination of *P. blakesleeanus* spores, leaving them as swollen cells. The effect of MgCl₂, GTP and ATP on the ADP-ribosylation of the endogenous proteins was studied. The presence of two additional [³²P]ADP-ribosylated proteins of 57 and 55 kDa was observed in the absence of MgCl₂. An increase in incorporation of radioactivity into the 55 kDa band was observed when the assay was performed in the presence of GTP or ATP. The addition of Mg²⁺ together with either or both nucleotides eliminated the appearance of the 57 and 55 kDa bands, but intensified the 37 kDa band. Photoaffinity-labelling of the soluble fraction with [α -³²P]GTP revealed a 55 kDa band together with other proteins of 32 and 17 kDa. These results suggest that among the five different endogenous substrates for the fungal mono(ADP-ribosyl)transferase, the 55 kDa protein may be a GTP-binding protein.

Keywords: ADP-ribosyltransferase, *Phycomyces blakesleeanus*, GTP-binding protein, dormant spores

INTRODUCTION

Mono-ADP-ribosylation is a post-translational modification of proteins by the enzymic transfer of ADP-ribose from NAD, resulting in alteration of the functional properties of the respective proteins. Mono(ADP-ribosyl)transferases have been identified in a range of eukaryotic cells and tissues. They have been purified from turkey red blood cells, hen and frog liver, and rabbit skeletal muscle. All of these enzymes catalyse the transfer

of ADP-ribose from NAD to arginine (Williamson & Moss, 1990). Also, a cysteine-specific ADP-ribosyltransferase has been purified from human erythrocytes (Jacobson *et al.*, 1990).

ADP-ribosylation is a reversible modification cycle of proteins, since ADP-ribosylarginine and ADP-ribosylcysteine hydrolases have been identified in animal tissues in which the respective transferases have been detected (Moss *et al.*, 1985; Williamson & Moss, 1990). The role of mono-ADP ribosylation in eukaryotes is less well characterized than in prokaryotes, although it has been suggested by different authors that this modification could

Abbreviations: MIBG, *m*-iodobenzylguanidine; NO, nitric oxide.

participate in a number of biological processes, for example stimulation of adenylyl cyclase activity, inhibition of Na^+ -dependent phosphate transport, increase in light sensitivity of salamander retinal rods, modulation of starvation and stress responses in cultured cells, regulation of Ca^{2+} release, inhibition of Ca^{2+} -dependent ATPase activity, and alteration in protein phosphorylation (reviewed by Williamson & Moss, 1990). It has been demonstrated that endogenous ADP-ribosylation is under the regulatory control of hormones and second messengers (Brüne & Lapetina, 1989; Molina y Vedia *et al.*, 1989; Brüne *et al.*, 1990). Therefore, these findings suggest a possible role of ADP-ribosylation in the signal-transduction pathways mediated by an agonist-receptor system. On the other hand, it has been reported that a brefeldin A-sensitive mono(ADP-ribosyl)transferase might be involved in membrane traffic (De Matteis *et al.*, 1994).

Phycomyces blakesleeanus is a zygomycete fungus whose spores show an endogenous dormancy, that is, they need an activation treatment to induce germination. The germination process involves a morphological transition from an elliptical spore to a spherical cell, with subsequent tube formation and development into mycelial cells in a suitable culture medium. Immediately after the activation treatment, the cytoplasmic cAMP levels are transiently elevated, suggesting that this second messenger might be the trigger of spore germination (for a review, see Van Laere *et al.*, 1987). Mutants that do not germinate show no increase in cAMP (Van Laere & Rivero, 1986; Rivero & Cerdá-Olmedo, 1987), strongly supporting the hypothesis that this nucleotide is a critical agent in breaking *P. blakesleeanus* spore dormancy. Other rapid metabolic changes include an increase in the concentration of fructose 2,6-bisphosphate, and in the enzymic activities of trehalase and glycerol-3-phosphatase. These enzymic activities are regulated by phosphorylation, probably through a cAMP-dependent protein kinase (Van Laere & Hendrix, 1983; Van Mulders & Van Laere, 1984; Van Laere *et al.*, 1987).

