The small GTP-binding protein Rho is expressed differentially during spore germination of *Phycomyces blakesleeanus*

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Evidence has been obtained for the presence of the small 22 kDa GTP-binding Rho protein in dormant spores of *Phycomyces blakesleeanus*. Immunoblotting with a polyclonal antibody against RhoA revealed a soluble and membrane-associated 22 kDa protein. When [³²P]ADP-ribosylated by *Clostridium botulinum* C3 exotoxin the protein had a pI of 5.7, a value similar to that reported for other RhoA proteins. The 22 kDa protein was expressed at all stages of growth investigated, but radiolabelling of the [³²P]ADP-ribosylated protein increased when tube-formation occurred and decreased as the hyphae branched. Localization of RhoA during spore germination studied by immunofluorescence microscopy revealed the presence of RhoA in the cell membrane of the spore. When the spore started to swell, RhoA was observed as patches in the cell membrane which become concentrated in the neck region of the site of the protuberation tube, but this protein was never observed at the point of growth of the hyphal tip. The above results suggest that RhoA associated with one or more membrane proteins could participate in the molecular mechanism involved in maintaining cell integrity during the extrusion of the germ-tube of *P. blakesleeanus*.

Keywords: GTP-binding proteins, Rho proteins, spore germination, *Phycomyces blakesleeanus*

INTRODUCTION

The activation of cell surface receptors leads to a multiplicity of responses, such as growth and morphogenesis, which are accompanied by changes in organization of the cytoskeleton. Cytoskeletal changes after cell activation have been attributed to changes in pH, Ca²⁺ or phosphorylation and also to the activation of small GTP-binding proteins of the Rho subfamily (Ridley & Hall, 1994). The Rho-subfamily proteins modulate signal transduction from plasma membrane receptors and trigger cellular responses related to polarity, motility, cell shape, and rearrangements of the actin cytoskeleton (reviewed by Machensky & Hall, 1996). Members of the Rho-subfamily, like other GTP-binding proteins, exhibit both GDP/GTP binding and GTPase activities (Nobes & Hall, 1994). They have GDP-bound inactive and GTP-bound active forms, which are interconvertible by GDP/GTP exchange and GTPase reactions mediated by different proteins (Nobes & Hall, 1994).

RhoA, RhoB and RhoC isoforms are unique among the many small GTP-binding proteins, including other members of the Rho-subfamily, in that they can be selectively ADP-ribosylated by the C3 exotoxin from *Clostridium botulinum*; Asn41 is the putative target domain of human Rho protein (Sekine *et al.*, 1989). The ADP-ribosylation of Rho inhibits several cell functions related to the actin-based cytoskeleton and thereby participates in the control of cell shape of higher organisms (Takai *et al.*, 1995).

Saccharomyces cerevisiae grows by budding for cell division. This polarized cell growth is initiated by signals from the cell surface that result in the realignment of the cytoskeleton and the biosynthetic machinery toward a targeting patch at the bud site. Cortical actin patches are localized at the site of bud emergence on unbudded cells, and in small and medium-sized buds in budding cells. Different small GTP-binding proteins have been associated with the bud emergence site of *Sacch. cerevisiae* (see reviews by Cid *et al.*, 1995; Cabib *et al.*,

1998). Of these, Rho1p has been localized at the periphery of the bud emergence sites, the staining sites overlapping with those of the cortical patches of actin, even though there is no evidence that the GTP-binding protein is connected with the regulation of the actin cytoskeleton (Yamochi *et al.*, 1994). On the other hand, it has been reported that Rho1p activates glucan synthase, and therefore is required for cell wall synthesis (Drgonová *et al.*, 1996). In *Schizosaccharomyces pombe*, it has been reported that Rho1p partially complements a geranylgeranyl transferase mutant in which cell wall synthesis is defective, particularly in the glucan synthase activity (Arellano *et al.*, 1996). These results, together with those from *Sacch. cerevisiae*, suggest that Rho plays a central role in yeast morphogenesis.

