Antonie van Leeuwenhoek (2007) 91:237–251
DOI 10.1007/s10482-006-9113-7

ORIGINAL PAPER

Mucor rouxii Rho1 protein; characterization and possible role in polarized growth

Silvia Argimón · Fiorella Galello · Elba Pereyra · Silvia Rossi · Silvia Moreno

Received: 19 April 2006 / Accepted: 10 August 2006 / Published online: 2 November 2006

Abstract We have previously shown that protein kinase A of the medically important zygomycete *Mucor rouxii* participates in fungal morphology through cytoskeletal organization. As a first step towards finding the link between protein kinase A and cytoskeletal organization we here demonstrate the cloning of the *RHO1* gene and the characterization of its protein product. The Rho1 protein primary sequence shows 70–85% identity with fungal *RHO1* or mammalian RhoA. Two protein kinase A phosphorylation sequences in adequate context are predicted, Ser73 and Ser135. The peptide IRRNSQKFV, containing Ser135 proved to be a good substrate for *M. rouxii* protein kinase A catalytic subunit. The over-expressed Rho1 fully complements a *Saccharomyces cerevisiae* null mutant. The endogenous protein was identified by western blot against a developed antibody and by ADP-ribosylation. Localization in germlings was visualized by immunofluorescence; the protein was localized in patches in the mother cell surface and excluded from the germ tube. Measurement of Rho1 expression during germination indicates that Rho1, at both the mRNA and protein levels, correlates with differentiation and not with growth. Rho1 has been shown to be the regulatory protein of the β-1,3-glucan synthase complex in fungi in which β-1,3-glucans are major components of the cell wall. Even though glucans have not been detected in zygomycetes, caspofungin, an echinochandin known to be an inhibitor of β-1,3-glucan synthase complex, is shown here to have a negative effect on growth and to produce an alteration on morphology when added to *M. rouxii* growth culture medium. This result has an important impact on the possible participation of β-1,3-glucans on the regulation of morphology of zygomycetes.

Keywords Caspofungin · Cloning · Gene expression morphology · *Mucor rouxii* · Rho1

Abbreviations
Mr Mucor rouxii
PKA protein kinase A

Introduction

Rho GTPases are molecular switches that control a wide variety of signal transduction pathways in

S. Argimón · F. Galello · E. Pereyra · S. Rossi · S. Moreno (✉)
Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, Pabellón 2, Piso 4, 1428 Buenos Aires, Argentina
e-mail: smoreno@qb.fcen.uba.ar

Present Address:
S. Argimón
Aberdeen Fungal Group, University of Aberdeen, Aberdeen AB25 2ZD, UK

all eukaryotic cells and can be regulated through direct phosphorylation or ubiquitination (Jaffe and Hall 2005). They are known principally for their pivotal role in regulating the actin cytoskeleton, but their ability to influence cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity is probably just as significant. In fungi, Rho1 GTPase has been identified as a master regulator of cell wall integrity signaling, not only because it receives the major inputs from the cell surface but also because it regulates a variety of outputs involved in cell wall biogenesis, actin organization, and polarized secretion (Levin 2005).

Cell polarity is central to cell function, acting to organize the biochemistry and the structures that result in the development of cells with specialized physiology. An extreme case of polar growth has evolved in filamentous fungi which are able to extend the tips of their tube-like cells (hyphae) for unlimited time, provided nutrients are available. Until recently the understanding of hyphal morphogenesis was based on the *Saccharomyces cerevisiae* model, in which several small GTP binding proteins have been associated with the bud emergence site (Harris and Momany 2004). However several features characteristic of filamentous fungi, such as the ability to maintain multiple axes of polarity and an extremely rapid extension rate, cannot be explained by simple extrapolation of yeast models (Harris and Momany 2004; Harris et al. 2005). Although the cortical landmark proteins that generate the positional signals in fungi are still mostly unknown, it is quite clear that, as in yeast, the positional information is relayed by Rho GTPases signalling modules to the morphogenetic machinery that remodels the cell surface (Harris and Momany 2004; Harris et al. 2005; Seiler and Plamann 2003). Actin microfilaments are part of this morphogenetic machinery, providing a structural framework around which cell shape and polarity are defined, as well as the driving force for cells to move and to divide. At present *Ashbya gossypii* has proven to be a suitable model to elucidate the regulatory networks that govern the functional differences between filamentous growth and yeast growth, and intensive work on this field is currently under study using this model (Wendland and Walther 2005).

Signalling systems controlling filamentous growth are multiple and conserved between distantly related fungi. In particular, cAMP signalling has been demonstrated to be involved in fungal development (Lee et al. 2003; Lengeler et al. 2000; Mahdani and Fink 1998). Two transcriptional regulators, Flo8 and Sf11, have been identified as target effectors for this pathway in *S.cerevisiae* (Gancedo 2001; Rua et al 2001). In mammalian systems Rho A (Rho1 in fungi) has been demonstrated to be a substrate for phosphorylation by protein kinase A (PKA) (Lang et al. 1996; Ellerbroek et al. 2003) and signal transduction by cAMP has been proposed to cross-talk with Rho A/Rho1 mediated pathways both in higher and lower eukaryotes (Wendland 2001; DeMali et al 2003).

We are particularly interested in looking for PKA targets so as to understand the signal transduction pathway all the way from the increase in cAMP levels, through the activation of PKA, up to the physiological effect the phosphorylation of the target protein might have as response. To attain this goal we have been using as a model system the dimorphic fungus *Mucor rouxii*, a medically important and understudied zygomycete. We have already demonstrated that PKA, cytoskeletal organization and integrin-like interactions are involved in polarized growth and branching (Pereyra et al. 1992, 2000, 2003). Due to the key role Rho1 has in fungal polarized growth, as stated above, we chose to clone this gene from *M.rouxii* as a first step in looking for links between PKA and fungal morphology. In this paper we describe the molecular cloning of *RHO1* gene, the comparison of the predicted Rho1 protein with other fungal and human sequences, and some of its properties including preliminary results on its possible role in polarized growth studying the effect an echinocandin, a known inhibitor of β-1,3-glucan synthase complex, has on *Mucor* morphology.

Materials and methods

Fungal strain, growth and microscopic observations

Spores of *M. rouxii* (NRRL 1894) (obtained and stored as described previously (Haidle and

Storck 1966), were inoculated at a concentration of 10^6 spores/ml in defined medium consisting of mineral salt solution supplemented with vitamins and 2% glucose (DMG) (Bartnicki-García and Nickerson 1962). Flasks were incubated in a gyratory shaker at 100 r.p.m. and at 28°C for variable periods of time. Where indicated, caspofungin acetate (Cancidas, Merck, provided by Merck Argentina) was added to the cultures at the beginning of germination from a filter-sterilized stock solution. Growth was continued for the times indicated in each case. For microscopic observations, 1- ml samples of cultures were withdrawn, observed and photographed using a Zeiss Axioplan epifluorescent microscope. About 100 cells were scored for each experiment. For growth measurements, 5- ml samples were collected from the cultures on pre-weighed nitrocellulose filters for dry weight determinations. The filters were rinsed with distilled water and dried at 90°C to constant weight.

Cloning strategy

To clone *M. rouxii RHO1* (Mr *Rho1*) gene, degenerate oligonucleotide primers RHO4 (5′-CCGAGGTGTACGTCCCCACNGTNTTY-GA-3′) and RHO8 (5′-GGG-TCTTGGCGG-AGCAYTCNWVRTA-3′) were designed based on an amino acid alignment of known eukaryotic RHO GTPase sequences and used, along with 1μg of Mr genomic DNA, in a touch-down PCR reaction (Rose et al. 2003). Gene-specific primers (RHO13, 5′-TTGATTCACCCGATTCATTGG-3′ and RHO16, 5′-CGACGTACT-TGTAAG-CACTAA-3′) were designed based on the sequence of the 540 bp genomic fragment obtained, and used to amplify the 5′ and 3′-ends of the cDNA by RACE-PCR (Clontech, Mountain View, CA, USA). The complete *RHO1* cDNA sequence was amplified by RT-PCR (Promega, Madison, WI, USA) and the genomic DNA sequence by PCR using as primers RhoBamHI, 5′-CGCGGATCCTATGGCTGAAATCAGA-3′, and RhoPstI, 5′-AACTGCAG-TTTTTT-TTTTTTTTT-3′. The PCR amplified fragments were cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing.

