The GTP-binding protein $G\alpha_s$ is present in dormant spores and expressed differentially during spore germination of the fungus *Phycomyces blakesleeanus*

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A DNA sequence homologous to a $G\alpha_s$ DNA probe, and the corresponding $G\alpha_s$ protein (stimulatory α -subunit of GTP-binding protein) were detected in *Phycomyces blakesleeanus*. The protein was demonstrated in membrane fractions of dormant spores of this fungus using three different experimental approaches. Photoaffinity-labelling experiments with [α - 32 P]GTP of the membrane fraction revealed two bands, of 56 and 32 kDa. The 56 kDa GTP-binding protein was detected by this method in all the stages of early development and growth investigated. Also, a spore protein of 56 kDa was ADP-ribosylated by cholera toxin, and a 56 kDa protein was detected by Western blotting with a specific antibody against mammalian $G\alpha_s$. These results indicate that $G\alpha_s$ (56 kDa) is present in dormant spores of *P. blakesleeanus*. Using the ADP-ribosylation and Western blotting assays, $G\alpha_s$ was detected during all stages of spore germination before the hyphae became highly branched, but it was not detected in the branched hyphae that formed 18 h after the initiation of spore germination. Therefore, $G\alpha_s$ is expressed differentially during *Phycomyces* development.

Keywords: GTP-binding protein, spore germination, *Phycomyces blakesleeanus*

INTRODUCTION

Dormant spores of the fungus *Phycomyces blakesleeanus* must be activated by heat shock (50 °C for 3 min) or monocarboxylic acids (acetate, propionate or butyrate) in order to induce germination and growth 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). For instance, mutants that do not germinate show no increase in cAMP (Van Laere & Rivero, 1986; Rivero & Cerdá-Olmedo, 1987), which strongly supports the hypothesis that this nucleotide is a critical agent in breaking *Phycomyces* spore dormancy.

We have demonstrated the presence of protein kinase C in crude membrane extracts of *Phycomyces* spores (Carrillo-

Rayas *et al.*, 1988). We found that when spores were inoculated in culture medium together with tetradecanoyl phorbol acetate, an activator of protein kinase C, germination was inhibited. However, the inhibition of germination disappeared if dibutyryl-cAMP was added to the culture medium. These results suggest that protein kinase C and eventually phosphoinositide hydrolysis may down-regulate the germination process by modulating the concentration of cAMP.

In higher organisms, a plasma membrane receptor and adenylate cyclase are separate proteins that do not physically interact. Rather, they are functionally coupled by heterotrimeric G proteins, which consist of three subunits, α , β , and γ . Subtle structural differences in the α -subunit distinguish G proteins from each other. Some α -subunits can be identified by their characteristic ADP-ribosylation catalysed by bacterial toxins, specifically cholera toxin and pertussis toxin. For instance, cholera toxin and pertussis toxin ADP-ribosylate $G\alpha_s$ and $G\alpha_o$ (also some forms of $G\alpha_i$), respectively. Both toxins ADP-ribosylate the α -subunit of transducin (Gilman,

Abbreviations: G-proteins, GTP-binding proteins; $G\alpha_s$, stimulatory α -subunit of GTP-binding protein; $G\alpha_i$, inhibitory α -subunit of GTP-binding protein; G_γ , transducin.

1987). The membrane effectors modulated by heterotrimeric G proteins include adenylate cyclase, phospholipase C, retinal cGMP-phosphodiesterase and ion channels (Kazirol *et al.*, 1991; Simon *et al.*, 1991).

Among lower organisms, it has been suggested by different authors that in *Dictyostelium discoideum*, some membrane receptors for cAMP are possibly linked to heterotrimeric G proteins, since cells lacking the $G\alpha_2$ gene cannot activate adenylyl cyclase, guanylyl cyclase or phospholipase C (reviewed by Gross, 1994). On the other hand, *Neurospora crassa* has been shown to possess a $G\alpha$ protein, Gna-1, of molecular mass 39 kDa which is a member of the $G\alpha_i$ family and is ADP-ribosylated by pertussis toxin (Turner & Borkovich, 1993).

Recently, the presence of GTP-binding proteins in the sporangiothore of *Phycomyces* has been reported (Ashktorab & Cohen, 1994). Demonstration of GTP-binding proteins in *Phycomyces* sporangiospores and early stages of development would help elucidate the signalling pathways involved in germination and growth. In the present work, we used a mixed-membrane fraction derived from dormant spores to examine whether *Phycomyces* contains $G\alpha_s$ at this developmental stage. We also investigated the expression of this membrane protein during spore germination. Additionally, we looked for DNA sequences homologous to rat $G\alpha_s$ genes.