We are interested in the elucidation of the biochemical events underlying spore germination in *P. blakesleeanus*. The purpose of this study was to characterize endogenous ADP-ribosylation in dormant spores in an attempt to identify substrate proteins in the fungus.

METHODS

Strain. The wild-type strain of *P. blakesleeanus* NRRL 1555 (–) (Northern Regional Research Laboratory, USDA, Peoria, IL, USA) was used throughout this work.

Media and growth conditions. The fungus strain was maintained and propagated in YPG medium (Bartnicki-Garcia & Nickerson, 1962). Sporangiospores were produced on YPG solidified with 2% (w/v) agar from cultures incubated at 24 °C for 6 d under diffused light.

To promote germination and growth, the spores were activated chemically by including ammonium acetate to a final concentration of 30 mM in Sutter medium composed of glucose and asparagine (Sutter, 1975). Benzamide, *m*-iodobenzylguanidine

(MIBG) or novobiocin were added to different culture flasks at the concentrations indicated in Results before spore inoculation. Germination was determined after 20 h growth and expressed as the percentage of cells exhibiting a germ tube (200 cells were counted).

Preparation of cell-free extracts. Cell-free extracts from dormant spores were obtained basically as described by Carrillo-Rayas *et al.* (1988), with some modifications. Briefly, the spores were washed and resuspended in 50 mM Tris/HCl buffer (pH 7.0) containing 10% (v/v) glycerol, 5 mM EGTA, 5 mM EDTA, and 5 µg antipain ml^{-1} . About 5 ml (10^{10} spores) of the suspension was mixed with an equal volume of glass beads (0.45–0.50 mm diam.) and broken in a Braun model MSK cell homogenizer for 180 s while cooling with a stream of CO_2 . The crude extract was centrifuged at 100 000 *g* (r_{av}) for 60 min and the supernatant (cytosol) was divided into aliquots and kept at –20 °C until use.

Assay of ADP-ribosylation. ADP-ribosylation was carried out in a reaction mixture (40 µl) composed of: 40 mM MES (pH 6.0), 1–10 µM [^{32}P]NAD (3–5 µCi per assay; 1 µCi = 37 kBq) and 75–100 µg protein (as indicated in Results). When indicated, 10 mM MgCl_2 , 10 mM DTT, 1 mM ATP and 1 mM GTP were added separately or combined. The reaction was terminated by adding 100 µl 50% (w/v) trichloroacetic acid (TCA). Precipitates were collected onto GF/B Whatman filters, washed, dried, and radioactivity was measured in a Beckman LS 7800 spectrophotometer. To visualize endogenous ADP-ribosylated proteins, assays were done as described above, but the reaction was terminated by the addition of SDS-PAGE solubilization buffer (2×). Samples were boiled for 3 min and subjected to SDS-PAGE (10%, w/v, acrylamide) (Laemmli, 1970). After electrophoresis the gels were stained with Coomassie blue, dried, and exposed (7–10 d) to X-OMAT films for autoradiography. For the two-dimensional gel electrophoresis, the reaction was terminated by the addition of lysis buffer (9.8 M urea, 2% NonidetP-40, 2% (v/v) ampholytes pH 7–9, 100 mM DTT) and separated by IEF-SDS-PAGE as described by Bravo (1984). When ADP-ribosyltransferase activity was assayed with 75 mM agmatine as exogenous substrate, samples were diluted fivefold with water after 90 min incubation at 30 °C. [^{14}C]ADP-ribosyl agmatine was separated from [^{14}C]NAD on a Partisil 10 SAX column (4.6 × 250 mm) used with a mobile phase of 25 mM potassium phosphate buffer (pH 4.7) with a flow rate of 1 ml min^{-1} (Moss *et al.*, 1983). The diluted fraction (250 µl) was injected onto the HPLC column and fractions of 0.5 ml were collected and radioactivity was measured as described above. Under these experimental conditions [^{14}C]ADP-ribosylated agmatine and [^{14}C]NAD had retention times of 5 min and 8 min, respectively.