In silico analysis

The translation of Mr Rho1 sequence, multiple alignments and phylogenetic tree were performed with DNAMAN software, version 4.1. The resulting protein sequence was analyzed for homology with other known protein sequences via the BLAST search engine at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The putative PKA phosphorylation sites in Mr Rho1 protein were determined analyzing the aminoacid sequence using the Scan site prediction program (http://www.scansite.mit.edu). For the prediction of the tertiary protein structure the sequence of the Mr Rho1 was modeled against human RhoA as template (1FTN) using Swiss Model, ''An Automated Comparative Protein Modelling Server'' (http://www.expasy.hcuge.ch/swissmod/SWISS-MODEL.html) facility (Peitsch 1995) which runs on the software ProMod II (Peitsch 1996).

Kinetic analysis

M. rouxii PKA holoenzyme was prepared from mycelial powder through DEAE-cellulose and $(NH_4)_2SO_4$ precipitation and the C subunit was isolated by dissociating sucrose gradient centrifugation with 0.5 M NaCl and 1 mM cAMP as described previously (Zaremberg et al. 2000). Phosphotransferase activity of the *M.rouxii* C subunit was assayed in a reaction mixture containing 50 mM Tris-HCl pH 7.4, 0.1 mM [γ^{32}P]ATP (300 cpm/pmol), 0.1 mM EGTA, 0.1 mM EDTA, 15 mM $MgCl_2$, 10 mM β-mercaptoethanol and variable amounts of the two substrate peptides assayed: kemptide (LRRASLG) and rhotide (IRRNSQKFV), which were synthesized by BioSynthesis Inc.. After 15-min incubation at 30°C aliquots from each reaction were processed by the phosphocellulose paper method (Roskoski 1983). Data from peptide substrates were fitted to Michaelis–Menten curves using GraphPad Prism 4.02 version to derive the K_m and V_{max} values.

Semi-quantitative RT-PCR

RNA was prepared from samples of *M. rouxii* cells grown up to different stages using standard proce-

dures. The RNA samples were stored in RNA storage solution (Ambion, Austin, TX, USA). Semiquantitative RT-PCR of Mr Rho1 RNA was performed using the housekeeping gene elongation factor EF-1α (TEF-1) as an internal standard. TEF-1 has been demonstrated to be constitutively expressed throughout germination in the phylogenetically highly related fungus *M. racemosus* (Linz et al. 1986). The RT-PCR amplification reaction was calibrated in order to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts while still keeping amplification for these genes in the log phase. The adequate number of cycles for the semiquantitative PCR reactions was 23. Oligonucleotide primers sense and antisense were synthesized according to Rho1 (Rho13 5′-TTGATTCACCCGA-TTCATTGG-3′ and Rho16 5′-GCTGCATGAACAATTGC-TTGATT-3′) and TEF1 (TEF1 5′-TCACG-TCGATTCCGGTAAGTC-3′ and TEF2 5′-TATCACCGTGCCAGCCAGA-3′) sequences. The expected PCR product lenghts were 260 bp for Rho1 and 550 bp for TEF1.

Complementation assay

pYOMrRHO1 was constructed by insertion of a PCR amplified Mr *RHO1* fragment into the *Eco*RI/*Bgl*II gap of pYO701, which consists of pRS314, the 0.2-kb *S.cerevisiae RHO1* promoter and its corresponding 0.1-kb terminator. The pYOMrRHO1 plasmid was introduced into a yeast strain (YOC706), which contained a *rho1* deletion and a plasmid (pYO774) expressing the yeast *RHO1* gene under the control of the *GAL1* promoter, generously provided by M. Yukawa from the University of Tokyo (Qadota et al. 1994). The YOC706 strain was grown and maintained in YP medium containing both 2% glucose and 2% galactose as carbon sources. For the complementation assays, the YOC706 strain, transformed with pYOMrRHO1, was grown in YPGlu at 30°C. Viability was assayed by comparison with the growth at the same temperature in YPGal.

Preparation of cell free extracts

Cell-free extracts were obtained from sporangiospores throughout all the germination stages. Cells of 200 mg wet weight were vortexed three times for 1 min at 4°C with 1.2 g glass beads (0.5–0.6 mm diameter) and 0.6 ml homogenization buffer (50 mM potassium phosphate, pH 6.8, 5 mM EDTA, 3 mM EGTA, 120 mM NaCl, 10 mM 2-mercaptoethanol, and complete protease inhibitor mix (Roche, Indianapolis, IN, USA), and the extract centrifuged briefly at 500 × g to remove glass beads. The supernatant, containing cytosol and mixed-membrane fractions was divided in aliquots and kept at – 20°C until use. For the ADP-ribosylation assay mixed membranes were separated from cytosol by 1 h centrifugation at 100,000 × g.

ADP ribosylation assay

ADP-ribosylation was carried out in a reaction mixture (40μl) composed of 50 mM Tris/HCl pH 7.4, 2 mM $MgCl_2$, 1 mM ATP, 0.2% Nonidet P40, 1 mM EDTA, 1 mM DTT, 2μM NAD, 4×10^6 dpm [^{32}P]NAD (1000 Ci/mmol, Amersham Pharmacia Biotech., Piscataway, NJ, USA), 20–50μg protein from either cytosol or mixed membranes fractions and 0.1μg *Clostridium botulinum* C3 exotoxin (Biomol, Plymouth Meeting, PA, USA). The reaction proceeded for 30 min at 37°C and was terminated by addition of 2 × SDS-PAGE solubilization buffer. After the ADP-ribosylation reaction, samples were analyzed by 14% SDS-PAGE. After electrophoresis the gels were stained, dried and exposed to X-OMAT-films for autoradiography.

Western blot analysis

After electrophoresis, proteins were transferred onto nitrocellulose and assayed for immunoreactivity against a polyclonal antibody (1/300) raised in rabbit by Bio-Synthesis against a peptide sequence speficic for Mr Rho1 (GTIEELRRNS). Sheep anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody and immunoreactivity revealed by chemiluminescence Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), visualization and scanning by digital imaging.

Fluorescence microscopy

Cells from 5 to 7 h of growth were fixed with 4% (v/v) formaldehyde in PBS for 24 h. After

permeabilization for 5 min with cold ethanol/toluene (3:1, v/v), fixed cells were incubated for 2 h with the primary antibody anti Mr Rho1 (1/100) followed by the addition of goat anti-rabbit IgG conjugated with Cy3 (Invitrogen, Carlsbad, CA, USA). Cells were analyzed by epifluorescence microscopy using a Olympus Fluoview scanning laser biological inverted microscope IX70; images were acquired with a Zeiss AxioCam digital camera, operated by the AxioVision 3.1 software (both from Carl Zeiss, Jena, Germany) and exported into Adobe Photoshop for processing.

Results and discussion

Cloning and sequencing of *M.rouxii RHO1*

The strategy used for cloning the gene coding for Rho1 small GTP binding protein from *M. rouxii* (Mr Rho1) can be summarized as follows: in a first step, a specific product was obtained by PCR amplification from genomic DNA using degenerate primers derived from conserved sequences in all the Rho1 proteins. Specific primers were further designed from the cloned fragment and the 5′ and 3′ termini from the cDNA were obtained by RACE. Independent amplification of genomic DNA and cDNA with the same set of 5′ and 3′ specific terminal primers yielded the complete sequence of Mr *RHO1* gene and cDNA shown in Fig 1. A DNA fragment of 937 base pairs was cloned. An ORF of 588 bases long was found, beginning with an ATG in a favorable context (the equivalent to the Kovak sequence for mammalian genes), derived from the comparison with the sequences around ATG of 30 genes cloned in *Mucor* species ($A_{40}/C_{30}A_{73}$ A_{57}/C_{33} A_{60} **ATG**). The ORF was interrupted by three introns spanning nt 197–250, 361–412 and 548–613. The cDNA, cloned and sequenced independently, confirmed the predicted introns and ORF. A protein of 195 amino acids, with a MW of 21.6 kDa, was translationally predicted. The high degree of homology of this protein with Rho1 from other fungi (see Fig 2) allowed us to establish that the cloned gene corresponds to a *bona fide RHO1* gene.