Phycomyces blakesleeanus is a zygomycete fungus whose spores need an activation treatment (50 °C for 3 min, or monocarboxylic acids) in order to induce germination and growth in a suitable culture medium. The germination process involves a morphological transition from an elliptical spore to a spherical cell, and following protrusion of the germ-tube, its elongation and branching (Van Laere *et al.*, 1987). In these stages of development, a dynamic reorganization of the actin cytoskeleton must take place, which may involve the Rho proteins. In the work described here we have demonstrated the presence of RhoA in dormant spores of *P. blakesleeanus*, and investigated its expression and cellular localization during the spore germination process.

METHODS

Chemicals and reagents. [32 P]NAD (1.11×10^{14} Bq mmol $^{-1}$) was purchased from Amersham. Nucleotides, NAD, Nonidet P40 and antipain were from Sigma. Materials for SDS-PAGE were from Bio-Rad. C3 exotoxin from *C. botulinum* was purchased from Upstate Biotechnology. Mouse monoclonal and rabbit polyclonal antibodies against RhoA were obtained from Santa Cruz Biotechnology. Other reagents were of the highest quality commercially available.

Strains. The wild-type strain NRRL 1555(–) of *P. blakesleeanus* from Northern Regional Research Labs, Peoria, IL, USA, a gift from Dr E. Cerda-Olmedo, was used throughout this work.

Media and cultivation conditions. *P. blakesleeanus* 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 YPG liquid medium and incubated as described by Martínez-Cadena *et al.* (1995).

Preparation of cell-free extracts. Cell-free extracts were obtained from spores or mycelia as described by Devezé-Alvarez *et al.* (1996). The crude extracts were centrifuged at 100 000 g (r_{av}) for 60 min, and the supernatant (cytosol) and mixed-membrane fractions were divided into aliquots and kept at –20 °C until use.

Assay of ADP-ribosylation. ADP-ribosylation was carried out in a reaction mixture (40 μ l) composed of 50 mM Tris/HCl

pH 7.4, 2 mM MgCl $_2$, 1 mM ATP, 0.2% (v/v) Nonidet P40, 1 mM EDTA, 1 mM DTT, 2 μ M NAD, 7.4×10^4 Bq [32 P]NAD ($\sim 1.11 \times 10^{14}$ Bq mmol $^{-1}$), 75–100 μ g protein and, when indicated, 0.1 μ g *C. botulinum* C3 exotoxin. The reaction proceeded for 30 min at 37 °C and was terminated by the addition of 2 $\times$ solubilization buffer [0.25 M Tris/HCl pH 6.8, 10% (v/v) β -mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS, 0.001% (w/v) bromophenol blue]. Samples were boiled for 3 min and subjected to 12% (v/v) SDS-PAGE (Laemmli, 1970). After electrophoresis the gels were stained with Coomassie blue, dried, and exposed (7–10 d) to X-OMAT films for autoradiography.

ATPase assay. H $^{+}$ -ATPase was determined by measuring the phosphate released from ATP by the method of Ames (1966) in 0.12 ml of 30 mM Tris/MES pH 6.5, 5 mM MgSO $_4$, 3 mM ATP, 1 μ M valinomycin. Different aliquots of the sucrose gradient containing 75–150 μ g protein were added to start the reaction. This was stopped with 1.88 ml of the molybdate reagent used to measure the released phosphate. The A_{820} was measured after 1 h. Vanadate-sensitive ATPase activity was measured in the presence and absence of 100 μ M sodium vanadate.

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

Western blot analysis. After electrophoresis, proteins were transferred onto nitrocellulose and assayed for immunoreactivity as described by Towbin *et al.* (1979). Polyclonal antibodies against RhoA were tested at a dilution of 1:2000. Sheep anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody and immunoreactivity revealed by chemiluminescence (ECL, Amersham).

Fluorescence microscopy. Cells from different stages of growth were fixed with 4% (v/v) formaldehyde in PBS pH 7.0 for 24 h. After permeabilization for 5 min with cold ethanol/chloroform (3:1, v/v), fixed cells were incubated for 2 h with the primary antibody mouse anti-RhoA followed by the addition of donkey anti-mouse-IgG conjugated with fluorescein isothiocyanate. Cells were analysed by epifluorescence microscopy using a Zeiss Axioscop microscope coupled with a no. 9 filter (excitation 450–490 nm, emission 510 nm).

