Small GTP-binding proteins are associated with chitosomes and vesicles carrying glucose oxidase from *Mucor circinelloides*

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Fractions enriched with chitosomes and vesicles carrying glucose oxidase (GOX) activity from the dimorphic zygomycete *Mucor circinelloides* were obtained using two successive sucrose gradients, the first a linear-log and the second an isopycnic gradient. Using an \[^{32}P\]GTP-binding assay, we detected the association of small GTP-binding proteins (21 and 17 kDa) with both types of vesicles. In addition, by ADP-ribosylation with C3 exotoxin, and Western blot analysis with specific antibodies, we identified the small GTPases RhoA (Rho1p) and Rab8, and a 17 kDa protein, with pI values of 6.0, 6.1, and 6.2 and molecular masses of 21, 21 and 17 kDa, respectively, associated with those vesicles carrying GOX activity. Rab and Cdc42 proteins with pI values of 6.1 and 6.2 and molecular masses of 21 and 17 kDa, respectively, were found associated with chitosomes. These data indicate the presence in *M. circinelloides* of low molecular mass G-proteins in chitosomes and in vesicles carrying GOX activity. The difference in association of Rho1 and Cdc42, with vesicles carrying GOX activity and chitosomes, respectively, indicates that each of these proteins probably controls formation, transport and specific plasma membrane site docking of the respective vesicles.

**INTRODUCTION**

During the first stages of the exocytic pathway, nascent proteins are translocated into the endoplasmic reticulum, transported through the *cis*, medial and *trans* (TGN network) cisternae of the Golgi apparatus, and sorted into secretory vesicles. In regulated secretion, some soluble proteins and lipids are carried by secretory vesicles, and the proteins are released to the cell surface, generally after the cells have received an appropriate signal. The process of exocytosis of these vesicles involves the directed transport, docking and fusion of the secretory or transport vesicles with the plasma membrane (see Burgess & Kelly, 1987). In filamentous fungi, the secretory vesicles carry certain proteins and precursors responsible for cell-membrane and cell-wall formation; these vesicles immediately move to and fuse with the plasma membrane, releasing their contents (Harris & Momany, 2004).

The formation of vesicles and the distinct intracellular transport pathways of these vesicles are regulated by heterotrimeric and small GTPase proteins. GTPases have been termed 'molecular switches', because their cycling between the GTP- and the GDP-bound forms results in dramatic changes in their three-dimensional structure (Takai et al., 2001). Heterotrimeric G-proteins participate during the formation of secretory vesicles (Leyte et al., 1992) and in intracellular trafficking pathways through the interaction of various small GTPases, for example, ARF (Colombo et al., 1995), Cdc42 (Harris & Momany, 2004) and Rab (Deneka et al., 2003), with their respective effector proteins.

Small GTP-binding proteins (G-proteins) of the highly conserved Rho family act as molecular switches regulating cell signalling, cytoskeletal organization and vesicle trafficking in eukaryotic cells. Rho1p (RhoA homologue), Rho3p (RhoC homologue) and Cdc42p act as key regulators of cell polarity in *Saccharomyces cerevisiae* by coordinating two important cellular processes of vesicular traffic: (1) delivery of vesicles depending on or independently of the actin cytoskeleton through four effectors – Myo2, a type V myosin (Pruyne et al., 1998), Bni1 (Dong et al., 2003), and Exo70 and Sec3, two subunits of the exocyst complex – through physical interaction (Robinson et al., 1999; Adamo et al., 1999, 2001; Zhang et al., 2001); and (2) docking and fusion of vesicles with the plasma membrane (Boyd et al., 2004; Roumanie et al., 2005). The Rab family, another group of small GTPases, first discovered in yeast, participates in all aspects of vesicular trafficking; however, the similarity between the yeast (Ypt) and mammalian homologue (Rab) suggests that their