The sequence of Mr Rho1 protein (Zygomycota) was compared and aligned with the available homologous sequences from 10 fungi (nine Ascomycota and one Basidiomycota) and with one representative of mammalian RhoA (Fig. 2A). It is already known that the RhoA proteins from mammalian origin are highly conserved with more than 90% of identity in their primary sequences. The first conclusion derived from this alignment is that this protein is highly conserved throughout the phylogenetic scale. The identity of Mr Rho1 with almost all of the fungal sequences ranges between 71.3 and 83.9% while the identity with the human RhoA is 72%. Fig. 2B shows a phylogenetic tree of the homologous Rho1 sequences. Two clusters are evident, one including the budding yeast ascomycetes (Saccharomycetes) and the other including the filamentous ascomycetes. Rho1 from *Schizosaccharomyces pombe*, an archiaeascomycete, and Rho1 from *Yarrowia lipolytica*, seem to be separated from these two clusters. *Y.lipolytica* is a fungus that although placed unambiguously among the saccharomycetes according to its rRNA sequence, displays some features shared with higher eukaryotes and filamentous fungi (Barth and Gallairdin 1997). Rho1 proteins from the basidiomycete (*F. neoformans*) and from the zygomycete *M.rouxii* are not clustered, as expected.

Importantly, fungal Rho1 proteins contain conserved residues or motifs unique to members of the Rho family (Paduch et al. 2001) (Fig. 2A). These include Asn42 (corresponding to human RhoA), the site for specific ADP-ribosylation by the C3 exoenzyme toxin from *Clostridium botulinum*, and the C-terminal CAAL (A is an aliphatic amino acid), the signature sequence for posttranslational modification by protein geranylgeranyltransferase and the poly(lysine) domain next to the CAAL motif, demonstrated to be involved in targeting of the Ras and Rho proteins to the plasma membrane (Hancock et al. 1991). The alignment also shows conserved sequences in the loops G1 to G5 belonging to the catalytic moiety of the protein (Paduch et al. 2001). The G2 and G3 loops correspond to the functional regions switch I and switch II that surround the γ-phosphate group of GTP. These regions, highly conserved among small GTP binding proteins and important for interaction with the effectors, undergo structural changes

```
1       actattaacaagATGGCTGAAATCAGACGAAAACTTGTGATTGTTGGAGATGGTGCTTGT
                    M  A  E  I  R  R  K  L  V  I  V  G  D  G  A  C    16

61      GGTAAAACCTGTTTGTTGATTGTCTTTTCAAAGGGTACTTTTCCTGAGTTCTATGTTCCC
         G  K  T  C  L  L  I  V  F  S  K  G  T  F  P  E  F  Y  V  P    36

121     ACCGTTTTTGAAAATTACGTAGCTGATGTCGAAGTCGATGGAAAACACGTGGAATTAGCT
         T  V  F  E  N  Y  V  A  D  V  E  V  D  G  K  H  V  E  L  A    56

181     TTATGGGATACAGCAGgtatatctatcacttagactattttgaaaaatgataacttacat
         L  W  D  T  A                                                  61

241     gtatacatagGCCAAGAAGATTATGATCGTCTCCGTCCCTTGTCTTACCCTGATTCTCAT
                  G  Q  E  D  Y  D  R  L  R  P  L  S  Y  P  D  S  H    78

301     GTTATCTTGATTTGTTTTGCTGTTGATTCACCCGATTCATTGGAAAACGTTCAAGAGAAG
         V  I  L  I  C  F  A  V  D  S  P  D  S  L  E  N  V  Q  E  K    98

361     gtaattaaaataaaacctatgcatacatgtatactcacatgtttaaaaatagTGGATCTC
                                                           W  I  S  101

421     TGAAGTACTCCACTTCTGTCAAGGTTTACCTATTGTTTTAGTTGGTTGTAAGAAAGATTT
           E  V  L  H  F  C  Q  G  L  P  I  V  L  V  G  C  K  K  D  L  121

481     AAGAAATGATCCTGGAACAATTGAAGAACTTAGAAGAAACTCTCAAAAACCTGTCAGTTC
           R  N  D  P  G  T  I  E  E  L  R  R  N  S  Q  K  P  V  S  S  141

541     AGAAGAGgtaaaaaaaaaaaaaaagaaaaaggggggcaaagagatgaagataataatctt
           E  E                                                        143

601     gtttttttttttagGGTGCTTCTATTGCTCAAAGAATTAGTGCTTACAAGTACCTTGAATG
                        G  A  S  I  A  Q  R  I  S  A  Y  K  Y  L  E  C  159

661     TTCTGCCAAGACTGGTGAAGGTGTACGTGAAGTATTTGAACACGCAACAAGAGCTGCATT
           S  A  K  T  G  E  G  V  R  E  V  F  E  H  A  T  R  A  A  L  179

9721     AATGGTTTCTAAGAAGAAAAAGTCAAAGAGTGGTGTCTGCAACCTTTTGTAAaaaaaaaa
           M  V  S  K  K  K  K  S  K  S  G  V  C  N  L  L  *           195

781     gaaataaaaaaaaaaataaaaataaatgcagcagccagcagcagcagcggacataaatac
841     acacacaaaacacaaatacaaacacaaacacacacacacaagcacatatctatattattc
901     taatttaataaacaaacaaaagggaaattctattatg
```

Fig. 1 Nucleotide sequence and deduced amino acid sequence of *Mucor rouxii* Rho1 (Accession N° AF487548). The coding nucleotides are in upper-case letters; non-coding nucleotides and introns are in lower-case letters. Nucleotide and amino acid sequences are numbered on the left- and right-hand sides, respectively

upon GTP binding and hydrolysis. G1, G2, G3 and G5 are identical or homologous between fungi and human RhoA. The consensus sequence for G4 in GTP binding proteins has been described to correspond to (N/T)(K/Q)D; however fungi, have a conserved C instead of the N/T residue.

Human RhoA protein has been demonstrated to be phosphorylated on Ser188 by PKA, both in vivo and in vitro (Forget et al. 2002). This phosphorylation causes the relocalization of RhoA from the plasma membrane to the cytosol due to the increase in its ability to interact with RhoGDI (Rho GDP-dissociation inhibitor). This enhanced interaction also protects RhoA, particularly its active form, from ubiquitin-mediated proteosomal degradation (Rolli-Derkinderen et al. 2005). An in silico prediction of PKA phosphorylation sites in Mr Rho1 was performed using the Scan site program (http://www.scansite.mit.edu). The search yielded Ser135 and Ser187 with good scores and solvent accesibilities, while Ser73 was predicted as a phosphorylation site with poorer parameters. Ser187 in Mr Rho1, as well as an equivalent residue in a good number of fungi (see Fig 2), seems to be equivalent to the human RhoA Ser188 since it is embedded in a context of basic lysine residues, suitable for phosphorylation

by PKA. The fungal homology shown in Fig. 2, shows that Mr Ser135, highly predicted as a putative substrate of PKA, is not conserved in mammalian RhoA; however some fungi, particularly *Y. lipolytica*, conserve this Ser in a favorable PKA context. Mr Rho1 Ser73 is a residue absolutely conserved not only in Rho proteins from lower and higher eukaryotes, but in all the small GTP binding proteins. There has been no report of this aminoacid being modifidied by phosphorylation by PKA, nor by any other protein kinase. Mr Rho1 sequence was modelled against the crystallized structure of human RhoA bound to GDP (1FTN). From this structure we can visualize that Ser73 has poor surface accesibility, while Ser135 is located in an exposed α-helix (A4) of the protein (data not shown). This helix is known to contain the residues characteristic for each member of the small GTP binding protein family for a certain species, and as judged by the low homology observed in this region it also contains species-specific residues. In order to have some experimental evidence of whether Ser135 could be a reasonable phosphorylation site for PKA we designed a peptide containing the aminoacids around Ser135, which we named rhotide (IRRNSQKFV) and assayed its behavior as a substrate for a partially purified preparation of *M.rouxii* PKA catalytic subunit in comparison with kemptide (LRRASLG), the peptide used typically as the specific substrate for PKA. The K_m for rhotide was 120 ± 14μM (n = 3) in comparison with 50 ± 7μM (n = 3) which was the value for kemptide. The relative V_{max} for kemptide was 2.3-fold higher than the one for rhotide. This gives a specificity constant (V_{max}/K_m) 5-fold higher for kemptide than for rhotide. These values are completely within the range of what could be acceptable for a specific substrate for a protein kinase.