RESULTS

C3-exotoxin-catalysed ADP-ribosylation of spore membrane and soluble fractions

Cytosol and mixed-membrane fractions from dormant spores of *P. blakesleeanus* were incubated under ADP-ribosylation conditions with C3 exotoxin and [32 P]NAD. After electrophoretic separation and subsequent autoradiography, a single [32 P]ADP-ribosylated band with a molecular mass of 22 kDa was observed in both fractions (Fig. 1a, lanes 3 and 4, respectively). When these fractions were incubated with [32 P]NAD, but without C3 exotoxin, no [32 P]ADP-ribosylated protein was observed in the cytosol or in the mixed-membrane fraction (Fig. 1a, lanes 1 and 2, respectively). No auto-ADP-ribosylation of the C3 exotoxin was observed when the toxin was incubated in the presence of [32 P]NAD and in the absence of the fungal protein (Fig. 1a, lane 5). Also, we incubated the soluble (Fig. 1b) or the mixed-membrane fraction (Fig. 1c) with increasing concentrations of non-radioactive NAD. The

Fig. 1. ADP-ribosylation of a 22 kDa protein by C3 exotoxin in *P. blakesleeanus*. (a) 100 µg cytosol (lanes 1 and 3) or mixed-membrane fraction (lanes 2 and 4) from spores of *P. blakesleeanus* was incubated under conditions for ADP-ribosylation in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of *C. botulinum* C3 exotoxin. As control, C3 exotoxin was incubated without the cellular extracts (lane 5). Samples were then subjected to PAGE and autoradiography. (b, c) The soluble (b) and mixed-membrane (c) fractions were incubated under ADP-ribosylation conditions in the presence of C3 exotoxin and 2, 25 or 100 µM non-radioactive NAD. Identical results were obtained in another five experiments.

intensity of the 22 kDa [32 P]ADP-ribosylated protein decreased with the increase of non-radioactive NAD. The above results with C3 exotoxin demonstrate the presence of Rho protein(s) in both cytosol and mixed-membrane fractions of the fungal spores.

Subcellular distribution of the Rho protein

Since both the cytosol and the mixed-membrane fraction from spores contained the Rho protein, we studied the distribution of this protein in different membrane fractions. We separated the spore homogenate on an isopycnic sucrose gradient (Fig. 2a) and looked for the C3 exotoxin substrates in different fractions of the gradient. Fig. 2(b), lane 6, shows that only fraction VI, corresponding to a density of 1.19–1.24 g ml $^{-1}$ contained the Rho protein. As expected, the Rho protein was also observed in pooled fraction I (Fig. 2b, lane 1) with a density corresponding to soluble proteins (1.05 g cm $^{-3}$). Vanadate-sensitive ATPase activity was used as a marker enzyme for plasma membrane and assayed in different aliquots of the sucrose gradient. Aliquots from fraction IV with densities from 1.14 to 1.16 g cm $^{-3}$ showed no vanadate-sensitive ATPase activity (1.80 vs 1.72 µmol mg $^{-1}$ h $^{-1}$ in the absence vs the presence of vanadate). Aliquots from fraction V with densities from 1.162 to 1.183 g cm $^{-3}$ showed a slight vanadate-sensitive

Fig. 2. ADP-ribosylation by C3 exotoxin in fractions of a spore homogenate separated on an isopycnic sucrose gradient. The homogenate from dormant spores was separated as follows: 3 ml of the extract was layered on top of a linear (20–60%, w/v) sucrose gradient which was centrifuged at 81 500 g for 22 h and fractionated from the top into 1 ml aliquots with an ISCO model 185 fractionator. Protein was determined by the Lowry method and density with an ATAGO refractometer. (a) Protein distribution in the sucrose gradient. Aliquots were pooled into six fractions with the following densities: I, 1.058–1.105; II, 1.110–1.124; III, 1.128–1.136; IV, 1.140–1.160; V, 1.162–1.183; VI, 1.192–1.240. (b) ADP-ribosylation in the presence of C3 exotoxin was assayed with 100 µg protein from fractions I–V (lanes 1–5, respectively), and 48 µg from fraction VI (lane 6). Identical results were obtained in another three experiments.

