Glyceraldehyde-3-phosphate dehydrogenase is negatively regulated by ADP-ribosylation in the fungus *Phycomyces blakesleeanus*

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Dormant spores of *Phycomyces blakesleeanus* contain a 37 kDa protein that is endogenously mono-ADP-ribosylated. This protein was purified and identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by N-terminal sequencing and homology analysis. GAPDH enzymic activity changed dramatically upon spore germination, being maximal at stages where ADP-ribosylation was nearly undetectable. The presence of glyceraldehyde 3-phosphate in this reaction affected the [^{32}P]ADP-ribosylation of the GAPDH. ADP-ribosylation of the GAPDH occurred by transfer of the ADP-ribose moiety from NAD to an arginine residue. A model for the regulation of GAPDH activity and its role in spore germination in *P. blakesleeanus* is proposed.

Keywords: regulation, spores, germination, zygomycete, GAPDH

INTRODUCTION

In many fungi the spores do not germinate when provided with conditions suitable for growth of the organism. Instead, they require activation via physical or chemical treatment. The spores of the zygomycete fungus *Phycomyces blakesleeanus* must be activated by heat shock or treatment with certain monocarboxylic acids in order to germinate and grow in a suitable culture medium (Van Laere, 1986).

Although dormant spores exhibit a very low metabolism, the components of most pathways are present and ready to operate (Van Laere *et al.*, 1987). Upon germination, rapid metabolic changes occur which include an increase in the concentration of fructose 2,6-bisphosphate, and in trehalase activity. The latter is regulated by phosphorylation, most probably through a cAMP-dependent protein kinase (Van Laere & Hendrix, 1983; Van Mulders & Van Laere, 1984; Van Laere *et al.*, 1987). In addition to trehalase, other enzymes associated with germination have been isolated and characterized in some detail. One of these proteins is glycerol-3-phosphatase. When measured under physiological conditions, glycerol-3-phosphatase is activated both *in vivo* and *in vitro* by a cAMP-dependent process (Van Schaftingen & Van Laere, 1985). More recently, we have

identified mono(ADP-ribosyl)transferase activity in spores of *P. blakesleeanus*, but its role in the germination process of this fungus remains unclear (Deveze-Alvarez *et al.*, 1996).

Mono-ADP-ribosylation, like phosphorylation, is a post-translational modification and is effected by the enzymic transfer of the ADP-ribose moiety from NAD⁺ with release of nicotinamide. ADP-ribosylation often results in activation of the target protein. Some of the transferases can also use an amino acid or water instead of the amino acid in the target protein as an acceptor. Some of the transferases also catalyse the covalent attachment of ADP-ribose to themselves. Mono(ADP-ribosyl)transferase enzymes have been identified in different eukaryotic cells and tissues. Most of the characterized enzymes catalyse the transfer of ADP-ribose from NAD to arginine (Williamson & Moss, 1990). A cysteine-specific ADP-ribosyltransferase has been purified from human erythrocytes (Jacobson *et al.*, 1990), but its endogenous substrates are poorly characterized.

ADP-ribosylation is a reversible modification of proteins. That is, both ADP-ribosyltransferases and ADP-ribosylhydrolases have been identified in animal tissues (Williamson & Moss, 1990; Moss *et al.*, 1985). The role of mono-ADP-ribosylation in eukaryotic cells is less well characterized than in prokaryotes, although it has been suggested by different authors that this modification is involved in a number of biological processes.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GA3P, glyceraldehyde 3-phosphate.

Mono-ADP-ribosylation in eukaryotes has been implicated in modulation of cell-cell interactions, signal transduction, the architecture of the cellular cytoskeleton, and vesicular transport (Koch-Nolte & Haag, 1997).

We are interested in revealing the biochemical and molecular mechanisms involved in spore germination. In the present study, we have identified the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as one of the substrates of the arginine-specific ADP-ribosyltransferase present in dormant spores of *P. blakesleeanus*. We have also shown that ADP-ribosylation inhibits the enzymic activity of GAPDH and that this inhibition is overruled by glyceraldehyde 3-phosphate (GA3P).