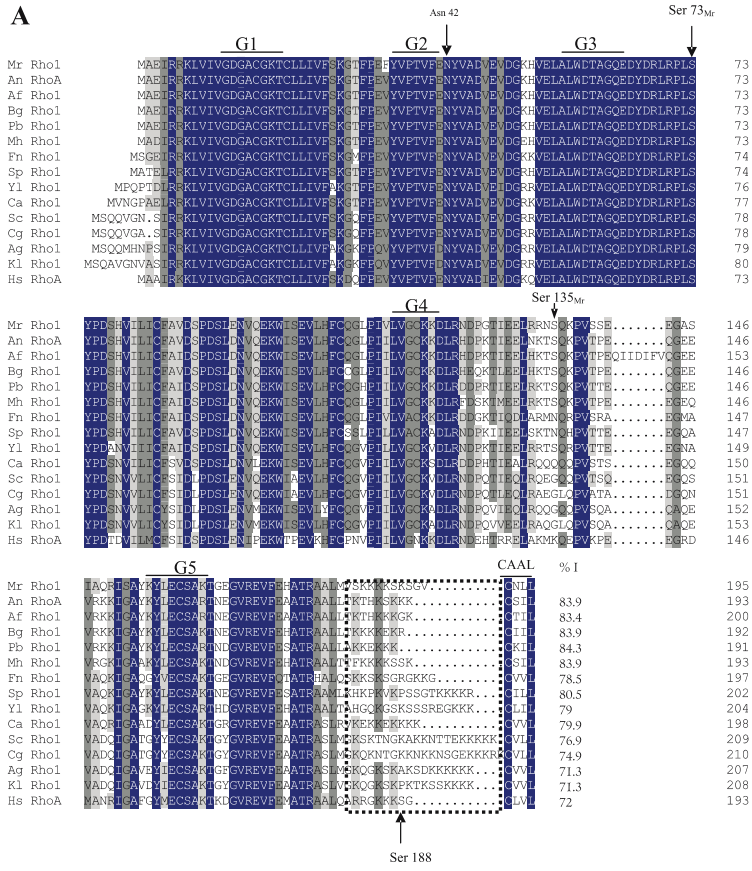
Complementation of the *S.cerevisiae rho1* null mutant by *M.rouxii* Rho1

Rho1 is a single copy gene, coding for an essential protein, and attempted deletions have been unsuccessful in all haploid systems tested. To determine whether Mr Rho1 would be a functional homolog of *S.cerevisiae* Rho1, we tested whether Mr Rho1 could rescue a *rho1* null mutant of *S.cerevisiae*. *RHO1* is an essential gene for yeast cell viability (Madaule et al. 1987). We constructed a plasmid that expressed the *Mr RHO1* cDNA under the control of the *S.cerevisiae RHO1* promoter and its terminator (pYOMrRHO1), by inserting the PCR-amplified *Mr RHO1* cDNA into plasmid pYO701. The *Mr RHO1* containing plasmid was introduced into a yeast strain (YOC706) expressing the yeast *RHO1* gene under control of the *GAL1* promoter. The growth of the transformants was then tested on plates containing glucose but no galactose (YPD), where expression of yeast *RHO1* was shut off. Figure. 3 shows that, while strains 1 and 3 representing two independent transformants of YOC706 with pYOMrRHO1 grew well at 30°C in both carbon sources, the host strain YOC706 (strain 2 in Fig 3) was unable to grow in glucose. This result shows that Mr Rho1 expression could rescue the *rho1* null mutant of *S.cerevisiae*, suggesting that this protein could share some of the functions of *S.cerevisiae* Rho1 in vivo, at least those regarding viability.

Identification of *M. rouxii* Rho1 in germlings

The presence of *M. rouxii* Rho1 in fungal extracts was determined by Western blot and ADP-ribosylation. The Western blot was performed using an antibody raised against a specific synthetic peptide (GTIEELRRNS) derived from a region of the translationally predicted Mr Rho1 primary sequence, that corresponds to a domain specific for Rho1 small GTP binding proteins and at a time species specific. The polyclonal antibody recognised a protein of ~ 22 kDa (Fig. 4A, lane 1). The specificity of the antibody was assayed by previous neutralization of the primary antibody with the specific peptide (Fig. 4A, lane 2).

ADP-ribosylation by C3 exotoxin from *C.botulinum*, specific for members of the Rho1 family of small GTP binding proteins, was assayed using cytosol and mixed-membrane fractions from germlings of *M.rouxii* using [^{32}P]NAD. After electrophoretic separation and subsequent autoradiography, a single [^{32}P] ADP-ribosylated band with a molecular mass of around 22 kDa, comigrating with the immunoreactive band, was