METHODS

Strains. The wild-type strain of *P. blakesleeensis* NRRL(-) 1555 was used throughout this work.

Media and cultivation conditions. The fungus strain was maintained and propagated in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose, w/v) adjusted to pH 4.5 with H_2SO_4 before autoclaving (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.

Spores were activated chemically by including 30 mM ammonium acetate in the culture medium. Spores were inoculated into YPG medium containing ammonium acetate in Erlenmeyer flasks at a final density of 5×10^5 spores ml^{-1} . Flasks were shaken aerobically at 24 °C for the times indicated in Results. The culture was then filtered and processed as stated below.

Preparation of cell-free extracts. Cell-free extracts were obtained as described by Carrillo-Rayas *et al.* (1988) with some modifications. Briefly, the spores or mycelium 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 of suspension (10^{10} spores or mycelium) was mixed with an equal volume of glass beads (0.45–0.50 mm diameter) and broken in a Braun model MSK cell homogenizer for 180 s while cooling with a stream of CO_2 . The homogenate was centrifuged at 100 000 g ($r_{av.}$) for 120 min and the mixed-membrane fraction was used throughout this study.

Assay of ADP-ribosylation. Cholera toxin (1 mg ml^{-1}) was activated by incubation with 40 mM dithiothreitol for 30 min at 30 °C. ADP-ribosylation was carried out in a reaction mixture (40 μl) composed of: 40 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 1 mM ATP, 1 mM GTP, 0.1% (w/v)

Triton X-100, 1–10 μM [^{32}P]NAD [3–5 μCi (111–185 kBq) per assay] and 50–75 μg membrane protein. The reaction was initiated by the addition of 4 μg activated toxin. Following incubation for 2 h at 30 °C, the reaction was stopped by adding 40 μl 2 $\times$ SDS-PAGE buffer A [0.125 M Tris/HCl, 4% (w/v) SDS, 20% glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Membrane proteins were separated by SDS-PAGE according to Laemmli (1970). Gels were dried and exposed (7–10 d) to X-Omat films for radioautography.

[α - ^{32}P]GTP photoaffinity-labelling of membrane proteins.

This procedure was essentially the same as that described by Basu & Modak (1987). The reaction mixture was made in a total volume of 40 μl and contained: 10 mM Tris/HCl (pH 7.8), 100 mM NaCl, 1 mM dithiothreitol, 2 mM $MnCl_2$, 10 μM ATP, 0.05 μCi (1.85 kBq) [α - ^{32}P]GTP μl^{-1} and 50–75 μg of membrane protein. Where indicated, the samples were preincubated for 10 min with different concentrations of non-radioactive GTP and GDP. Samples were maintained on ice for 10 min and then irradiated for 10 min with the short wavelength of a UV-lamp (model UVGL-58, UVP San Gabriel, CA), at a distance of 5 cm. Irradiated samples were then combined with 40 μl 2 $\times$ buffer A and subjected to SDS-PAGE. After drying, gels were exposed to X-Omat films for 2–5 d.

Western blotting. Membranes (50 μg) were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane (Towbin *et al.*, 1979). Blots were immersed for 60 min at room temperature in 10 ml blocking solution that contained 5% (w/v) dried milk in buffer B [20 mM Tris/HCl, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6]. Blots were washed with three changes of 10 ml buffer B, followed by incubation for 1 h with anti- $G\alpha_s$ antibody (diluted 1:3000 in 10 ml buffer B). After washing with three changes of 10 ml buffer B, a secondary antibody conjugated with horseradish peroxidase (diluted 1:5000 in 10 ml buffer B) was added to each blot. Again blots were washed with four changes of 10 ml buffer B. Immunoreactivity was monitored by an enhanced chemiluminescence method (Amersham).