ATPase activity (1.02 vs 0.72 µmol mg $^{-1}$ h $^{-1}$ in the absence vs the presence of vanadate) and aliquots from fraction VI with densities from 1.192 to 1.24 g cm $^{-3}$ showed a 66.5% inhibition of the ATPase activity by vanadate (1.613 vs 0.539 µmol mg $^{-1}$ h $^{-1}$ in the absence vs the presence of vanadate). As a 22 kDa band that was [32 P]ADP-ribosylated by the C3 exotoxin was detected in fraction VI, we conclude that Rho is a plasma membrane protein.

Identification of the Rho protein present in the spores

Members of the Rho subfamily, specifically RhoA, RhoB and RhoC, which are all substrates for C3 exotoxin, have different pIs; for example, RhoA from mammalian cells has a pI of 5.9–6.1 (Janlink *et al.*, 1994), RhoB a pI

Fig. 3. Electrophoretic separation of isoelectrofocussed soluble fractions. (a) Nine of the 20 fractions separated by isoelectric focusing were ADP-ribosylated in the presence of C3 exotoxin and subjected to SDS-PAGE. (b) Western blot analysis of RhoA of the pH 5.7 fraction previously ADP-ribosylated (lane 1); autoradiography of the nitrocellulose membrane containing [32 P]ADP-ribosylated-Rho (lane 2); incubation of the pH 5.7 fraction in the absence of the primary and in the presence of the secondary antibody (lane 3). Identical results were obtained in another two experiments.

of 5.3 (Yeremian *et al.*, 1987) and RhoC a pI of 6.8 (Nemoto *et al.*, 1992). To investigate the pI of the Rho protein of *P. blakesleeana*, the cytosol was loaded into a Rotofor Cell (Bio-Rad) for fractionation in a wide-range pH gradient (pH 3–10) and separated by preparative isoelectric focusing. Each of the 20 fractions obtained after the isoelectric focusing was ADP-ribosylated by C3 exotoxin in the presence of [32 P]NAD, and afterwards subjected to SDS-PAGE and autoradiography. One band corresponding to 22 kDa was detected in a fraction electrofocused at pH 5.7 (Fig. 3a). This pI corresponds approximately to that reported for the RhoA protein. To corroborate the above results, immunocytochemical studies were performed using a polyclonal antibody against the RhoA protein. Fig. 3(b), lane 1, shows that the antibody recognized a 22 kDa protein from the same fraction of pH 5.7 that was ADP-ribosylated by C3 exotoxin (Fig. 3b, lane 2). This band was not observed when the blot was incubated only with the secondary antibody (Fig. 3b, lane 3). The same result

was obtained if the mixed-membrane fraction was isoelectrofocussed (data not shown). These results demonstrate that *P. blakesleeana* spores contained the RhoA protein with a pI of 5.7.

Rho protein is present in different developmental stages of *P. blakesleeana*

The expression of the Rho protein was investigated during different stages of spore germination. Chemically activated spores were incubated in liquid complex medium and aliquots were withdrawn from the culture at different times of the differentiation process. 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. At 9 h, the germ-tube was approximately three times the size of the swelled spore, and at 18 h hyphal branching had occurred (Fig. 4a). Both cytosol and mixed-membrane fractions were obtained from the cells incubated at the times indicated above and assayed for [32 P]ADP-ribosylation with C3 exotoxin. The Rho protein was expressed in all stages studied, but the intensity of radiolabelling of the [32 P]ADP-ribosylated protein increased in the cytosol when the tube formation occurred and afterwards the intensity of the band decreased as the hyphae branched (Fig. 4b). In the mixed-membrane fraction (Fig. 4c), the intensity of the Rho band increased after the band from the cytosolic fractions started to decrease, as expected for a protein that is synthesized first in the cytosol and afterwards processed for integration into the membrane. In addition, cytosol and mixed-membrane fractions from the above cells were separated by SDS-PAGE and then electrotransferred to nitrocellulose. Blots were subsequently incubated with the polyclonal antibody against RhoA. As shown in Fig. 4(d, e), the antibody recognized a 22 kDa band in both cytosol (Fig. 4d) and membrane (Fig. 4e) fractions. Under the conditions of the immunodetection used, this band was not detected in all stages of growth investigated. If the blots were incubated with the primary antibody for longer periods, the 22 kDa band was observed in all stages. This band was not detected at any stage of growth when fractions were tested solely with the secondary antibody (data not shown).