METHODS

Preparation of cytosolic fractions (S31K). The wild-type strain of *P. blakesleeanus* NRRL 1555(–) (Northern Regional Research Laboratory, Peoria, IL, USA) was used throughout this study; it was maintained on slants of YPG medium (Bartnicki-García & Nickerson, 1962). Spores were harvested as described by Martínez-Cadena & Ruiz-Herrera (1987). To promote germination and growth, the spores were heat shocked (48 °C, 15 min) before inoculation. Spores were resuspended in 50 mM Tris/HCl buffer (pH 7.0) containing 10% (v/v) glycerol, 10 µg antipain ml⁻¹, 10 µg soybean trypsin inhibitor ml⁻¹, 1 mM PMSF, 5 mM EGTA and 5 mM EDTA, and disrupted in a Braun homogenizer for 180 s while cooling with a stream of CO₂. The crude extract was centrifuged at 100 000 g (*r*_{av}) for 60 min at 4 °C. The supernatant (S31K) was used as cytosolic fraction for the ADP-ribosylation assays.

ADP-ribosylation assay. The S31K fraction (100 µg) was added to the incubation mixture (final volume 40 µl) containing 40 mM Tris/HCl (pH 7.4), 10 mM DTT, 5 µM [³²P]NAD (5 µCi per assay; 1 µCi = 37 kBq), 10 mM MgCl₂, 1 mM ATP, 1 mM GTP. Incubation was carried out for 1.5 h at 37 °C and the reaction was terminated with 4×SDS gel loading buffer [0.25 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol]. Samples were boiled for 3 min and separated by 10% SDS-PAGE (Laemmli, 1970). Gels were fixed, stained with Coomassie brilliant blue R-250, destained and dried. Dry gels were exposed for 1 week to X-OMAT films for autoradiography.

GAPDH activity assay. GAPDH activity of the S31K fraction and of partially purified preparations was monitored by following the reduction of NAD⁺ to NADH at 340 nm as described by Shu-Chan & Moulday (1990). All assays were carried out in 30 mM pyrophosphate buffer, pH 8.4, in a final volume of 1 ml, containing 12 mM sodium arsenate, 1 mM NAD⁺ and 1.5 mM GA3P. One milliunit (mU) of enzyme activity is defined as the production of 1 µmol NADH min⁻¹ (mg protein)⁻¹ using 6.22 mM⁻¹ cm⁻¹ as the absorption coefficient for NADH.

Partial purification. The cytosolic fraction (50 ml) containing 30 mg total protein was separated by isoelectric focusing in a Rotofor Cell (Bio-Rad) for an initial fractionation in a wide-range pH gradient (pH 3–10; ampholytes were used at a final concentration of 2%). Constant power (15 W) was applied for 5 h at 4 °C. Runs were terminated when the voltage had stabilized for about 30 min. Twenty Rotofor fractions were collected, and each fraction was assayed for ADP-ribosyl-

transferase activity. [³²P]ADP-ribosylated proteins were analysed by SDS-PAGE. Fractions in which [³²P]ADP-ribosylated proteins were detected were pooled and further separated by a second isoelectric focusing in the Rotofor Cell using a narrow pH gradient (pH 5.7–6.0). An ADP-ribosylation assay was carried out on each of the 20 Rotofor fractions, and those with activity were pooled. The pool was divided into two parts, one of which was loaded onto a Superdex 200 HR 10/30 column. The column was equilibrated with 50 ml (two column volumes) of elution buffer (50 mM Tris/HCl, pH 7.5). The sample (0.5 ml) was loaded and eluted with the same buffer into 0.3 ml fractions. Protein content was estimated by absorbance at 280 nm. The collected fractions were analysed by 10% SDS-PAGE. The other part of the pool was used for N-terminal sequencing. Basically, the proteins were incubated with [³²P]NAD and separated by preparative SDS-PAGE followed by transfer to a PVDF membrane and staining with Coomassie blue. The 37 kDa [³²P]ADP-ribosylated protein was visualized by autoradiography and N-terminally sequenced by Bio-Synthesis (Lewisville, TX, USA).

Chemical stability of the ADP-ribose-protein bond. This was determined as previously described (Aktories *et al.*, 1988). One sample was incubated for 60 min under ADP-ribosylation conditions as described above, and then divided into four portions. Each of these was treated with 1 M hydroxylamine (pH 7.5), 2 mM HgCl₂, 50% formic acid or water. Incubation was carried out for 60 min at 30 °C and samples were processed for electrophoresis.