Isolation of total DNA and Southern blotting. Total DNA was isolated as described by Van Heeswijck & Roncero (1984) with some modifications. Spores were activated to induce germination as described above and incubated for 12 h. Germlings were harvested by filtration and exhaustively washed with 1 l distilled water, then with 50–100 ml 10 mM Tris/HCl pH 8.0 containing 1 mM EDTA, air-dried, and stored at –70 °C. Frozen cells were disrupted with liquid nitrogen until a fine powder was obtained. This powder was carefully resuspended in 10 ml 200 mM Tris/HCl (pH 8.0) containing 10 mM EDTA; 0.2% SDS (final concentration) and 100 μg proteinase K ml^{-1} were then added. The mixture was incubated for 30 min at 68 °C, and then for 30 min at room temperature. Afterwards, the sample was centrifuged at 7400 g ($r_{av.}$) for 10 min and the supernatant (10 ml) was mixed with 0.6 ml 8 M potassium acetate. After incubation for at least 60 min at 0 °C, the sample was centrifuged at 11 300 g ($r_{av.}$) for 15 min. DNA was then precipitated with 2 vols absolute ethanol, and washed twice with 80% (v/v) ethanol. After centrifugation, the precipitate was air-dried, resuspended in sterile distilled water, and the proteins were extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). DNA was recovered in the aqueous phase and precipitated with 2 vols ethanol and 7.5 M ammonium acetate (1/10 of the volume) and centrifuged at 4 °C for 10 min. Then, the pellet was washed twice by centrifugation with 80% ethanol, air-dried for 10 min and resuspended in 0.5 ml distilled water.

Total DNA was digested with restriction endonucleases and electrophoresed in a 1% (w/v) agarose gel. The DNA was

depurinated with 0.25 M HCl, denatured with 0.4 M NaOH, and transferred to a nylon filter according to the manufacturer's instructions (DuPont). Hybridization to Digoxigenin UTP oligonucleotide 3'-end-labelled G α_s probe was performed under medium-stringency conditions (55 °C). The hybridization bands were revealed by chemiluminescence (Boehringer-Mannheim).

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

RESULTS

Detection of genomic sequences homologous to G α_s genes

In order to provide further evidence for the presence of G-proteins in *P. blakesleeanus*, Southern blotting was performed to search for the presence of sequences with homology to a specific probe derived from the rat G α_s gene (Fig. 1). When different endonucleases were used to restrict *P. blakesleeanus* DNA, one major hybridization

Fig. 1. Southern blot analysis of *P. blakesleeanus* DNA. *P. blakesleeanus* (lanes 1–2) or *S. cerevisiae* (lane 3) DNA was denatured, transferred to a nylon filter, and hybridized to a Digoxigenin UTP 3'-end-labelled G α_s oligonucleotide probe under medium-stringency conditions (55 °C). Each lane contained 10 µg DNA. Restriction endonucleases used were *Hind*III (lanes 1 and 3) and *Xba*I (lane 2). This is one experiment of two performed with the same results.

Fig. 2. Photoaffinity-labelling by [α - 32 P]GTP in *P. blakesleeanus* membrane fractions from different stages of growth. Photoaffinity-labelling was assayed by irradiating the membranes with UV for 10 min in the presence of [α - 32 P]GTP. Photolabelled samples were subjected to 10% SDS-PAGE and autoradiography. Lanes: 1, dormant spores; 2, 30 min after activation; 3, 4 h growth; 4, 7 h growth; 5, 11 h growth; 6, 18 h growth. The 56 kDa band observed at all stages of growth investigated is marked with an asterisk. This is one experiment of three performed with the same results.

signal of 2.2 kbp with *Hind*III and another of 0.5 kbp with *Xba*I were observed (Fig. 1, lanes 1 and 2, respectively), suggesting that there is a region homologous to the G α_s gene in the fungus DNA. *Saccharomyces cerevisiae* DNA restricted with *Hind*III was used as a control. Although a broad band of high molecular mass was observed as a consequence of partial restriction, a hybridization band of 4.6 kbp can be distinguished (Fig. 1, lane 3).

[α - 32 P]GTP photoaffinity-labelling of membranes

One property of G-proteins is their capacity to bind guanine nucleotides. To study this, a spore membrane fraction was incubated with [α - 32 P]GTP and irradiated as described in Methods. This fraction was subjected to electrophoresis and autoradiography. Fig. 2 (lane 1) shows two bands, with molecular masses of 56 and 32 kDa, that bound [α - 32 P]GTP. When the membranes were pre-incubated with different concentrations of non-radioactive GTP or GDP, no bands were detected when 1000 µM GDP or 100 µM GTP was added to the reaction mixture (data not shown). We also investigated whether the expression of these proteins was constitutive or if they

Fig. 3. Cholera-toxin-catalysed ADP-ribosylation of membrane fractions from different stages of growth of *P. blakesleeenans*. Mixed-membrane fraction (100 µg) was incubated under conditions for ADP-ribosylation in the presence of cholera toxin, followed by electrophoresis and autoradiography as described in Methods. Lanes: 1, dormant spores; 2, 30 min after activation; 3, 4 h growth; 4, 7 h growth; 5, 11 h growth; 6, 18 h growth. The 56 kDa band is marked with an asterisk. Identical results were obtained in at least four other experiments.