Immunolocalization of Rho protein from different developmental stages of *P. blakesleeana*

Since Rho was expressed differentially during spore germination, we investigated its localization during this process by immunofluorescence using a polyclonal antibody against RhoA. The presence of the RhoA protein in the spore membrane periphery and inside the cell was observed (Fig. 5a). After the spore started to swell, RhoA could be observed as patches in the cell membrane and also distributed inside the cell (Fig. 5b). When the germ-tube extruded, RhoA was observed in a zone flanking this protuberance, and this patch remained as the germ-tube continued growing (Fig. 5c, d). Fluorescence was also observed in the cytosol,

Fig. 4. Expression of Rho protein during the development of *P. blakesleeanus*. Cellular fractions (100 µg protein) were separated by electrophoresis and proteins were transferred onto nitrocellulose and assayed for immunoreactivity against RhoA or were incubated under conditions for ADP-ribosylation in the presence of C3 exotoxin followed by electrophoresis and radioautography. Lanes: 1, dormant spores; 2, spores 30 min after activation; 3, swelling spores; 4, cells starting to extrude a germ-tube; 5, cells with a germ-tube three times the size of the spore; 6, mycelia. (a) Morphology of the cells used for the detection of Rho (bar, 40 µm); (b) soluble [32 P]ADP-ribosylated-Rho proteins; (c) mixed-membrane [32 P]ADP-ribosylated-Rho proteins. (d) Western blot analysis of RhoA of soluble proteins; (e) Western blot analysis of RhoA of membrane proteins. Identical results were obtained in another four experiments.

confirming the experiments described above showing the presence of RhoA in this subcellular fraction. When cells were incubated without anti-Rho, but with the secondary antibody, these cells showed negligible fluorescence (Fig. 5e).

DISCUSSION

Rho has been found in the cytoplasm as a GDP-bound inactive form and in the plasma membrane as a GTP-active form (reviewed by Takai *et al.*, 1995). In yeast cells, it has been demonstrated that Rho1p, a homologue of mammalian RhoA, is present in both the cytosolic and particulate fractions (McCaffrey *et al.*, 1991; Yamochi *et al.*, 1994). We found also that in dormant spores of *P. blakesleeanus* a Rho protein is present in the cytosol as well as in the plasma membrane. By isoelectric focusing and Western blot analysis with polyclonal antibodies against RhoA, it was found that the antibody recognized a 22 kDa protein with a pI of 5.7 which was also a substrate for C3 exotoxin, demonstrating that *P. blakesleeanus* spores contained at least the RhoA protein.

The Rho protein was expressed in all stages of spore germination studied. Although Rho was found in both the cytoplasm and the particulate fraction, the distribution and intensity of radiolabelling of the [32 P]ADP-ribosylated protein in the two fractions were different. Active Rho increased in the cytosolic fraction (after the extrusion of the germ-tube) prior to its increase in the membrane (at the stage when the germ-tube was three times the size of the spore). In all organisms studied to date Rho is synthesized in the cytoplasm and after its post-translational modification and activation is translocated to the membrane, where it interacts with its effectors (Takai *et al.*, 1995). It appears that, in *P. blakesleeanus*, after Rho is synthesized in the cytoplasm this protein is modified and translocated to the cell membrane, where it may interact with a putative effector necessary for the protuberance of the germ-tube.