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

RESULTS

Identification of a 37 kDa ADP-ribosylated protein from the S31K fraction in *P. blakesleeanus*

Soluble extracts of spores of *P. blakesleeanus* presented a broad band of a [³²P]ADP-ribosylated protein with a molecular mass of 37 kDa which was separated into three major proteins of 38/6.9, 37/8.1 and 36/4.1 kDa/pI after two-dimensional electrophoresis (Deveze-Alvarez *et al.*, 1996). Two additional [³²P]ADP-ribosylated proteins of 55 and 57 kDa were observed when these extracts were incubated under ADP-ribosylation conditions in the absence of MgCl₂ (Deveze-Alvarez *et al.*, 1996). In order to purify and characterize the 37 kDa protein, the S31K proteins were separated by two sequential isoelectrofocusing steps. The fractions which presented a [³²P]ADP-ribosylated protein with a molecular mass of 37 kDa were pooled, concentrated, and N-terminally microsequenced. The obtained sequence of this region of the protein (1 VKVGVNGFGR-IGRLV15) was identical to the GAPDH N-terminal sequence in human, mouse and rabbit, and showed strong homology (86% identity) with sequences from other fungi such as *Neurospora crassa* (Sahni & Kinsey, 1997), *Podospora anserina* (Ridder & Osiewacz, 1992), *Cryphonectria parasitica* (Choi & Nuss, 1990), *Cochliobolus heterostrophus* (Van Wert & Yoder, 1992), *Candida albicans* (Gil-Navarro *et al.*, 1997), *Claviceps purpurea* (Jungehulsing *et al.*, 1994), *Schizophyllum commune* and *Agaricus bisporus* (Harmsen *et al.*, 1992).

Table 1. ADP-ribosylation of GAPDH measured by ^{14}C incorporation from [*nicotinamide*- ^{14}C]NAD or [*adenine*- ^{14}C]NAD

Proteins (100 µg) were incubated with [*nicotinamide*- ^{14}C]NAD ([*nic*- ^{14}C]NAD) or [*adenine*- ^{14}C]NAD ([*ade*- ^{14}C]NAD) under ADP-ribosylation conditions as described in Methods. Incubation was carried out for 1.5 h at 37 °C and the reaction was terminated by the addition of 20 % TCA (final concentration). Samples were filtered through Whatman paper filters and incorporated radioactivity was counted in a spectrophotometer.

Sample	[<i>nic</i> - ^{14}C]NAD (a)	[<i>ade</i> - ^{14}C]NAD (b)	Ratio (a)/(b)
S31K	0.1×10^{-10}	1.1×10^{-10}	0.09
IEF fraction	0.72×10^{-10}	5.36×10^{-10}	0.13

NAD-dependent post-translational modification of GAPDH

McDonald & Moss (1993) reported that nitric oxide (NO) induces a covalent binding of NAD to GAPDH through a non-enzymic process. To study the mechanism involved in the modification of GAPDH, we incubated the fraction enriched for the 37 kDa protein with either [*adenine*- ^{14}C]NAD or [*nicotinamide*- ^{14}C]NAD. Table 1 shows that a significant level of radioactivity was incorporated into the protein when the fraction was incubated with [*adenine*- ^{14}C]NAD, but not when it was incubated with the [*nicotinamide*- ^{14}C]NAD. Furthermore, the labelling of the 37 kDa protein did not occur when the fraction was heat-denatured, suggesting an enzymic modification of the protein (data not shown). These results demonstrate that the GAPDH is modified by a mono(ADP-ribosyl) transferase activity present in *P. blakesleeana* spores.

Arginine is the amino acid acceptor of the ADP-ribose in the GAPDH

We have previously reported the presence of an arginine-specific ADP-ribosyltransferase in spores of *Phycomyces* (Deveze-Alvarez *et al.*, 1996). An examination of the stability of the bond between the ^{32}P -label and the 37 kDa protein (GAPDH) of *P. blakesleeana* revealed that the intensity of the labelling was not reduced by treatment with 2 mM HgCl_2 , 1 M Tris/HCl pH 9.0 or formic acid, but was slightly diminished with NH_2OH (data not shown). These results established that the [^{32}P]ADP-ribosylation of GAPDH does not occur through a thiol linkage to a cysteine, which is a common site for NO-catalysed covalent modification of this enzyme by radiolabelled NAD^+ (McDonald & Moss, 1993). We can also rule out the amino acid that is involved in bonding of ADP-ribose-GAPDH as reported by De Matteis *et al.* (1994) since this bond is sensitive to formic acid. We conclude that the GAPDH of *P. blakesleeana* is *in vitro* post-translationally ADP-

Fig. 1. Effect of GA3P on the ADP-ribosylation of the 37 kDa protein. The S31K fraction was incubated with [^{32}P]NAD in the absence (lane 2) or presence (lane 3) of GA3P under ADP-ribosylation conditions. The proteins were separated by SDS-PAGE, stained with Coomassie blue (a), dried, and analysed by autoradiography (b). Lane 1, molecular mass markers.

ribosylated on an arginine residue. This is the first report of a modification by this enzyme on an arginine residue of GAPDH.