were present in different stages of spore germination and outgrowth. Chemically activated spores were incubated in growth medium and aliquots withdrawn from the culture at different times were processed as stated in Methods. Under the conditions used, spore swelling occurs during the first 4 h and germ-tube formation starts at about 7 h of incubation. At 11 h, the germ-tube is three times the size of the spore, and at 18 h, hyphal branching has occurred. Fig. 2 (lanes 2–6) shows the presence of different proteins that bind [α - 32 P]GTP; for example, at 30 min the presence of three radiolabelled bands, of 72, 56 and 48 kDa, can be observed; at 4 and 7 h, three bands, of 81, 72 and 56 kDa; at 11 h, four bands, of 86, 65, 56 and 32 kDa, and at 18 h, five bands, of 56, 35, 32, 22 and 21 kDa. A radiolabelled band corresponding to a molecular mass of 56 kDa was detected at all stages of growth investigated.

ADP-ribosylation of membranes by cholera toxin

When the spore mixed-membrane fraction was incubated with [32 P]NAD in the presence of cholera toxin we found five ADP-ribosylated proteins, of 85, 56, 44, 32 and 29 kDa (Fig. 3, lane 1). These bands did not appear if non-radioactive NAD (1 mM) was added together with the toxin or if this fraction was incubated with [32 P]NAD in the absence of cholera toxin (data not shown). Fig. 3 (lanes 2–6) shows the pattern of ADP-ribosylation by cholera toxin in membranes obtained from different stages of spore germination and outgrowth. Different [32 P]ADP-

Fig. 4. Immunodetection of $G\alpha_s$ during *P. blakesleeenans* spore germination. Chemically activated spores were inoculated in complex medium and incubated at 24 °C. Samples were withdrawn at different times and processed as described in Methods. Membrane proteins (100 µg) were electrophoresed and transferred to nitrocellulose. Strips were then incubated with anti- $G\alpha_s$ serum and immunodetection, based on chemiluminescence, was performed with a secondary antibody conjugated with horseradish peroxidase. Lanes: 1, dormant spores; 2, 30 min after activation; 3, 4 h growth; 4, 7 h growth; 5, 11 h growth; 6, 18 h growth. The 56 kDa band is marked with an asterisk. The same result was obtained in three other experiments.

ribosylated proteins were detectable in all the stages of early development and growth tested, except that at 18 h of incubation, when hyphal branching had occurred, no ADP-ribosylated band was detected. Therefore, *Phycomyces* contains substrates for cholera toxin at different developmental stages, although the substrates differ from one stage to another. The ADP-ribosylated proteins included one of 56 kDa, the same molecular mass as the protein detected by the [α - 32 P]GTP photoaffinity method.

Immunodetection of $G\alpha_s$ in *Phycomyces* membranes

The results presented above indicated that the 56 kDa membrane protein has biochemical characteristics that are common to the α -subunit of G_s . We therefore investigated whether one or more of these proteins presented immuno-reactivity with an antiserum against $G\alpha_s$. Membrane proteins from the different stages of growth were separated by SDS-PAGE and then electrotransferred to nitrocellulose. Blots were subsequently incubated with a rabbit antibody raised against the carboxy-terminal decapeptide of $G\alpha_s$ and immunodetection made with an anti-rabbit immunoglobulin conjugated with horseradish peroxidase. As shown in Fig. 4 (lane 1), anti- $G\alpha_s$ serum

recognized, among others, a protein of 56 kDa. As a control, when mouse brain membrane proteins (which contain 45 and 48 kDa proteins recognized by anti-G α_s serum) were incubated overnight with the anti-G α_s serum, and the mixture then incubated with the fungus membrane blots, no band (56 kDa) was immunodetected. Also, blots incubated solely with the secondary antibody did not show any band (data not shown). Fig. 4 (lanes 2–6) shows that G α_s is present during all stages of spore germination, i.e. in swollen and germinated cells. After 18 h of growth, it was not immunodetected. This result agrees with that of ADP-ribosylation by cholera toxin. Therefore, *P. blakesleeanus* G α_s is present in dormant spores and in early stages of germination.