A
Asn 42
Ser 73Mr
G1
G2
G3
Mr Rho1 MAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGKHVELALWDTAGQEDYDRLRPLS 73
An RhoA MAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGKHVELALWDTAGQEDYDRLRPLS 73
Af Rho1 MAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGKHVELALWDTAGQEDYDRLRPLS 73
Bg Rho1 MAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGKHVELALWDTAGQEDYDRLRPLS 73
Pb Rho1 MAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGKHVELALWDTAGQEDYDRLRPLS 73
Mh Rho1 MADIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGEHVELALWDTAGQEDYDRLRPLS 73
Fn Rho1 MSGEIRGKLVIVGDGACGKTCLLIVFSKGCFPEVYVPTVFENYVADIEVDGKKVELALWDTAGQEDYDRLRPLS 74
Sp Rho1 MATELRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGRHVELALWDTAGQEDYDRLRPLS 74
Yl Rho1 MPQPTDIRGKLVIVGDGACGKTCLLIVFAKGTFPEVYVPTVFENYVADIEIDGRRVELALWDTAGQEDYDRLRPLS 76
Ca Rho1 MVNGPAEIRGKLVIVGDGACGKTCLLIVFSKGTFPEVYVPTVFENYVADIEVDGRKVELALWDTAGQEDYDRLRPLS 77
Sc Rho1 MSQQVGN.SIRGKLVIVGDGACGKTCLLIVFSKGCFPEVYVPTVFENYVADIEVDGRRVELALWDTAGQEDYDRLRPLS 78
Cg Rho1 MSQQVGA.SIRGKLVIVGDGACGKTCLLIVFSKGCFPEVYVPTVFENYVADIEVDGRRVELALWDTAGQEDYDRLRPLS 78
Ag Rho1 MSQQMHNPSIRGKLVIVGDGACGKTCLLIVFAKGKFPEVYVPTVFENYVADIEVDGRRVELALWDTAGQEDYDRLRPLS 79
Kl Rho1 MSQAVGNVASIRGKLVIVGDGACGKTCLLIVFPKGCFPEVYVPTVFENYVADIEVDGRRVELALWDTAGQEDYDRLRPLS 80
Hs RhoA MAAIRGKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDTAGQEDYDRLRPLS 73
G4
Ser 135Mr
Mr Rho1 YPDSHVILICFAVDSPDSLENVCEKWISEVLHFCQGLPIVLVGCKKDLRNDPGTIEELRRNSQKEVSSE.....EGAS 146
An RhoA YPDSHVILICFAVDSPDSLDNVCEKWISEVLHFCQGLPILLVGCKKDLRHDPKTIEELNKTSQKEVTPE.....CGEE 146
Af Rho1 YPDSHVILICFAIDSPDSLDNVCEKWISEVLHFCQGLPILLVGCKKDLRHDPKTIEELHKTSQKEVTPEQIIDIFVQGEE 153
Bg Rho1 YPDSHVILICFAVDSPDSLDNVCEKWISEVLHFCQGLPILLVGCKKDLRHEQKTLEELHKTSQKEVTPE.....CGEE 146
Pb Rho1 YPDSHVILICFAIDSPDSLDNVCEKWISEVLHFCQGHPILLVGCKKDLEDDPRTIEELRKTSQKEVTTE.....CGEE 146
Mh Rho1 YPDSHVILICFAIDSPDSLDNVCEKWISEVLHFCQGLPILLVGCKKDLKTDSKTMEELRKTSQKEVTPE.....CGEQ 146
Fn Rho1 YPDSHVILICFAIDSPDSLDNVCEKWISEVLHFCQGLPIVLVGCKKDLRDDGKIIQDLARMNQREVSRA.....ECMA 147
Sp Rho1 YPDSHVILICFAVDSPDSLDNVCEKWISEVLHFCGSLPILLVGCKADLRNDPKTIEELSKTNQHPVTTE.....EGQA 147
Yl Rho1 YPDANVILICFAIDSPDSLDNVCEKWISEVLHFCQGVPILLVGCKQDLRNDPKTIEELRKTSQRPVTTE.....EGNA 149
Ca Rho1 YPDSNVILICFSVDSPDSLDNVLEKWISEVLHFCQGVPILLVGCKSDLRDDPHTIEALRQQQQQPVSTS.....EGQQ 150
Sc Rho1 YPDSNVVLICFSIDLPDSLDNVCEKWAEVLHFCQGVPILLVGCKVDLRNDPQTIEQLRQEGQQPVTSQ.....EGQS 151
Cg Rho1 YPDSNVVLICFSIDLPDSLDNVCEKWAEVLHFCQGVPILLVGCKVDLRNDPQTLEQLRAEGLQPVATA.....DGQN 151
Ag Rho1 YPDSNVVLICYSIDLPDSLDNVWEKWISEVLYFCQGVPILLVGCKADLRNDPQVIEQLRQQGQQPVSQA.....CAQE 152
Kl Rho1 YPDSNVVLICYSIDLPDSLDNVWEKWISEVLHFCQGVPILLVGNKADLRNDPQVVEELRAQGLQPVSQA.....CAQE 153
Hs RhoA YPDTEVILLCFSIDSPDSLENIPEKWTPEVKHFCPNVPILLVGNKRDLRNDEHTRRELAKMKQEPVKPE.....EGRD 146
G5
CAAL
% I
Mr Rho1 TAQRLSAYGYLECSAKTGEGVREVFEEATRAALMVRKMKSKSGV.....GNIL 195
An RhoA VRKKICAYGYLECSAKTNEGVREVFEEATRAALLVKTHKSKKK.....GSIL 83.9 193
Af Rho1 VRKKICAYGYLECSAKTNEGVREVFEEATRAALLVKTHKKKGK.....GTIL 83.4 200
Bg Rho1 VRKKICAYGYLECSAKTNEGVREVFEEATRAALLTKKKKEKR.....GIIL 83.9 192
Pb Rho1 VRKKICAAGYLECSAKTNDGVREVFEEATRAALLAKKEKKK.....GKIL 84.3 191
Mh Rho1 VRGKICAAGYLECSAKTNDGVREVFEEATRAALLTFKKKKSSK.....GSIL 83.9 193
Fn Rho1 VAQKICAQGYVECSAKTGEGVREVFQEATRHALQEKKSKSGRGKKG.....GVVL 78.5 197
Sp Rho1 VAQKICASGYLECSAKTNEGVREVFESATRAAMLKHKPKVKPSSGTKKKKR....GILL 80.5 202
Yl Rho1 VAQKICASKYLECSAKTHDGVREVFEATRAALTAHGQKGSKSSSREGKKK....GLLL 79 204
Ca Rho1 VAQRICAADYLECSAKTGRGVREVFEAATRASLRVKEKKEKKKK.....GVVL 79.9 198
Sc Rho1 VADQICAIGYVECSAKTGYGVREVFEAATRASLMEKSKTNGKAKKNTTEKKKKK.GVLL 76.9 209
Cg Rho1 VADQICAIGYVECSAKTGYGVREVFEAATRASLMEKQKNTGKKNKKNSGEKKKRKGVLL 74.9 210
Ag Rho1 VADQICAVEYLECSAKTGFGVREVFEAATRASLMEKQGKSKAKSDKKKKKK....GVVL 71.3 207
Kl Rho1 VADQICAVDYLECSAKTGYGVREVFEAATRASLVEKQGKSKPKTKSSKKKK....GVVL 71.3 208
Hs RhoA MANRICAEGYVECSAKTKDGVREVFEAATRAALQARRGKKKSG.....GLVL 72 193
Ser 188
B
0.05
Ca Rho1
Sc Rho1
Cg Rho1
Ag Rho1
Kl Rho1
Mr Rho1
An RhoA
Af Rho1
Bg Rho1
Pb Rho1
Mh Rho1
Sp Rho1
Yl Rho1
Fn Rho1
Hs RhoA
89
100
99
99
42
92
74
76
77
86
53
62

◄ **Fig. 2** **A**) Aminoacid sequence alignment of *M. rouxii* Rho1 with the Rho1 from 10 other fungi. Alignment with human RhoA is added as representative of higher eukaryotic species. Mr (*Mucor rouxii*, AAM03110), An (*Aspergillus nidulans*, Q9C3Y4), Af (*Aspergillus fumigatus*, AAG12155), Bg (*Blumeria graminis*, AAK94951), Pb (*Paracoccidioides brasiliensis*, AAQ93069), Mh (*Monacrosporium haptotylum*, AAU06192), Fn (*Filobasidiella neoformans*, AAF91317), Sp (*Schizosaccharomyces pombe*, BAA07377), Yl (*Yarrowia lipolytica*, AAG01806), Ca (*Candida albicans*, BAA24262), Sc (*Saccharomyces cerevisiae*, AAA34977), Cg (*Candida glabrata*, CAG60553), Ag (*Ashbya gossypii*, AAG41249), Kl (*Kluyveromyces lactis*, AAN05733), Hs (*Homo sapiens*, AAM2117). The fungi used for the comparison are ascomycetes with the exception of Mr (zygomycete) and Fn (basidiomycete). Aminoacids are numbered on the right-hand side. %I, identity. The conserved domains among Rho-type GTPases: G1, G2, G3, G4, G5, and the geranylgeranylation site CAAL are overlined. The conserved ADP-ribosylation residue (Asn 42 in Hs RhoA) is indicated by an arrow. Ser 188 in Hs RhoA and Ser 73 and 135 from Mr Rho1 are also indicated by arrows. The lysine rich domain in the Rho proteins is boxed with a dotted line. **B**) Neighbor-joining phylogenetic tree of Rho1 protein sequences shown in A). The numbers along the branches show the percentage of occurrence of nodes in 1000 boostrap replicates. Hs RhoA was used as outgroup taxon

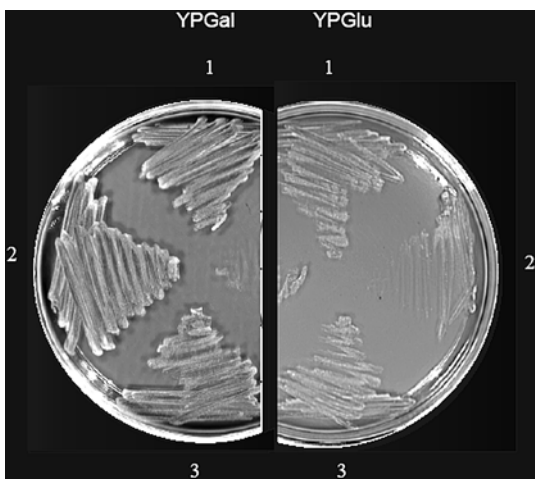


Fig. 3 Complementation of a *rho1* null mutant of *S. cerevisiae* by *M. rouxii* Rho1 protein. *S. cerevisiae* YOC706 strain containing a *rho1* deletion and a plasmid expressing the yeast *RHO1* gene under the control of the *GAL1* promoter was transformed with pYOMrRHO1, expressing Mr Rho1 under the *S.cerevisiae RHO1* promoter. Growth of transformants was tested on YPGal (left) and YPGlu (right) plates. Samples 1 and 3 represent two independent transformants and sample 2 the strain without transformation

observed in both fractions (Fig. 4B, lanes 1 and 2). 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 (not shown). The above results demonstrate on one hand that the protein belongs to the Rho1 familiy of small GTP binding proteins, since these are the only members of this family that are ADP-ribosylated by C3 exotoxin (Cabib et al. 1998), and on the other hand that Mr Rho1 is present both in cytosol and mixed membrane fractions, as has been described for Rho proteins.