[α - ^{32}P]GTP photoaffinity-labelling of soluble proteins. This procedure was the same as that described by Martínez-Cadena *et al.* (1995).

Protein determination. Protein concentration was estimated by the Lowry method, using serum bovine albumin as standard.

RESULTS AND DISCUSSION

A soluble mono(ADP-ribosyl)transferase activity was detected in extracts of dormant spores of *P. blakesleeanus*. This enzymic activity increased linearly up to 200 µg protein and 90 min incubation, after which no change of activity was observed. Additionally, this enzymic activity showed an optimum pH of 6.5, and used casein and albumin as substrates. The activity was lost after in-

Fig. 1. Two dimensional electrophoresis of [32 P]ADP-ribosylated soluble proteins from *P. blakesleeana*. The cytosolic fraction (100 μ g protein) was incubated under ADP-ribosylation conditions as described in Methods. Proteins were separated by two-dimensional IEF-SDS-PAGE followed by autoradiography. The same results were observed in three other independent experiments.

cubation at 95 °C for 5 min, therefore discounting the addition of ADP-ribose adducts to the protein (data not shown). When the soluble extracts were investigated for the presence of endogenous substrates, a broad band with molecular mass of ~ 37 kDa was observed by SDS-PAGE and autoradiography to be a very good substrate for this enzyme (Fig. 1). This modified protein was subjected to isoelectric focusing gel separation and three major bands of 38/6.9, 37/8.1 and 36/4.6 kDa/pI were observed (Fig. 1).

Meyer *et al.* (1988) have reported different chemical treatments which can determine the amino acid bound to the ADP-ribose; for example, hydroxylamine and HgCl_2 have been used to determine arginine and cysteine modification, respectively. Thus, we used these chemical treatments to determine the amino acid modified by the *Phycomyces* enzyme. Fig. 2 shows that no significant decrease in radioactivity was observed in the presence of 1 mM HgCl_2 . The radioactivity was partially released from the endogenous [32 P]ADP-ribosylated substrates after 30 min and totally after 60 min of incubation in the presence of 3 M hydroxylamine (pH 7.0). It has been reported that the polymers of ADP-ribose (ADP-ribose-glutamate) are hydroxylamine-labile and totally released after a short period (10 min) of incubation with 3 M hydroxylamine (pH 7.0) (Riquelme *et al.*, 1979). Our data with the hydroxylamine treatment eliminate the possibility of a poly(ADP-ribosyl)synthetase under our experimental conditions. Additionally, this mono(ADP-ribosyl)transferase used as an ADP-ribose acceptor a compound containing a guanidino moiety such as agmatine (data not shown). Smets *et al.* (1990) have reported that MIBG is a high-affinity substrate for the mono(ADP-ribosyl)transferase of turkey erythrocyte membranes and of cholera toxin. Under our experimental conditions, the fungal transferase did not use

Fig. 2. Analysis of the ADP-ribose amino acid acceptor. The cytosolic fraction (100 μ g protein) from *P. blakesleeana* was incubated under ADP-ribosylation conditions. After 1 h at 30 °C, samples were heated at 95 °C for 3 min and divided into three aliquots. Where indicated, 3 M hydroxylamine (pH 7) or 1 mM HgCl_2 was added. Samples were withdrawn after 30 and 60 min incubation and [32 P]ADP-ribosylated proteins were separated by 10% SDS-PAGE followed by autoradiography. Lanes: 1, control 30 min; 2, control 60 min; 3, HgCl_2 30 min; 4, HgCl_2 60 min; 5, 3 M hydroxylamine 30 min; and 6, hydroxylamine 60 min. The same results were obtained in three other independent experiments.