DISCUSSION

Recent developmental molecular biology techniques have revealed the presence of at least nine genes for the G protein α -subunits in mammalian cells (reviewed by Kaziroti *et al.*, 1991). The predicted amino acid sequences for these genes have established 98–100% homology between these proteins. G protein α -subunits have been divided into three subfamilies: G α_s , G α_i and G α_t (Kaziroti *et al.*, 1991). The use of different bacterial toxins has played an important role in identifying these proteins. For example, cholera toxin ADP-ribosylates G α_s and G α_t and pertussis toxin ADP-ribosylates G α_i and G α_t (Gilman, 1987). By cross-hybridization experiments with rat G α cDNAs, two genes (*GP41* and *GP42*) were isolated from *S. cerevisiae* (Nakafuku *et al.*, 1987, 1988). Although the GP1 α and GP2 α predicted proteins have approximately 100 more amino acids than mammalian G α proteins, the overall structural similarities with G α_i and G α_s are remarkable. Recent studies have shown that GP1 α is involved in mating-factor-mediated signal transduction (Miyajima *et al.*, 1987; Dietzel & Kurjan, 1987). On the other hand, genetic and biochemical experiments suggest that GP2 α together with Ras may participate in regulation of the intracellular levels of cAMP (Nakafuku *et al.*, 1988). In this work, we found by Southern blotting a major band of hybridization with a G α_s DNA probe, which suggests the presence of at least one gene for G α_s .

We found in *P. blakesleeanus* a protein of 56 kDa that was ADP-ribosylated by cholera toxin and bound GTP, as revealed by photoaffinity assays. In addition, this protein was recognized by a specific polyclonal anti-G α_s antibody. These data indicate the presence of G α_s in this fungus, with a molecular mass similar to that predicted for *S. cerevisiae* G-protein (Nakafuku *et al.*, 1987, 1988). Ashktorab & Cohen (1994) detected in sporangiotheca membranes of *Phycomyces* two substrates for cholera toxin, with molecular masses of 51 and 40 kDa. The difference in molecular mass observed could be that, as stated before, cholera toxin can ADP-ribosylate different G-proteins; furthermore, these authors used an antibody common to G α proteins. In this work we used specific antibodies for a motif only present in G α_s . The presence of G α_s in dormant spores could imply its participation in spore activation after physical or chemical treatment. As stated

above, if this protein functionally corresponds to that found in *S. cerevisiae* (Nakafuku *et al.*, 1988), it could participate in the regulation of cAMP synthesis by modulating adenylate cyclase activity. G α_s was also detected after chemical activation and immediately after the appearance of the germ-tube; this suggests its possible participation in other cellular processes associated with growth and/or germination. It is difficult to conclude whether different amounts of G α_s protein are present in the various stages of spore germination, since the intensity of the bands varied between the different detection systems. These variations can be explained by the fact that the assays done in this work detect different G proteins. For example, the experiments involving ADP-ribosylation by cholera toxin can detect both G α_s and G α_t , and we do not know if the fungus during spore germination is expressing a G α_t -like protein or another cholera toxin substrate of the same molecular mass as that found in dormant spores. The photolabelling experiments with [α - 32 P]GTP will detect those G-proteins that bind this nucleotide, therefore other GTP-binding proteins could be present in the different stages tested, for example at 11 and 18 h of growth. However, of the three biochemical approaches used to identify G-proteins, only the immunodetection assures that the band observed in the different development stages corresponds to G α_s . Little is known about *Phycomyces* metabolism after spore activation and during germination (Van Laere *et al.*, 1987). These findings indicate that different signal transduction pathways involving G proteins may participate in each of these differentiation stages.

We also observed other proteins (molecular masses of 85, 80, 72 and 32 kDa) that were ADP-ribosylated by cholera toxin. The 85 and 80 kDa proteins were recognized by anti-G α_s . These results could suggest that these are G α_s -like proteins. However, it cannot be discounted that in the membrane fraction, which contains cell walls, protein aggregates can be formed by the mechanical method used to disrupt the spores, and that these aggregates are resistant to the denaturing conditions used to separate the proteins by electrophoresis. On the other hand, a 32 kDa protein present in dormant spores and in growing cells (18 h) was ADP-ribosylated by cholera toxin and bound [α - 32 P]GTP. This protein was not recognized by the anti-G α_s antibody, so it may correspond to a novel GTP-binding protein or it may originate from hydrolysis of the carboxy-terminal segment of G α_s and therefore not be recognized by the antibody. However, this is difficult to conclude since this protein was observed in all samples tested.

Little is known about the triggers which cause the increase in cAMP levels after spore activation or the processes involved in germ-tube formation (Van Laere *et al.*, 1987). The occurrence of G-proteins in dormant spores and in distinct stages of differentiation might imply their participation in the signal pathway involved in spore activation and germination of *P. blakesleeanus*. On the other hand, Cohen *et al.* (1980) detected a GTP-sensitive adenylate cyclase in *P. blakesleeanus* sporangiothecae. Therefore, it is of interest to study whether the adenylate

cyclase from dormant and germinating spores is regulated by the heterotrimeric GTP-binding proteins. Work is in progress in this direction.

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