In order to determine the cellular expression of Mr Rho1, immunochemical localization was undertaken in cells around the stage of germ tube emission (Fig. 4C). The fluorescence of Rho1 was detected as patches in the cell membrane periphery and inside the cell (Fig. 4C, middle panel). Cells with extruded germ-tubes maintain the Rho1 fluorescence in a zone flanking the protuberance, but not along the new germ tube (Fig. 4C, left panel). Cells probed with pre-immune rabbit serum or without anti-Rho1 showed only the minimal, commonly observed auto-fluorescence (Fig. 5C, right panel). The same distribution pattern has been described for the Rho protein from another zygomycete, *Phycomyces blakesleeanus*, whose Rho1 protein, although not cloned, has been visualized using commercially available anti-RhoA antibodies (Ramírez-Ramírez et al. 1999).

Rho1 protein is present in different developmental stages of *M.rouxii*

The expression of the Rho1 protein was investigated during different stages of spore germination. *Mucor rouxii* sporangiospores 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 and isodiametric growth occurred during the first 3 h and germ-tube formation started at about 4–5 h of incubation at 30°C. We initially examined the levels of Rho1 mRNA transcripts along the germination (Fig. 5A). To quantitate transcript levels we used semi-quantitative RT-PCR with two different

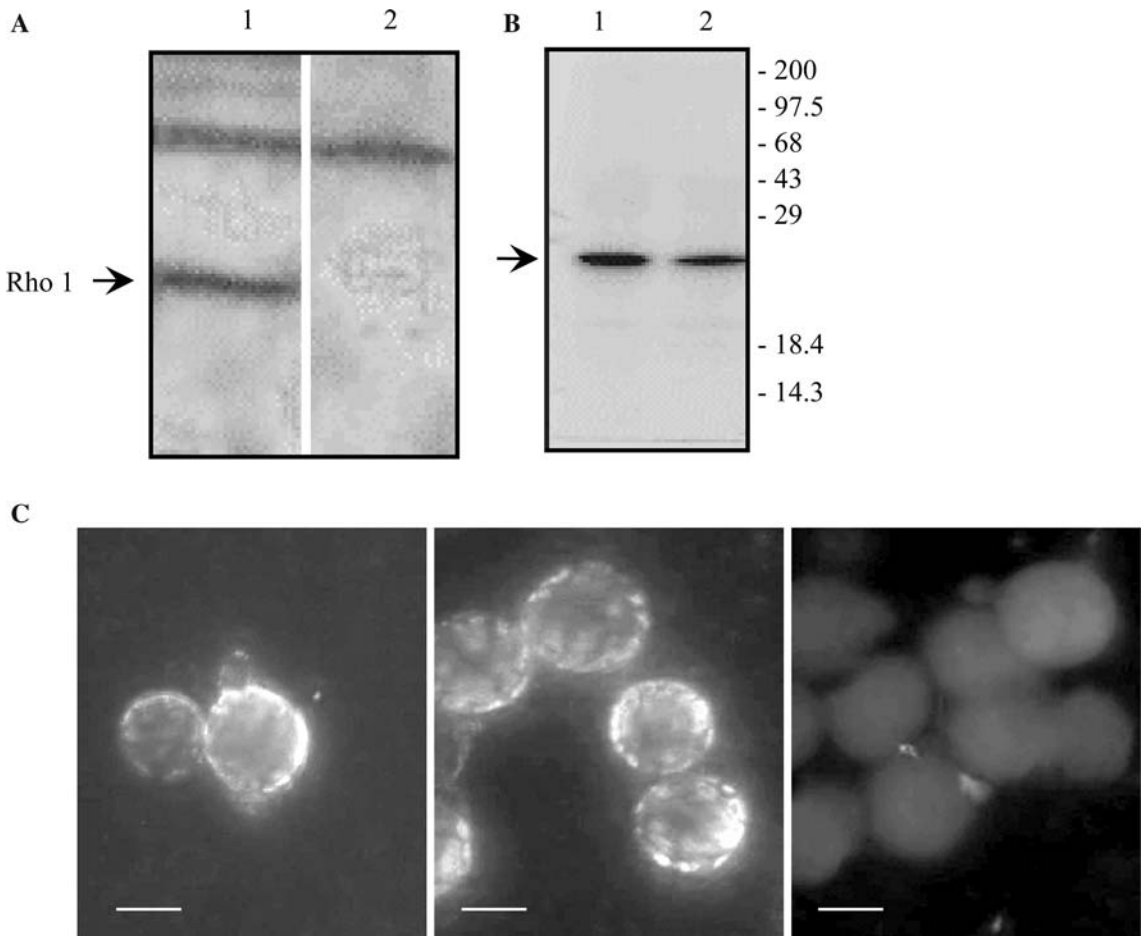


Fig. 4 Detection of endogenous *M. rouxii* Rho1 protein. **A**) Western blot of crude extract (40μg) from germlings, electrophoresed on 14% SDS-PAGE, developed with anti-Mr Rho1 antibody produced against a specific peptide (see Materials and Methods). Lanes 1 and 2 represent samples without and with preincubation with the antigenic peptide (7 mM) respectively. **B**) Mr Rho 1 ADP-ribosylation. Cytosol (lane 1) and mixed membranes (lane 2) fractions from germling extracts were submitted to ADP-ribosylation by C3-exotoxin and [^{32}P] NAD. Samples (20μg) were electrophoresed on 14% SDS-PAGE and developed by autoradiography. **C**) Immunocytochemistry. *M. rouxii* germs from 5 h of growth were stained with anti- Mr Rho1 and a secondary antibody coupled to Cy3. Cells were visualized by epifluorescence microscopy. Left and middle panels show representative examples of a germling (left) and rounded cells (middle), while the right panel is a control without the addition of first antibody. Bar: 7μm

primer pairs: one pair specific for Rho1 and the other specific for translation elongation factor TEF1, which is reported to increase proportionally with the whole protein during fungal growth (Linz et al. 1986). The Rho protein was expressed in all stages studied; however the level of expression did not parallel that of a constitutive protein such as TEF1, but began to increase more steadily after 2 h of growth, and remained stable at 3–6 h (Fig. 5A, compare lanes 1–2 with 3–6). This difference in expression level was corroborated at the protein level, by Western blot using anti Mr Rho1 of samples from spores and from germlings (Fig. 5B). This expression pattern is analogous to the one displayed by another small GTP binding protein, MRas1 from *M. racemosus*, a protein that has been shown to be involved during morphogenesis of this fungus (Roze et al. 1999). In both cases, the mRNA and proteins are expressed at low levels during spherical growth and then accumulate during polar growth (germ tube emergence and elongation).

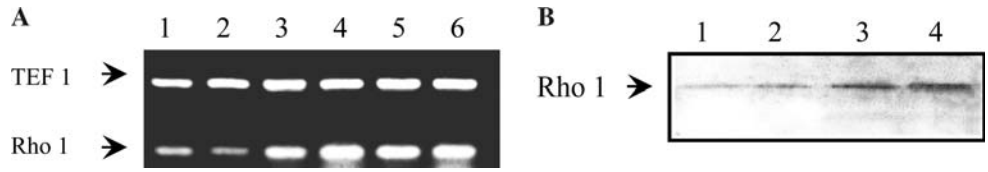


Fig. 5 Rho 1 expression during *Mucor rouxii* germination. **A**) Expression of Rho1 mRNA detected by semi-quantitative RT-PCR. Total RNA was extracted from cell cultures grown up to 0 (spores), 1, 2, 3, 4 and 5 h (lanes 1 – 6). A representative RT-PCR reaction is shown. TEF1 mRNA was used as control. **B**) Expression of Rho1 protein detected by immunoblot of extracts from spores (lanes 1 and 2, 20 and 40μg) and from germlings of 5 h lanes 3 and 4, 20 and 40μg) using anti-Mr Rho1 antibody as in Fig. 5