10 mM MIBG as a substrate. These results indicate that the enzyme is an NAD:arginine-specific (ADP-ribosyl)transferase.

Biochemical characteristics of the ADP-ribosyltransferase

It has been reported that Mg^{2+} stimulates the enzymic activity of ADP-ribosyltransferases detected in different types of cells (Soman *et al.*, 1984). We investigated the ADP-ribosylation of endogenous proteins in the absence or presence of MgCl_2 alone or in combination with GTP or ATP. Fig. 3(a) shows the presence of two additional [32 P]ADP-ribosylated proteins, a predominant and a minor band with molecular masses of 55 and 57 kDa, respectively, when the assay was performed in the absence of Mg^{2+} . An increase in incorporation of radioactivity into the 57 kDa band was observed when the enzymic assays were performed in the presence of GTP or ATP. The addition of Mg^{2+} together with either or both nucleotides eliminated the ADP-ribosylation of the 57 and 55 kDa bands, but intensified the radiolabelling in the 37 kDa band. When casein was used as a substrate for ADP-ribosylation, it was observed that MgCl_2 and GTP added independently stimulated the [32 P]ADP-ribose incorporation, whereas ATP caused inhibition of the enzymic activity (data not shown).

In mycelia of *Neurospora crassa*, Hasunuma (1991) reported at least seven different guanine-nucleotide-binding proteins which were endogenously ADP-ribosylated. There-

Fig. 3. Endogenous ADP-ribosylation and photoaffinity-labelling by [α -³²P]GTP of soluble proteins. (a) The cytosolic fraction (100 μ g protein) of *P. blakesleeanus* was incubated under ADP-ribosylation conditions and 10 mM MgCl₂, 1 mM GTP or/and 10 mM ATP were added as indicated. (b) Photoaffinity-labelling was assayed by irradiating the soluble proteins (100 μ g) with UV light for 10 min in the presence of [α -³²P]GTP. In both (a) and (b), proteins were then separated by 10% SDS-PAGE followed by autoradiography. The position of the 55 kDa protein (see text) is marked by an asterisk. The same results were obtained in three other independent experiments.

fore, the cytosolic fraction was incubated with [α -³²P]GTP and the presence of GTP-binding proteins was detected by photoaffinity labelling as described in Methods. Fig. 3(b) shows that three major bands with molecular masses of 55, 32, and 17 kDa bound [α -³²P]GTP; these results suggest that the 55 kDa protein which is a substrate for the (ADP-ribosyl)transferase may be a GTP-binding protein.

Sodium nitroprusside (Brüne & Lapetina, 1989) and benzamide (Tanigawa *et al.*, 1984; Banasik *et al.*, 1992) have been described as positive and negative effectors of ADP-ribosylation, respectively. When the effect of these compounds on the enzymic activity was tested in the presence of MgCl₂, 0.1 mM sodium nitroprusside enhanced the activity by a factor of 1.23, whereas 4 mM benzamide inhibited activity by almost 50% (data not shown). These results agree with those observed for other ADP-ribosyl transferases (Tanigawa *et al.*, 1984).

Furthermore, we investigated the effect of sodium nitroprusside, which is a NO-releasing agent, and benzamide on the [³²P]ADP-ribosylation of the 37 kDa protein; the cytosolic proteins were incubated under ADP-ribosylation conditions in the presence of different concentrations of these compounds. Fig. 4(a) shows that the intensity of the labelled band increased with the sodium nitroprusside concentration and decreased when the benzamide concentration was increased. Densitometric analysis of the radiolabelled bands revealed a 300% stimulation with 250 μ M sodium nitroprusside and a 50%