**Abbreviations**: CHS, chitin synthase; GOX, glucose oxidase; G-protein, GTP-binding protein; IEF, isoelectric focusing.
Mucor circinelloides is a dimorphic fungus that presents yeast and mycelial growth depending upon the environmental conditions in which it is cultivated. That is, it can grow as yeast cells under anaerobic conditions or as mycelia under aerobic conditions, and switch to either morphology when environmental conditions are changed. It produces highly specialized structures called spores, which are non-motile, non-flagellated, elliptical in shape, and resistant to many adverse conditions. Upon germination, spore diameter increases several-fold via isotropic growth, a process that is dependent upon macromolecular synthesis and accompanied by a series of biochemical events. The swollen spore has the potential to develop into either the hyphal or yeast phase, depending on environmental conditions (see Orlowski, 1991). Thus, the germination process involves a morphological transition from an elliptical spore to an isotropic cell, followed by transformation into a budding yeast cell or the protrusion of the germ tube with its elongation and branching. For either form a dynamic reorganization of the actin cytoskeleton must take place (reviewed by Orlowski, 1991).

Recently we reported the presence of the small GTPases RhoA (Rho1p homologous) and RhoC (Rho3p homologous) at all stages of growth investigated (De la Cruz et al., 2007). However, the association of these G-proteins with chitosomes and secretory vesicles in M. circinelloides remained unclear.

In this study, we obtained two different populations of vesicles: chitosomes and vesicles carrying glucose oxidase (GOX) activity, using two consecutive sucrose gradients from mycelial cells of M. circinelloides. Under the experimental conditions used, in both types of vesicles we distinguished the presence of different G-proteins. Various small GTPases such as Rab8 (Sec4p) and RhoA (Rho1p) and a 17 kDa protein were found to be associated with vesicles carrying GOX activity, whereas Rab8 (Sec4) and Cdc42p were found associated with chitosomes. The difference in association of Rho1 and Cdc42 with vesicles carrying GOX activity and with chitosomes, respectively, probably reflects the specific role they play in the formation, transport and docking of these two different vesicles to the plasma membrane.

**METHODS**

**Strains.** M. circinelloides R7B strain (Leu<sup>−</sup>) and UPO1171, an R7B transformed strain in which the promoter of the gene gpdl encoding glycereraldehyde-3-phosphate dehydrogenase is fused to the open reading frame of the gene gox of Aspergillus niger, were used throughout this study. The product of this gene, GOX, is secreted and thus the protein must be transported in vesicles from the Golgi to the plasma membrane. This strain was kindly provided by Dr José Arnau (University of Copenhagen).

**Media and culture conditions.** M. circinelloides strain UPO1171 was maintained and propagated in YPG medium (Bartnicki-García & Nickerson, 1962). Spores were produced on YPG solidified with 2% (w/v) agar from cultures incubated at 28 °C for 6 days under diffused light. Spores were harvested with sterile distilled water, washed by centrifugation, and resuspended in sterile distilled water. They were counted with a haemocytometer and kept at 4 °C for no more than 1 week. Mycelia were obtained by inoculating 5 × 10<sup>5</sup> spores ml<sup>−1</sup> in liquid YPG, pH 4.5, and incubating for 12 h under aerobic conditions at 28 °C.

**Preparation of cell-free extracts.** The cells were filtered through Whatman no. 1, washed and resuspended in 50 mM Tris/HCl, pH 7.8 containing 10% sucrose. About 20 ml of the resuspended cells was mixed with an equal volume of glass beads (0.45–0.50 mm diameter) and the cells were broken in a Braun model MKS cell homogenizer for 1 min while cooling with a stream of CO<sub>2</sub>. The homogenate was centrifuged at 3000 g for 5 min to remove cell walls and unbroken cells. The cell-wall-free material (crude extract) was centrifuged at 54 500 g for 45 min; the pellet, a mixed-membrane fraction, was discarded and the supernatant was saved for the purification of the vesicles carrying GOX and chitin synthase (CHS) activity.