Regulation of β-1,3-glucan synthesis by *M.rouxii* Rho1

It has been well established, first for *S. cerevisiae* (Inoue et al. 1996; Cabib et al. 1998) and subsequently for several fungi (Odds et al. 2003; Ruge et al. 2005) that one of the roles of Rho1p is to be a regulatory protein of the β-1,3-glucan synthase complex, involved in the synthesis of β-1,3-glucans, a major component of cell walls from most fungi. It has been described that Rho1 proteins are involved in polar growth, branching and cell wall synthesis (Guest et al. 2004). However, glucans have not been detected in zygomycetes, such as *M. rouxii*, at least as major components (Bartnicki-García and Reyes 1968; Dow and Rubery 1977). In order to evaluate whether there is also a β-1,3-glucan synthase complex in *M. rouxii*, and thus infer that Rho1 might be participating in this complex, we assayed the effect of an echinochandin, caspofungin acetate, in cell growth and morphology. Echinocandins are antifungal agents which inhibit the 1,3-β-D-glucan synthase complex. Caspofungin acetate (registered name, Cancidas, from Merck) is a member of this family of lipopeptides consisting of cyclic hexapeptides N-linked to a fatty acyl side chain (Letscher-Bru and Herbrecht 2003). As a first step we assessed the effect of caspofungin on growth by addition of the drug to cell cultures simultaneously with sporangiospores inoculation. The effect on growth was followed by dry weight measurement. The inhibition of growth attained was of 18, 26 and 92% for 10, 100 and 300μg/ml of caspofungin, respectively. This result in itself indicates that the drug target is present in this organism. These values are much higher than those reported for ascomycetes, but more in the range of those reported recently for another zygomycete, *Rhizopus oryzae* (Ibrahim et al. 2005). This was the first zygomycete to be reported to respond to this antifungal agent, through the inhibition of its 1,3-β-D-glucan synthase activity. As a second step we analyzed the effect caspofungin had on *M. rouxii* morphology. It has been reported that some fungi are more susceptible to morphological effects of caspofungin than to its fungicidal or fungistatic activity (Arikan et al. 2001). In fact, in the case of *M. rouxii*, a concentration of only 10μg/ml, which had an effect on growth of only 18% inhibition, produced profound morphological alterations, as visualized by microscopic examination (Fig 6). As compared to control cells between 6 and 9 h of growth (Fig 6 A-C), hyphae from cells cultured in the presence of 10μg/ml caspofungin (Fig 6 D–F) grew abnormally, short and stubby, with highly branched tips and swollen germ tubes. This morphological effect was dose-dependent, with more severe effects seen at higher concentrations. As a result of the drug-induced altered growth, the short, highly branched filaments produce very compact clumps (Fig 6D), making it possible to score the effect macroscopically. The observed morphological aberrations are similar to those described for the effect of caspofungin on *Aspergillus fumigatus* (Kurtz et al. 1994).

Conclusions

Rho GTPases are involved in transmission of signals from cell surface receptors to regulate

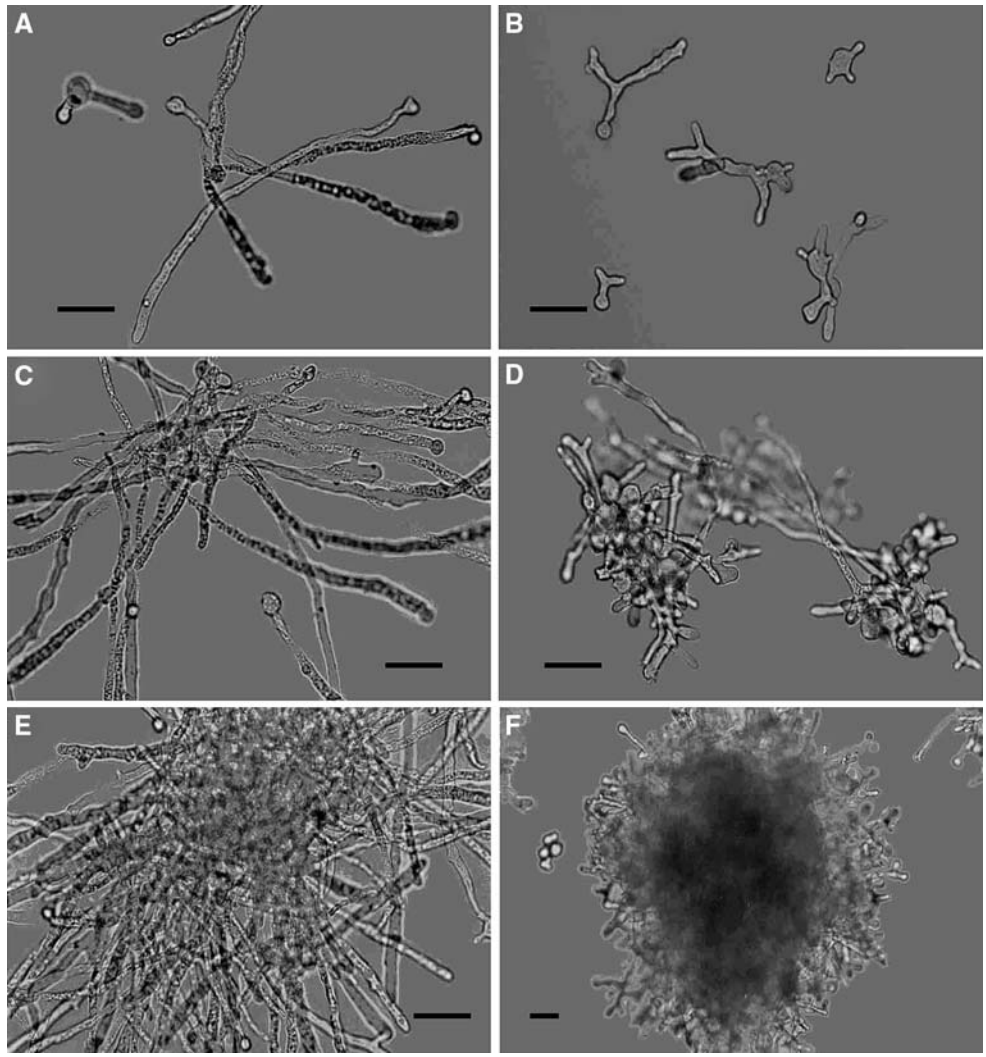


Fig. 6 Morphology of *Mucor rouxii* cells grown in defined medium in the presence of caspofungin. Control cells (**A**, **C** and **E**) and cells treated with 10μg/ml caspofungin (**B**, **D** and **F**) were grown for 6, 9 and 12 h respectively. Representative images are shown. Magnification bar: 25μm. Panel F is shown at a lower magnification in order to appreciate a whole clump

many important processes within the cell including organization of the actin cytoskeleton, gene transcription, cell cycle progression, and membrane trafficking (Jaffe and Hall 2005; Cabib et al. 1998). In this study a Rho GTPase of *M.rouxii* was cloned, characterised and identified as a Rho1 protein. The predicted protein shared the common motifs present in members of the Rho GTPase superfamily including the phosphate binding loop, the switch I and II regions, charged residues underlying specificity of interaction with downstream effector proteins, and residues present in the active site and modified by bacterial toxins. Western blot analysis, ADP-ribosylation and immunofluorescence indicate that Mr Rho1 is expressed within the cell. The measurement of Mr Rho1 mRNA levels during fungal germination indicates that the expression of this gene correlates with the differentiation process and not with growth. Western blot of germling extracts corroborate that the levels of Mr Rho1

in this stage are greater than those observed in sporangiospore extracts.

Two lines of evidence presented in this work, suggest that Mr Rho1 is part of the glucan synthase complex, as occurs in other fungi. Firstly Mr Rho1 complements *rho1* deletion mutants in *S. cerevisiae*. Not all the Rho1 or RhoA proteins complement fully this deletion. For example, the complementation reported to be produced by human RhoA is partial, but is fully restituted with a chimera encoding a protein with 27 residues derived from yeast and all other regions from the human RhoA (Qadota et al. 1994). This 27 residue region (from aa 90–117 of the Mr Rho1 numbering), corresponding to the α 3 chain and loop 7, is almost absolutely identical in fungal Rho proteins, suggesting they may all share the same functions.

The second evidence is the change in cell morphology observed in *M. rouxii*, when grown in the presence of caspofungin, an antifungal agent which mechanism of action is to inhibit the glucan synthase complex, in which Rho1 is involved. This result implicates β-1,3-glucans in *M. rouxii* morphogenesis, especially in branch formation pattern. The requirement of this polymer for normal wall composition for hyphal formation is clearly not critical since the cells do indeed form tubular hyphae despite alterations in branching. This behaviour suggests the Rho1 protein might participate in the cell wall integrity pathway in *M. rouxii*. Recent results from our group (Pereyra et al. 2006) indicate that sustained activation of PKA leads to a profound alteration of the cell wall. It will be interesting to investigate whether β-1,3-glucans have a regulatory role in fungal morphology and whether there is a cross talk with PKA signalling. From the primary protein sequence of Mr Rho1 we could predict two putative PKA phosphorylation sites, Ser135 and Ser187. Ser187 is equivalent to Ser188 already described to be a target of PKA in higher eukaryotes, negatively regulating signal transduction by Rho (Lang et al. 1996; Forget et al. 2002). It is interesting that Ser135, conserved also in some other fungi, is well exposed in the protein surface, and is included within an helix that contains the residues characteristic of each small GTP binding protein. According to an interface array study of small GTP binding proteins and their effectors, almost all surface residues mediate recognition of effector proteins (Corbett and Alber 2001). So it can be predicted that the phosphorylation of Ser135 by PKA, if it occurs, could have an impact on the interaction of Mr Rho1 with an upstream or downstream effector. The postranslational modification of Mr Rho1 by PKA is a possible point of cross-talk between PKA signalling and *M. rouxii* morphology, and is presently under study.