Yeast and fungal cells produce and maintain a large osmotic gradient across their plasma membrane, resulting in turgor pressure which is apparently used to force wall extension, allowing cell growth (Ortega *et al.*, 1989). However, the presence of a large internal pressure presents a biophysical barrier for cell growth. Together

Fig. 5. Immunolocalization of RhoA protein during the development of *P. blakesleeenae*. The cells were grown at 24 °C in YPG medium. Cells were then fixed and processed for reactions against RhoA monoclonal antibodies and secondary antibody coupled with fluorescein. (a, a') Dormant spore; (b, b') swelling spore; (c, c') cells starting to extrude a germ-tube; (d, d') cells with germ-tubes three times the size of the spore; (e, e') cell incubated with the secondary antibody and without RhoA monoclonal antibody. (a–e) Fluorescence microscopy; (a'–e') light microscopy. Bar, 10 µm. Identical results were obtained in another four experiments. Two hundred cells from each stage were observed (75 % of these cells presented the fluorescence shown).

with the turgor pressure, the breaking of covalent bonds necessary for the insertion of new ones creates a predisposition to cell lysis. In *Sacch. cerevisiae*, Mulholland *et al.* (1994) reported that the cortical actin patch consists of a finger-like invagination of the plasma membrane, around which actin and associated proteins are organized. These cortical actin patches containing membrane invaginations were observed at the points of bud emergence as well as at the site of septum formation. Also, these authors suggested that the cell turgor pressure may be reduced by invaginating the cell surface wherever new wall material is inserted. Therefore, the cortical actin cytoskeleton plays a primary role in establishing and maintaining localized membrane invaginations necessary for polarized growth. The small GTP-binding protein Rho1p has been co-localized at the periphery of yeast cells together with the cortical actin patches. Both are found in the site of bud emergence, the tip of the growing buds, and the mother-bud neck region of the cells prior to cytokinesis (Yamochi *et al.*, 1994), although no direct association of Rho with the actin cytoskeleton has been reported.

During spore swelling and protrusion of the germ-tube, *P. blakesleeanus* must produce a large osmotic gradient across its plasma membrane, resulting in turgor pressure which is apparently used for wall growth. In this fungus, the extension of the cell wall during germination requires localized deposition and insertion of cell wall under osmotically prohibitive conditions. Immunofluorescence microscopy showed that RhoA is localized in a zone flanking the site of the tube protuberance as well as in the cytoplasm. This distribution of RhoA in the surface of the swelling spore and after tube protrusion suggests that RhoA together with other associated proteins could participate in reducing the turgor pressure of *P. blakesleeanus* by a similar mechanism as proposed for *Sacch. cerevisiae*.

Three targets of Rho1p have been reported. One target was shown to be Pkc1p, a homologue of mammalian protein kinase C (Nonaka *et al.*, 1995), which regulates cell wall integrity through activation of the MAP kinase cascade (Levin & Errede, 1995). A second target is the 1,3- β -glucan synthase (Drgonová *et al.*, 1996; Qadota *et al.*, 1996), which is involved in cell wall synthesis. A third target of Rho1p is Bni1p, known to be implicated in cytokinesis or establishment of cell polarity in *Sacch. cerevisiae* (Kohno *et al.*, 1996). Kohno *et al.* (1996) suggested that Bni1p probably links Rho1p with the actin cytoskeleton. *P. blakesleeanus* does not show 1,3- β -glucan synthase activity during vegetative growth. It is therefore difficult to conceive that Rho could participate in the regulation of 1,3- β -glucan synthase during spore germination. However, β -glucan is present in the cell wall of dormant spores, and the synthase might be the target of Rho during spore formation in the sporangia. Another enzyme (possibly chitin synthase) involved in the synthesis of the cell wall could be the target of Rho during the germination process.

We have previously reported the presence of protein kinase C activity in dormant spores of *P. blakesleeanus*

(Carrillo-Rayas *et al.*, 1988). We do not know if this enzyme is involved in the maintenance of cell wall integrity of *P. blakesleeanus*, but the experiments we reported with TPA (12-O-tetradecanoyl phorbol-13-acetate) suggested that protein kinase C could participate in the breakdown of spore dormancy in this fungus. Therefore, this enzyme could be considered as a good candidate as a target of Rho and it may regulate spore germination via the MAP kinase cascade. Other Rho-associated proteins such as the actin cytoskeleton could participate upstream of protein kinase C so that the swelling of the spore, tube protuberance and overall growth can take place.

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