**Purification of vesicles carrying GOX and CHS activity.** The enriched fractions of vesicles with GOX and CHS activity were obtained using two successive sucrose gradients. The purification of chitosomal CHS was done as described by Martínez-Cadena et al. (1987). Basically, the supernatant obtained in the preparation of cell-free extracts was concentrated to about 3 ml with Aquacide 11A. The concentrated sample was layered on the top of a 36 ml ‘linear-log’ sucrose gradient (Brakke & Van Pelt, 1970). This was centrifuged at 81 500 g for 2 h and fractionated from the top into 1 ml aliquots with an ISCO model 183 density-gradient fractionator. In each fraction, protein concentration and activity of GOX and CHS were measured. Fractions with maximal activities of GOX and CHS were pooled; 50 mM Tris/HCl, pH 7.8, was added up to 19 ml and mixed with 19 ml sucrose 65% with a density-gradient former to make a 25–65% isopycnic sucrose gradient. This second gradient was centrifuged at 81 500 g for 18 h and fractionated from the top into 1 ml aliquots. In each fraction, the density, GOX and CHS activity, and protein concentration were measured.

**GOX activity assay.** GOX activity was measured by a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Commercial GOX from A. niger was used as an enzymic source to make a standard curve. Usually a 50 μl sample or standard was mixed with 1445 μl reaction buffer [10 mg ABTS, 1 ml glucose (20%), 10 μl peroxidase (50 units ml<sup>−1</sup>), 16 ml 50 mM sodium phosphate buffer, pH 6.5, up to 20 ml H<sub>2</sub>O<sub>2</sub>]. The reactions were incubated for 15 min at room temperature and the absorbance measured at 420 nm. One unit is the amount of enzyme that oxidizes 1 μmol glucose in 15 min.

**CHS activity assay.** CHS activity was measured by the filtration method as described by Ruiz-Herrera et al. (1984). Zymogenic CHS was activated with 10 μg ml<sup>−1</sup> trypsin included in the reaction mixture. One unit of CHS is the amount of enzyme that polymerizes 1 nmol N-acetylglucosamine (GlcNAc) in 30 min.

**Electron microscopy.** Negatively stained specimens were examined by electron microscopy (Bracker et al., 1976). The samples were placed on carbon-coated Formvar membranes on grids that had been previously treated by a glow discharge to make the carbon film hydrophilic. Samples were rinsed with distilled water and then negatively stained with 2.5% aqueous uranyl acetate. Grids were
examined by electron microscopy. Chitin microfibres were synthesized in vitro by incubating the chitosomal fraction with UDP-GlcNAc and trypsin as described earlier, before processing them for electron microscopy.

**[α-32P]GTP-binding proteins assay.** GTP proteins were detected by a photoaffinity labelling method described by Singh et al. (1995), as follows. The enriched fractions of chitosomes or vesicles carrying GOX activity (75–100 μg) were incubated in a reaction mixture containing 10 mM Tris/HCl, pH 7.8, 100 mM NaCl, 1 mM DTT, 2 mM MnCl₂, 10 μM ATP and 0.25 μCi [α-32P]GTP ml⁻¹ and incubated in ice for 10 min. Afterwards, the microcentrifuge tubes were exposed to UV light (280 nm) for 10 min and the reaction was stopped by the addition of sample buffer. Where indicated, these fractions were incubated for 10 min with the appropriate non-radioactive nucleotide before [α-32P]GTP addition. The proteins were subjected to SDS-PAGE or isoelectric focusing (IEF)-SDS-PAGE, stained with Coomassie blue and exposed to X-OMAT film (Kodak).

Another GTP-binding assay used in this study was that described by Lapetina & Reep (1987). In this method the proteins of the fractions enriched with chitosomes or vesicles carrying GOX activity were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane for 21 h at 25 mA. Blots were washed with three changes of 4 ml GTP-binding buffer containing 50 mM Tris/HCl (pH 7.4), 1% Tween 20, 5 mM MgCl₂ and 10 μM ATP. After washing, nitrocellulose strips were incubated in 4 ml GTP-binding solution containing 2 μCi (74 kBq) [α-32P]GTP ml⁻¹; after incubation for 10 min, strips were washed twice with 4 ml GTP-binding buffer, dried and exposed to X-OMAT film (Kodak).

**ADP-ribosylation by C3.** ADP-ribosylation was carried out in a reaction mixture (100 μl) composed of 50 mM Tris/HCl pH 7.4, 2 mM MgCl₂, 1 mM ATP, 2 μM NAD, 1 mM DTT, 1 mM EDTA, 2 μCi (74 kBq) [32P]NAD, 100 μg enriched-vesicle fraction and, when indicated, 15 ng Clostridium botulinum C3 exotoxin. The reaction proceeded for 60 min at 30 °C and was terminated by the addition of 2× 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 days) to X-OMAT films for autoradiography.