Acknowledgements This study was supported by grants from the University of Buenos Aires, grant X-270, from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) grant PIP 5239 and from ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica) grant PICT 01-08793.

References

Arikan S, Lozano-Chiu M, Paetznick V, Rex JH (2001) In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. Antimicrob Agents Chemother 45:327–330

Barth G, Gaillardin C (1997) Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMS Microbiol Rev 19:219–237

Bartnicki-Garcia S, Reyes E (1968) Polyuronides in the cell wails of *Mucor rouxii*. Biochim Biophys Acta 170:54–62

Bartnicki-García S, Nickerson WK (1962) Nutrition, growth and morphogenesis of *Mucor rouxii*. J Bacteriol 83:841–858

Cabib E, Drgonova J, Drgon T (1998) Role of small G proteins in yeast cell polarization and wall biosynthesis. Annu Rev Biochem 67:307–333

Corbett KD, Alber T (2001) The many faces of Ras: recognition of small GTP-binding proteins. Trends Biochem Sci 26:710–716

DeMali KA, Wennerberg K, Burridge K (2003) Integrin signaling to the actin cytoskeleton. Comparison of morphogenetic networks of filamentous fungi and yeast. Curr Opin Cell Biol 15:572–582

Dow JM, Rubery PH (1977) Chemical fractionation of the cell walls of mycelial and yeast-like forms of *Mucor rouxii*: a comparative study of the polysaccharide and glycoprotein components. J Gen Microbiol 99:29–41

Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. J Biol Chem 278:19023–19031

Forget MA, Desrosiers RR, Gingras D, Beliveau R (2002) Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes. Biochem J 361:243–254

Gancedo JM (2001) Control of pseudohyphae formation in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:107–23

Guest MG, Lin X, Momany M (2004) *Aspergillus nidulans* RhoA is involved in polar growth, branching and cell wall synthesis. Fungal Genet Biol 41:13–22

Haidle CW, Storck R (1966) Control of dimorphism in *Mucor rouxii*. J Bacteriol 92:1236–1244

Hancock JF, Cadwallader K, Paterson H, Marshall CJ (1991) A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. EMBO J 10:4033–4039

Harris SD, Momany M (2004) Polarity in filamentous fungi: moving beyond the yeast paradigm. Fungal Genet Biol 41:391–400

Harris SD, Read ND, Roberson RW, Shaw B, Seiler S, Plamann M, Momany M (2005) Polarisome meets spitzenkorper: microscopy, genetics, and genomics converge. Eukar Cell 4:225–229

Ibrahim AS, Bowman JC, Avanessian V, Brown K, Spellberg B, Edwards JE Jr, Douglas CM (2005) Caspofungin inhibits *Rhizopus oryzae* 1,3-beta-D-glucan synthase, lowers burden in brain measured by quantitative PCR, and improves survival at a low but not a high dose during murine disseminated zygomycosis. Antimicrob Agents Chemother 49:721–727

Inoue SB, Qadota H, Arisawa M, Anraku Y, Watanabe T, Ohya Y (1996) Signaling toward yeast 1,3-beta-glucan synthesis. Cell Struct Funct 5:395–402

Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21:247–269

Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J and Douglas C (1994) Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob Agents Chemother 38: 1480–1489

Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J (1996) Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J 15:510–519

Lee N, D'Souza CA, Kronstad JW (2003) Of smuts, blasts, mildews, and blights: cAMP signaling in phytopathogenic fungi. Annu Rev Phytopathol 41:399–427

Lengeler KB, Davidson RC, D'Souza CA, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev 64:746–785

Letscher-Bru V, Herbrecht R (2003) Caspofungin: the first representative of a new antifungal class. J Antimicrob Chemother 51:513–521

Levin DE (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 69:262–291

Linz JE, Katayama C, Sypherd PS (1986) Three genes for the elongation factor EF-1α in *Mucor racemosus*. Mol Cell Biol 6:593–600

Madaule P, Axel R, Myers AM (1987) Characterization of two members of the rho gene family from the yeast *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 84:779–783

Madhani HD, Fink GD (1998) The control of filamentous differentiation and virulence in fungi. Trends Cell Biol 8:348–335

Odds FC, Brown AJ, Gow NA (2003) Antifungal agents: mechanisms of action. Trends Microbiol 11:272–279

Paduch M, Jeleń F, Otlewski J (2001) Structure of small G proteins and their regulators. Acta Biochim Pol 48:829–850

Peitsch MC (1995) Protein modeling by E-mail. Bio/Technology 13:658–660

Peitsch MC (1996) ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. Biochem Soc Trans 24:274–279

Pereyra E, Mizyrycki C, Moreno S (2000) Threshold level of protein kinase A activity and polarized growth in *Mucor rouxii*. Microbiology 146:1949–1958

Pereyra E, Argimón S, Jackson SL, Moreno S (2003) RGD-containing peptides and cAMP have antagonistic roles in the morphology of *Mucor rouxii*. Protoplasma 222:23–30

Pereyra E, Zaremberg V, Moreno S (1992) The effect of dibutyryl-cAMP on growth and morphology of germinating *Mucor rouxii* sporangiospores. Exp Mycol 16:93–101

Pereyra E, Ingerfeld M, Neal A, Jackson SL, Moreno S (2006) *Mucor rouxii* ultrastructure: cAMP and actin cytoskeleton. Protoplasma in press

Qadota H, Anraku Y, Botstein D, Ohya Y (1994) Conditional lethality of a yeast strain expressing human *RHOA* in place of *RHO1*. Proc Natl Acad Sci USA 91:9317–9321

Ramirez-Ramirez N, Garcia-Soto J, Gonzalez-Hernandez A, Martinez-Cadena G (1999) The small GTP-binding protein Rho is expressed differentially during spore germination of *Phycomyces blakesleeanus*. Microbiology 145:1097–1104

Rolli-Derkinderen M, Sauzeau V, Boyer L, Lemichez E, Baron C, Henrion D, Loirand G, Pacaud P (2005) Phosphorylation of serine 188 protects RhoA from ubiquitin/proteasome-mediated degradation in vascular smooth muscle cells. Circ Res 96:1152–1160

Rose TM, Henikoff JG, Henikoff S (2003) CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res 31:3763–3766

Roskoski R (1983) Assays of protein kinase. Methods Enzymol 99:3–6

Roze LV, Mahanti N, Mehigh R, McConnell DG, Linz JE (1999) Evidence that MRas1 and MRas3 proteins are associated with distinct cellular functions during growth and morphogenesis in the fungus *Mucor racemosus*. Fungal Genet Biol 28:171–189

Rua D, Tobe BT, Kron SJ (2001) Cell cycle control of yeast filamentous growth. Curr Opin Microbiol 4:720–727

Ruge E, Korting HC, Borelli C (2005) Current state of three-dimensional characterisation of antifungal targets and its use for molecular modelling in drug design. Int J Antimicrob Agents 26:427–441

Seiler S, Plamann M (2003) The genetic basis of cellular morphogenesis in the filamentous fungus *Neurospora crassa*. Mol Biol Cell 14:4352–4364

Wendland J (2001) Comparison of morphogenetic networks of filamentous fungi and yeast. Fungal Genet Biol 34:63–82

Wendland J, Walther A (2005) *Ashbya gossypii*: a model for fungal developmental biology. Nature Rev Microbiol 3:421–429

Zaremberg V, Donella-Deana A, Moreno S (2000) Mechanism of activation of cAMP-dependent protein kinase: in *Mucor rouxii* the apparent specific activity of the cAMP-activated holoenzyme is different than that of its free catalytic subunit. Arch Biochem Biophys 381:74–82