Regulation of GAPDH activity by its substrate and by ADP-ribosylation

The influence of ADP-ribosylation on GAPDH activity in the S31K fraction was examined. GAPDH activity was measured in the absence or presence of ADP-ribosylation conditions. GAPDH activity (1417 ± 149 units; $n = 3$) was inhibited approximately 50 % (637 ± 64 units; $n = 3$) when assayed under ADP-ribosylation conditions.

We also studied the effects of GA3P, the substrate for the GAPDH, on the ADP-(ribosyl)transferase activity. Fig. 1 shows that the presence of GA3P in the reaction affected the [^{32}P]ADP-ribosylation of the GAPDH. These findings suggest that both ADP-ribosylation and GA3P may play regulatory roles in determining activity of GAPDH.

ADP-ribosylation during the germination process of *P. blakesleeana*

ADP-ribosylation of proteins has a role in the development of different organisms (Eastman & Dworkin, 1994; Huh *et al.*, 1996; Shima *et al.*, 1996). Previously, we reported that different ADP-ribosylation inhibitors affected the germination of *P. blakesleeana* spores,

Fig. 2. Endogenous ADP-ribosylation of *P. blakesleeanus* proteins during the germination process. The S31K fraction was obtained from cultures at the times of germination indicated below. Samples (100 µg) from each time point were ADP-ribosylated as described in Methods. (a) Coomassie stain; (b) autoradiography. The 37 kDa band corresponding to GAPDH is arrowed. Lanes: 1, molecular mass markers; 2, dormant spores; 3, spores 0.5 h after activation; 4, swelling spores (4 h after activation); 5, cells with a germ-tube with half the size of the spore (5.5 h after activation).

leaving them as swollen cells (Deveze-Alvarez *et al.*, 1996). Bearing this in mind, we analysed the expression pattern of the ADP-ribosylated proteins during different stages of the cellular differentiation of *P. blakesleeanus*. Samples used for the ADP-ribosylation assays corresponded to dormant spores, and spores 0.5 h, 4 h and 5.5 h after activation (Fig. 2). Under the conditions used, spore swelling occurred during the first 4 h and germ-tube formation started at about 5.5 h of incubation at 24 °C. Analysis of labelled (ADP-ribosylated) cellular proteins from the different stages of differentiation showed that (i) several proteins are ribosylated throughout germination (Fig. 2b), and (ii) the GAPDH is ADP-ribosylated to a variable level (Fig. 2b). Remarkably, the level of ADP-ribosylation of GAPDH decreased 0.5 h after spore activation, but then reached a level comparable to the dormant spore after 4–5 h (Fig. 2b). Two [³²P]ADP-ribosylated proteins, of 79 and 83 kDa, respectively, were detected 30 min after activation and afterwards. We do not know the nature of these proteins (Fig. 2b).

GAPDH activity during germination in *P. blakesleeanus*

As shown above, the GAPDH activity from spore extracts was inhibited when assayed under ADP-ribosylation conditions. We therefore examined GAPDH activity during spore germination. The enzymic activity was measured in samples obtained from the same time points as above. The GAPDH activity in spores was 1250 ± 250 mU and it increased strongly at 0.5 h (3500 ± 700 mU); at 4 and 5.5 h of growth, this activity decreased to levels (1050 ± 200 mU) comparable to those found in

dormant spores. Therefore, a correlation between the ADP-ribosylation and activity of GAPDH exists during this stage of the germination. Thus, ADP-ribosylated GAPDH is associated with a decrease in its enzymic activity. Additionally, activity is only detected at low levels of ADP-ribosylation of GAPDH.

DISCUSSION

Our work has identified the enzyme GAPDH as one of the substrates of the arginine specific ADP-(ribosyl)-transferase of *Phycomyces*. Brüne & Lapetina (1989) reported that NO stimulates the auto-ADP-ribosylation of a 39 kDa protein in platelets. Subsequently, the 39 kDa protein was identified as GAPDH (Dimmeler *et al.*, 1992). These authors also reported that NO-mediated ADP-ribosylation of GAPDH inhibited this enzyme activity. Several investigators have questioned whether or not auto-ADP-ribosylation of GAPDH occurs as a result of stimulation by NO (McDonald & Moss, 1993; Itoga *et al.*, 1997).