**Western blotting.** The fractions enriched with vesicles carrying GOX activity and chitosomes were subjected to SDS-PAGE or IEF-SDS-PAGE and electrotransferred into a nitrocellulose membrane for 1 h at 100 mA (Towbin et al., 1979). Blots were immersed for 60 min at room temperature in 10 ml blocking solution which contained 3% albumin in TBS-T [20 mM Tris/HCl, 100 mM NaCl, 0.2% (v/v) Tween-20, pH 7.6]. Blots were washed with three changes of 10 ml TBS-T, followed by incubation with the first antibody with an appropriate dilution prepared in 10 ml of TBS-T. For RhoA, the primary antibody was diluted 1 : 2000 and incubated for 12 h; for the detection of Rab8, the primary antibody (a rabbit polyclonal antibody raised against the full-length Rab8A of human origin) was diluted 1 : 1500 and incubated for 5 h; and for Cdc42p the primary antibody was diluted 1 : 1500 and incubated for 5 h. After washing with three changes of 10 ml TBS-T, a secondary antibody, anti-rabbit IgG, conjugated with horseradish peroxidase diluted 1 : 2000 in 10 ml TBS-T, was added to all nitrocellulose membranes. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology. Immunoreactivity was monitored by the enhanced chemiluminescence method (Amersham).

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

**RESULTS**

**Analysis of GOX production in M. circinelloides UPO1171**

Our goal in this study was to investigate the presence of GTP-binding proteins in vesicles carrying different cargo proteins. For this, we used a transformed strain from M. circinelloides carrying the open reading frame of the gox gene of A. niger fused to the gpd1 gene promoter. Before we started to work with an enriched fraction carrying the vesicles with GOX activity, we studied the activity secreted into the medium at various development stages of M. circinelloides. As shown in Fig. 1, GOX activity was detected in the extracellular medium after 24 h growth and the activity continued to increase up to 48 h; afterwards, the activity detected did not change significantly. M. circinelloides R7B strain, the parental untransformed strain, was used as a negative control and, as can be observed in Fig. 1, no GOX activity was detected (Fig. 1). This result indicates, as expected, that the transformed strain is able to secrete GOX activity (Larsen et al., 2004). Therefore, we used this strain to study the presence of GTP-binding proteins in fractions enriched in vesicles carrying GOX activity and vesicles carrying CHS or chitosomes.

**Enrichment of two types of vesicles: chitosomes and vesicles carrying GOX activity**

To obtain fractions enriched with vesicles carrying GOX activity or with chitosomes, we followed the procedure described for the purification of chitosomal CHS (Martínez-Cadena et al., 1987), that is, using two successive sucrose gradients, the first ‘linear-log’ and the second an isopycnic gradient. In the first ‘linear-log’ sucrose gradient, we obtained a peak from fraction 7 to 13, containing both GOX and CHS activities (Fig. 2a). These fractions were

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**Fig. 1.** GOX activity measurements. Spores (5×10⁶ ml⁻¹) from strains R7B (●) and UPO1171 (●) were inoculated in YPG medium and incubated for 92 h at 28 °C. Samples were withdrawn at the times shown and GOX activity was determined in cell-free medium as described in Methods. One unit of GOX oxidizes 1 μmol glucose in 15 min. Similar results were obtained in two other experiments.
pooled, mixed with sucrose (65%) and subjected to an isopycnic sucrose gradient (Fig. 2b). In this gradient, three peaks of GOX activity were obtained, with densities of 1.10, 1.13 and 1.18 g ml$^{-1}$. Moreover, one peak of CHS activity with a density of 1.14 g ml$^{-1}$ was obtained. The same density for chitosomes of other fungi has been reported (see Bracker et al., 1976; Ruiz-Herrera et al., 1984; Martinez-Cadena et al., 1987). Therefore, this procedure was used to separate the vesicles carrying GOX activity from the chitosomes.