Fig. 4. Effect of nitroprusside, benzamide, MIBG and novobiocin on *P. blakesleeanus* ADP-ribosyltransferase activity. (a) The cytosolic fraction (100 μ g protein) was incubated under ADP-ribosylation conditions and, where indicated, different concentrations of sodium nitroprusside (NP) or benzamide (BzA) were added to the reaction mixture. (b) The cytosolic fraction (100 μ g protein) was incubated under ADP-ribosylation conditions and, where indicated, different concentrations of MIBG or novobiocin (NB) were added to the reaction mixture. After 1 h incubation proteins were separated by 10% SDS-PAGE followed by autoradiography. The same results were obtained in two other independent experiments.

inhibition with 8 mM benzamide. A NO-dependent endogenous cysteine-specific ADP-ribosylation of a 41 kDa protein has been reported in *Dictyostelium discoideum* (Tao *et al.*, 1992). McDonald & Moss (1993) reported that the NO-releasing agent sodium nitroprusside produces the covalent binding of NAD through non-enzymic S-nitrosylation of cysteine residues in those proteins where stimulation occurred. We observed that heated (95 °C, 3 min) soluble extracts incubated in either the absence or the presence of 200 μ M sodium nitroprusside failed to show detectable NAD incorporation into the 37 kDa protein (data not shown). These observations eliminate the possibility of non-enzymic addition of NAD adducts in *P. blakesleeanus* protein. Banasik *et al.* (1992) reported an IC₅₀ value of 4.5 mM for the effect of benzamide on the mono(ADP-ribosyl)transferase from hen heterophils, which agrees with our data on inhibition of the fungal enzyme.

Also, the effect of two other strong mono-ADP-ribosylation inhibitors, MIBG and novobiocin, was studied. Fig. 4(b) shows that the intensity of ADP-ribosylation of the 37 kDa band was decreased by incubation of the extracts with either MIBG or novobiocin; densitometric analysis of the bands indicated a 25% inhibition with

Fig. 5. Effect of different inhibitors on the germination of *P. blakesleeenii* spores. Spores ($5 \times 10^5 \text{ ml}^{-1}$) were inoculated in flasks containing liquid Sutter medium (Sutter, 1975), 30 mM ammonium acetate and different concentrations of benzamide (▲), MIBG (●) or novobiocin (■). After 20 h growth, germination was followed microscopically. Two hundred cells were counted. Similar results were obtained in three different experiments.

0.4 mM MIBG and a 36% inhibition with 0.5 mM novobiocin (data not shown). These data are somewhat higher than those reported for the mono(ADP-ribosyl)-transferase activity (IC_{50} 0.1 mM for MIBG and 0.28 mM for novobiocin) from turkey erythrocyte membranes (Smets *et al.*, 1990; Banasik *et al.*, 1992).

Since we observed an inhibitory effect on the ADP(ribosyl)-transferase activity of spore extracts, we investigated the effect of these drugs on the germination process of *P. blakesleeenii*. Fig. 5 shows that the concentrations necessary to inhibit 50% of the spore germination were 0.05 mM, 0.2 mM and 8 mM for novobiocin, MIBG and benzamide, respectively. All the above inhibitors stopped the germination process, preventing germ tube protrusion and leaving the spores as swollen cells. These data suggest that ADP-ribosylation may be involved in the germination process of *P. blakesleeenii*, particularly in germ-tube formation.

The results presented in this work indicate the presence of a soluble NAD:arginine ADP-ribosyltransferase in dormant spores of *P. blakesleeenii* which could be involved in the germination of the spores, particularly in germ-tube extrusion, and of five major soluble endogenous substrates, of 57, 55, 38, 37 and 36 kDa. They also suggest that the 55 kDa protein could be a G protein. Work is in progress to identify the nature of these proteins.

ACKNOWLEDGEMENTS

M.A.D.A. is a predoctoral student with a scholarship from CONACyT. This work was supported by the Consejo Nacional de Ciencia y Tecnología de México (grants N305 and N3032) and the Third World Academy of Sciences (grant BC90-111). We gratefully thank Dr J. Félix Gutiérrez-Corona for critical reading of the manuscript.

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Received 22 February 1996; revised 12 May 1996; accepted 12 June 1996.