De Matteis *et al.* (1994) reported that in rat basophilic leukaemia cells, brefeldin A, a fungal metabolite, stimulates the ADP-ribosylation of two cytosolic proteins of 38 and 50 kDa, p38 and p50, respectively. p38 appears to be identical with an isoform of GAPDH. They assessed the level of chemical reactivity of the brefeldin A-stimulated linkage of radiolabel from [³²P]NAD with GAPDH and reported that the incorporated radioactivity was stable to treatment with NH₂OH or HgCl₂, but was sensitive to acid and base, suggesting that brefeldin A induced a novel type of amino acid modification, different from those previously reported. Kots *et al.* (1993) reported that the ADP-ribosylation of

the GAPDH is activated not only by NO, but also by its substrate GA3P, and suggested that a lysine ϵ -amino group is the target for the ADP-ribose. In this work we found that the ADP-ribosylation of the GAPDH in spores of *P. blakesleeanus* appears to be mediated by a specific binding of ADP-ribose to an arginine residue. Recently, we reported the presence of three proteins of molecular masses of 38, 37 and 36 kDa in spores of *P. blakesleeanus* as possible substrates of an arginine-specific mono(ADP-ribosyl)transferase (Deveze-Alvarez *et al.*, 1996). In this work, we have identified the 37 kDa protein as GAPDH.

Although ADP-ribosylation by bacterial toxins has been extensively elucidated, mammalian ADP-ribosyltransferases have only recently been characterized (Koch-Nolte & Haag, 1997). The best-studied substrates have been the elongation factor 2, guanine nucleotide binding proteins, and structural proteins.

Bacterial ADP-ribosyltransferases are usually described as exotoxins whose targets are eukaryotic proteins. Evidence is being reported that there is a family of bacterial ADP-ribosyltransferases that function as endogenous regulators. For example, ADP-ribosylation plays a key role in the regulation of dinitrogen fixation in *Rhodospirillum rubrum* (Fu *et al.*, 1990) and in the development of *Streptomyces coelicolor* (Ochi *et al.*, 1992; Penyge *et al.*, 1992) and *Myxococcus xanthus* (Eastman & Dworkin, 1994). Reich & Schoolnik (1996) have suggested that the ADP-ribosyltransferase of *Vibrio fischeri* may participate in signalling during its symbiotic association with *Euprymna scolopes*.

The physiological importance of ADP-ribosylation in fungi is not known. In this work, we found that when soluble extracts of *Phycomyces* spores were incubated under ADP-ribosylation conditions before the GAPDH assay, a decrease of this enzymic activity was observed. This inhibition was overcome by the addition of GA3P to the ADP-ribosylation assay. This result suggests that probably the interaction of GA3P with GAPDH may affect the binding of ADP-ribose to the enzyme, consequently allowing GAPDH to remain active.

On the other hand, the profile of protein [32 P]ADP-ribosylation during germination indicates that the ADP-ribosylation of GAPDH is physiologically regulated in *P. blakesleeanus* and that the ADP-ribosylation of this enzyme could influence the energy metabolism and non-glycolytic functions of the fungus. The first common effect of different spore-activating treatments in *P. blakesleeanus* is a transient rise in cAMP content (Van Mulders & Van Laere, 1984). cAMP transiently activates trehalase and glycerol-3-phosphatase in spores (Van Laere *et al.*, 1987). The activation of these enzymes causes a quick decrease in trehalose with an associated increase in glycerol. Production of glycerol would be the most protective strategy, as glycerol can leak out of the membrane without damage and, unlike ethanol and lactate, it does not inhibit, but rather protects enzymic activity (Van Laere, 1986). We postulate that in the dormant spore, all the trehalose can be converted to

glycerol. This can result from activation of glycerol-3-phosphatase and reduced levels of glycolysis caused by inactivation of GAPDH by ADP-ribosylation. Glucose can then be transported and some converted to GA3P, which will protect GAPDH from ADP-ribosylation, allowing glycolysis to continue at a faster rate. Our current working model predicts that ADP-ribosylation and GA3P regulate the metabolic flux of glucose towards glycerol, glycolysis, pentose phosphate or GAPDH activity through non-glycolytic functions. Further work is required to determine whether this is correct.

Although GAPDH has been characterized as a glycolytic enzyme, it is in fact a multifunctional protein and has been implicated in several cellular activities, including the nuclear export of tRNA (Sing & Green, 1993), the regulation of actin filaments in microfilaments (Fuchtbauer *et al.*, 1986; Huiterol & Pantaloni, 1985), DNA repair (Meyer-Siegle *et al.*, 1991), and the regulation of calcium release from the endoplasmic reticulum (Kim *et al.*, 1990). The physiological role of the ADP-ribosylation of GAPDH needs further exploration. Since this enzyme plays an important role in glycolysis and in a number of non-glycolytic pathways as described above, variations in GAPDH activity could influence many other cellular activities, facilitating adaptation to different environmental challenges or growth conditions.

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