To determine whether the fractions contained chitosomes or vesicles carrying GOX activity, the samples were negatively stained and observed in the electron microscope. In the fraction with CHS activity (density 1.14 g ml$^{-1}$) was obtained. The same density for chitosomes of other fungi has been reported (see Bracker et al., 1976; Ruiz-Herrera et al., 1984; Martinez-Cadena et al., 1987). Therefore, this procedure was used to separate the vesicles carrying GOX activity from the chitosomes.

vesicles of 25–30 nm in diameter (Fig. 2e). These fractions were unable to synthesize chitin microfibrils in vitro, indicating no contamination with chitosomes. Mitochondrial contamination of both fractions was investigated by Western blot analysis with an anti-CORE I antibody, a marker of inner mitochondrial membranes. Both fractions, chitosomes and secretory vesicles, were free from inner mitochondrial membrane fragments, indicating no mitochondrial contamination (data not shown).

G-proteins are present in vesicles carrying GOX activity and in chitosomes

GTP-binding proteins (G-proteins) have been reported to associate with vesicles, regulating their transport or secretion (Adamo et al., 1999; Ridley, 2001). To determine whether the vesicles carrying GOX activity and chitosomes had G-proteins attached, an [$\alpha^{32}$P]GTP-radiolabelling assay was used as described by Lapetina & Reep (1987). Autoradiography showed that in the chitosomal fraction
(Fig. 3a, lane Ctrl), and in the fraction enriched with GOX activity (density 1.13 g ml$^{-1}$) (Fig. 3b, lane Ctrl), four proteins, with molecular masses of 17, 21, 48 and 65 kDa, respectively, were revealed. In addition, two proteins with molecular masses of 39 and 85 kDa, respectively, were observed in the fraction corresponding to chitosomes, and two other proteins with molecular masses of 31 and 70 kDa, respectively, were observed in the GOX-activity-enriched fraction (Fig. 3b, lane Ctrl). The two other peaks with GOX activity and densities of 1.10, 1.18 g ml$^{-1}$, respectively, showed the same GTP-binding proteins as the fraction stated above (data not shown). These results indicate that both monomeric and putative heterotrimeric G-proteins are associated with both vesicles.

To determine if the binding of GTP was specific, competition experiments were carried out between unlabelled nucleotides and $[^{32}\text{P}]$GTP. For these experiments, we used the photoaffinity radiolabelling of G-proteins with $[^{32}\text{P}]$GTP as described in Methods. Fig. 3 shows that either unlabelled GTP or GDP blocked the binding of $[^{32}\text{P}]$GTP to the 17, 21, 39, 48, 65 and 85 kDa proteins from chitosomes (Fig. 3a) and 17, 21, 31, 48, 65 and 70 kDa proteins from vesicles carrying GOX activity (Fig. 3b). This inhibition was found at 100 nmol l$^{-1}$ for either GTP or GDP. Unlabelled nucleotides such as GMP did not decrease the intensity of $[^{32}\text{P}]$GTP labelling in any of these proteins; other nucleotides such as ATP and UTP, tested at 100 nmol l$^{-1}$, had no significant effect on $[^{32}\text{P}]$GTP binding, indicating that the radioactive proteins observed in both types of vesicles are G-proteins.

**Difference in association of small G-proteins with vesicles carrying GOX and chitosomes**

$[^{32}\text{P}]$GTP-radiolabelled proteins of both vesicles were separated by two-dimensional gel electrophoresis to determine whether they correspond to one protein or multiple proteins with the same molecular mass. The autoradiography of vesicles carrying GOX activity (Fig. 4a) shows three small G-proteins with pI values of 6.0, 6.1 and 6.2, and molecular masses of 21, 21 and 17 kDa, respectively. The autoradiography of chitosomal proteins (Fig. 4b) shows only two small G-proteins with pI values of 6.1 and 6.2, and molecular masses of 21 and 17 kDa, respectively. The proteins corresponding to high molecular mass G-proteins were not detected under these experimental conditions. These results indicate that two small G-proteins (pI of 6.1 and 6.2, with molecular masses 21 and 17 kDa) are present in both vesicles. However, an additional small G-protein (pI of 6.0, molecular mass 21 kDa) was detected associated with vesicles carrying GOX activity.

![Fig. 3. $[^{32}\text{P}]$GTP binding of proteins from chitosomes and vesicles carrying GOX activity. Proteins from fractions from the chitosomal peak of density 1.14 g ml$^{-1}$ (a) and from vesicles carrying GOX activity of density 1.13 g ml$^{-1}$ (b) were assayed for $[^{32}\text{P}]$GTP binding. The reaction mixture was incubated with $[^{32}\text{P}]$GTP and irradiated with UV light or not irradiated (w/UV), separated by electrophoresis, and after electrophoretic separation exposed to X-ray film. The autoradiography of both vesicles shows G-proteins (arrows). In addition, the influence of unlabelled nucleotides on the $[^{32}\text{P}]$GTP binding of both vesicles was studied. For this, the fractions of chitosomes (a) and vesicles carrying GOX activity (b) were processed as described before, except that they were pre-incubated for 10 min with the indicated non-radiolabelled nucleotide (100 nM) before $[^{32}\text{P}]$GTP addition. Similar results were obtained in three other experiments.](https://www.microbiologyresearch.org)
RhoA is present in vesicles carrying GOX activity

Rab and Rho proteins are members of the Ras superfamily of small G-proteins that have been reported to associate with vesicles, acting as key regulators of polarized trafficking (Adamo et al., 1999; Takai et al., 2001). Members of the Rho subfamily, RhoA, RhoB (pI of 6.1 and 6.2, with molecular masses of 21 and 17 kDa) and RhoC, are substrates for the ADP-ribosyltransferase activity of the exotoxin C3 of C. botulinum (Sekine et al., 1989). To investigate whether one of the G-proteins detected in chitosomes or the vesicles carrying GOX activity corresponded to Rho proteins, these fractions were incubated with C3 exotoxin and [γ-32P]NAD. Fig. 5(a) shows that a Rho protein with a molecular mass of 21 kDa was detected in those fractions corresponding to vesicles carrying GOX activity, but Rho protein was not detected in fraction 14, which corresponds to the major peak with CHS activity, indicating the presence of Rho only in vesicles carrying GOX activity. Furthermore, Rho1p (RhoA homologue) and Rho3p (RhoC homologue) have been reported associated with vesicles (Symons & Rusk, 2003; Adamo et al., 2001; Roumanie et al., 2005). To investigate if both Rho proteins were associated with the vesicles carrying GOX activity, proteins from the fraction with a density of 1.13 g ml⁻¹ corresponding to one of the major peaks with GOX activity were first ADP-ribosylated with exotoxin C3, then separated by two-dimensional gel electrophoresis, and processed for autoradiography. Only one spot was detected, with a pI of 6.0 and molecular mass of 21 kDa, indicating that RhoA (Rho1p) protein is associated specifically with the vesicles carrying GOX activity.

Association of Cdc42p with chitosomes

Cdc42p, another member of the Rho subfamily of G-proteins, has also been found to associate with secretory vesicles. It has been reported that this protein acts as a positive allosteric regulator of the late secretory apparatus delivery of vesicles to the plasma membrane (Roumanie et al., 2005). To determine if this protein is associated with chitosomes or vesicles carrying GOX activity, we used polyclonal antibody anti-human Cdc42p. This antibody detected a protein with a molecular mass of 17 kDa only in the fraction enriched with chitosomes (Fig. 6a), whereas in the fraction of vesicles carrying GOX activity, we were unable to identify this G-protein (Fig. 6b), even though an [γ-32P]GTP-binding protein with a similar molecular mass was detected (see Fig. 4). This protein, identified as Cdc42p in chitosomes, showed a pI of 6.2 when separated by two-dimensional gel electrophoresis (Fig. 6c); this pI was detected earlier by an [γ-32P]GTP-binding assay of the chitosomal fraction. This result indicates that Cdc42p is associated specifically with chitosomes.

Association of Rab protein with secretory vesicles and chitosomes

The first evidence of the role of Rab family members in vesicular trafficking was reported in yeast in 1988 (Goud et al., 1988). These proteins participate in all aspects of vesicular trafficking; e.g. Rab8 (Sec4p) plays a key role in both fusion and membrane transport, directly linked to the
actin cytoskeleton (Jordens et al., 2005). We used rabbit polyclonal antibody anti-Rab8 of human origin to detect whether this protein is one of the G-proteins associated with chitosomes (Fig. 7a) and/or vesicles carrying GOX activity (Fig. 7b). In both vesicles, this antibody was able to detect a band of a molecular mass of 21 kDa (Fig. 7a, b). When the proteins from the enriched fractions of both vesicles were separated by two-dimensional gel electrophoresis, only one protein, with a pI of 6.1 and molecular mass of 21 kDa, was recognized by this antibody in both vesicle populations (data not shown). Even though this antibody can cross-react with other proteins from the Rab family, only one spot was detected after a two-dimensional gel electrophoresis, which indicates the presence of only one Rab protein associated with these vesicles (data not shown). As stated earlier, this protein was detected by the [α-32P]GTP-binding assay associated with the vesicles carrying GOX activity and with chitosomes. Therefore, these results indicate that Rab8 (Sec4p) might play a direct role in the transport or fusion of both vesicles with their target membranes, independently of their cargo proteins (GOX or CHS activities).

**DISCUSSION**

The cell wall of the dimorphic zygomycete *M. circinelloides* is composed, among other components, of chitin which is synthesized from UDP-GlcNAc as a result of the enzymic activity of CHS. This enzyme is compartmentalized in vesicles or chitosomes (see Bracker et al., 1976). Since this fungus is unable to secrete GOX through its various secretory vesicles, we used a *M. circinelloides* transformed strain in which the promoter of the gene *gpd1* is fused to the open reading frame of the gene *gox* of *A. niger*. We investigated whether G-proteins were associated with these vesicles depending on the function of their cargo: those carrying CHS, necessary for new cell-wall synthesis during polarized growth, and those carrying GOX activity, a secreted non-essential enzyme. Vesicles were purified by a
method based on two consecutive sucrose gradients (Martínez-Cadena et al., 1987).

Using an [z-32p]GTP-binding assay, ADP-ribosylation by C3 exotoxin, or Western blot analysis with specific antibodies, we detected the association of high molecular mass G-proteins (48, 65 and 70 kDa) in both types of vesicles and a differential association of small GTPases; for example, Rab8, RhoA (Rho1p) and a 17 kDa protein were associated with those vesicles carrying GOX activity, and Cdc42 and Rab8 proteins with chitosomes.

As stated before, Rab8 is one of the members of the Rab family associated both with chitosomes and with those vesicles carrying GOX activity. Members of the Rab family are involved in all steps of vesicle trafficking: vesicle formation, transport, docking, membrane remodelling and fusion (Titus, 1997; Segev, 2001; Langford, 2002; Molendijk et al., 2004). Rab8 acts in two stages of vesicular trafficking: transport and fusion of the secretory vesicle. During vesicle transport, the Rab8 protein acts on a component of the exocyst (Sec15) promoting the transport along actin cables by the type V myosin protein, Myo2p (Boyd et al., 2004). Fusion of the vesicle is promoted by the strong Rab8GTP–Sec15p interaction and protein–protein interactions through various subunits of the exocyst up to Sec3p, which marks the sites of exocytosis on the plasma membrane (Guo et al., 1999). Since we found the association of Rab8 (Sec4) with both types of vesicles, independent of vesicle cargo (a secreted enzyme or an enzyme necessary for cell formation), we suggest that this protein participates in transport and/or fusion of these vesicles with the plasma membrane.

Two members of the Rho family were identified: RhoA was found associated with the vesicles carrying GOX activity, and Cdc42 with chitosomes. The identification of RhoA or Rho1p (pI 6.0, 21 kDa) was done by immunological studies and an ADP-ribosylation assay with exotoxin C3 from C. botulinum, as described by various authors.

The Rho GTPases are important regulators of polarity in eukaryotic cells. In yeast, they are involved in regulating the transport, docking and fusion of secretory vesicles with the

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**Fig. 6.** Identification of Cdc42p associated with chitosomes. Proteins from fractions of chitosomes (density 1.14 g ml\(^{-1}\)) (a) and vesicles carrying GOX activity (density 1.13 g ml\(^{-1}\)) (b) were separated by SDS-PAGE, electro-transferred and the membranes incubated with human polyclonal antibody anti-Cdc42p for 5 h (dil. 1 : 2000); afterwards, the blots were incubated with a secondary antibody conjugated with horseradish peroxidase. The chitosomal fraction was subjected to two-dimensional electrophoretic separation, and processed as stated earlier (c). Identical results were obtained in two other experiments.

**Fig. 7.** Identification of Rab protein associated with vesicles carrying GOX activity and chitosomes. Proteins from the fraction of chitosomes (a) and from that of the vesicles carrying GOX activity (b) were separated by SDS-PAGE, electro-transferred and the membranes incubated with human polyclonal antibody anti-Rab8 (dil. 1 : 1500); afterwards, the blots were incubated with a secondary antibody conjugated with horseradish peroxidase. Similar results were obtained in three other experiments.
cell surface (Roumanie et al., 2005). Five effectors for Rho1 have been described: Pkc1 protein kinase, 1,3-β-glucan synthase (GS), the Bni1 and Bnr1 formin proteins, the Skn7 transcription factor, and the Sec3 exocyst component. Evidence is mounting to suggest that each Rho1-effector pair is regulated separately by a different complement of GAPs and perhaps different GEFs as well. Together, these effectors regulate synthesis of cell-wall glucans and chitin, expression of genes important for cell-wall biogenesis, polarization of the actin cytoskeleton, and perhaps exocytosis (Drees et al., 2001).

The RhoA isoform homologue of Rho1p is one of the most important proteins associated with secretory vesicles; it participates in vesicular trafficking and appears to function as a landmark for exocytic targeting on the plasma membrane when associated with various effectors such as Sec3 and Bni1, through physical interactions between Rho and the NH2-terminal domain of these effectors, to promote the polarization of fungi (Robinson et al., 1999; Guo et al., 2001).

As stated before, Cdc42 was found associated only with chitosomes. Cdc42 is an important regulator of many biological processes, including cell polarization, morphogenesis, membrane traffic, cell growth, and development in eukaryotic cells (reviewed by Johnson, 1999). Like many other members of the Ras superfamily of small GTP-binding proteins, Cdc42 has multiple downstream effectors, among them Sec3p (Zhang et al., 2001). Through this and other effectors, Cdc42 coordinates various cellular activities to carry out specific biological functions. A major function of Cdc42 is to control the polarized organization of the actin cytoskeleton, which is involved in polarized exocytosis. Zhang et al. (2001) demonstrated that Cdc42 competes with Rho1 in binding to Sec3p, suggesting that these two small G-proteins bind to similar or overlapping regions of this protein (Zhang et al., 2001). Different Rho family proteins carry out different yet related functions. Many work coordinately in a cascade of related cellular processes (Chant & Stowers, 1995). Both Cdc42 and Rho1 are involved in cell polarization and actin cytoskeletal organization. Their interactions with formin family proteins may be important in controlling actin organization (for review, see Jaffe & Hall, 2005). It is possible that the exocyst requires signalling from both these proteins for polarized localization. It is also possible that Cdc42 and Rho1 work at different time points during polarized growth. While Cdc42 is primarily involved in the early establishment of cell polarity, Rho1 may be mainly involved in the maintenance of polarized growth (Cabib et al., 1998). For example, rho1-104 mutant cells can initialize budding but stop further growth at tiny or small budded stages, suggesting that polarized growth cannot be sustained in this mutant (Yamochi et al., 1994). In addition, GFP-exocyst proteins lose their polarity in many rho1-104 cells at the restrictive temperature, even though the cells have a polarized budding pattern (Guo et al., 2001). The difference in association of Rho1 and Cdc42, with vesicles carrying GOX activity and with chitosomes, respectively, found in this study indicates that each of these proteins probably controls the formation, transport and specific plasma membrane site docking of the respective vesicles. Further genetic, biochemical and cytological experiments are needed to explore the relationship between Rho1 and Cdc42 during polarized cell growth and secretion in M. circinelloides.

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