JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7734–7741 Vol. 179, No. 24
0021-9193/97/$04.00+0

Cloning of the *RHO1* Gene from *Candida albicans* and Its Regulation of β-1,3-Glucan Synthesis

OSAMU KONDOH,[1]* YUKAKO TACHIBANA,[1] YOSHIKAZU OHYA,[2] MIKIO ARISAWA,[1] AND TAKAHIDE WATANABE[1]

Department of Mycology, Nippon Roche Research Center, Kamakura, Kanagawa 247,[1] and Department of Biological Sciences, Tokyo University, Bunkyuo-ku, Tokyo 113,[2] Japan

Received 11 August 1997/Accepted 8 October 1997

The *Saccharomyces cerevisiae RHO1* gene encodes a low-molecular-weight GTPase. One of its recently identified functions is the regulation of β-1,3-glucan synthase, which synthesizes the main component of the fungal cell wall (J. Drgonova et al., Science 272:277–279, 1996; T. Mazur and W. Baginsky, J. Biol. Chem. 271:14604–14609, 1996; and H. Qadota et al., Science 272:279–281, 1996). From the opportunistic pathogenic fungus *Candida albicans*, we cloned the *RHO1* gene by the PCR and cross-hybridization methods. Sequence analysis revealed that the *Candida RHO1* gene has a 597-nucleotide region which encodes a putative 22.0-kDa peptide. The deduced amino acid sequence predicts that *Candida albicans* Rho1p is 82.9% identical to *Saccharomyces* Rho1p and contains all the domains conserved among Rho-type GTPases from other organisms. The *Candida albicans RHO1* gene could rescue a *S. cerevisiae* strain containing a *rho1* deletion. Furthermore, recombinant *Candida albicans* Rho1p could reactivate the β-1,3-glucan synthesis activities of both *C. albicans* and *S. cerevisiae* membranes in which endogenous Rho1p had been depleted by Tergitol NP-40–NaCl treatment. *Candida albicans* Rho1p was copurified with the β-1,3-glucan synthase putative catalytic subunit, *Candida albicans* Gsc1p, by product entrapment. *Candida albicans* Rho1p was shown to interact directly with *Candida albicans* Gsc1p in a ligand overlay assay and a cross-linking study. These results indicate that *Candida albicans* Rho1p acts in the same manner as *Saccharomyces cerevisiae* Rho1p to regulate β-1,3-glucan synthesis.

The small-molecular-weight Rho (stands for Ras homologous) GTPases first identified in *Aplysia californica* (26) function as molecular switches or regulators in the cells: they are "on" in the GTP-bound form and "off" in the GDP-bound form (for reviews, see references 13, 42, and 43). In the budding yeast *Saccharomyces cerevisiae*, five Rho-type GTPases, Rho1p (27), Rho2p (27), Rho3p (29), Rho4p (29), and Cdc42p (1, 18), have been identified. *Saccharomyces RHO1* is essential for yeast cell viability, and an immunofluorescence microscopic study indicated that Rho1p is localized at the cell periphery in the growth site (budding tip and mother bud neck region), where cortical actin patches are localized, suggesting that Rho1p is involved in the regulation of actin cytoskeleton reorganization and cell wall biosynthesis (44). In recent years, various proteins, such as Pkc1p (19, 35), Rom7p (also known as Bem4p) (15), Bni1p (24), and the β-1,3-glucan synthase putative catalytic subunit (10, 38), have been identified as downstream targets of Rho1p in *S. cerevisiae*. These effector proteins function in the maintenance of yeast cell morphology during the cell cycle. Pkc1p, a homolog of mammalian protein kinase C, maintains cell wall integrity through the activation of the mitogen-activated protein kinase cascade; Rom7p/Bem4p is thought to be related to bud emergence, and Bni1p is involved in the regulation of actin cytoskeleton reorganization. The regulation of β-1,3-glucan synthesis by Rho1p has also been demonstrated in *Schizosaccharomyces pombe* (2).

The fungal cell wall structure, its surface area, and its composition are constantly changing in accordance with fungal cell cycle stages and in response to outside signals (8, 22). The cell wall consists mainly of β-glucans, mannoproteins, and a small amount of chitin, all of which are interconnected, providing cells with their rigidity and protecting them from osmotic pressure (8). β-1,3-Glucan is the major component of the fungal cell wall. The enzyme that synthesizes this polymer, β-1,3-glucan synthase (UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyltransferase; EC 2.4.1.34), has been purified (16), and the gene encoding its putative catalytic subunit has been cloned from *S. cerevisiae* (5, 9, 11, 16, 39), *Aspergillus nidulans* (21), and *Candida albicans* (31). The *S. cerevisiae* β-1,3-glucan synthase putative catalytic subunit is encoded by *GSC1* and *GSC2* (also known as *FKS1* and *FKS2*, respectively), a pair of closely related genes which, when both are disrupted, provoke synthetic lethality. These genes encode proteins of approximately 200 kDa with 16 transmembrane domains, the sizes of which are identical to the molecular weights of the dominant proteins in the purified enzyme (16). In *S. cerevisiae* Rho1p was identified as a regulator of β-1,3-glucan synthase on the basis of the following observations (10, 30, 38): the purified β-1,3-glucan synthase obtained by product entrapment contains *Saccharomyces cerevisiae* Rho1p and a β-1,3-glucan synthesis defect in *rho1* mutants could be restored in vitro by the addition of recombinant *Saccharomyces cerevisiae* Rho1p.

C. albicans, a dimorphic asexual pathogenic fungus, is a major cause of deep mycoses in immunocompromised patients. The dramatic increase in the number of *C. albicans* infections during the last two decades has been a result of several factors, including larger numbers of patients being treated with immunosuppressants for organ transplant, being given chemotherapy for cancer and enduring long-term catheterization, taking broad-spectrum antibiotics, and surviving longer in immunologically compromised states. *C. albicans* has two vegetative growth forms: budding growth, which is similar to that of *S. cerevisiae*, and hyphal growth, which can be induced by several stimuli, such as high temperature, alkaline pH, the supply of *N*-acetylglucosamine, and the supply of se-

* Corresponding author. Mailing address: Department of Mycology, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247, Japan. Phone: 81-467-47-2223. Fax: 81-467-46-5320. E-mail: osamu.kondoh@roche.com.

rum. Both morphologies are generally observed in disseminated infections. To overcome the limited efficacies, considerable side effects, and toxicities of existing therapeutic regimens for disseminated candidiasis in particular, the development of drugs with different modes of action against the *Candida* target is desirable. In *C. albicans*, only one β-1,3-glucan synthase gene (*GSC1*) has been cloned and no cells containing this disrupted gene could be isolated, suggesting that this gene is essential for growth, which is not the case for *S. cerevisiae* (31). For the development of a β-1,3-glucan synthase inhibitor specific towards pathogenic fungi, especially towards *C. albicans*, the identification and characterization of the regulatory component of β-1,3-glucan synthase, Rho1, in this fungus would be valuable.

We describe here the isolation and characterization of the *RHO1* gene from *C. albicans*. The identity of *Candida albicans RHO1* was confirmed by its ability to complement the growth defect of an *S. cerevisiae rho1* deletion mutant. We also showed a direct interaction between *Candida albicans* Rho1p and a β-1,3-glucan synthase putative catalytic subunit, *Candida albicans* Gsc1p, by ligand overlay and cross-linking studies.

MATERIALS AND METHODS

Bacterial strains, plasmids, and other techniques. The *C. albicans* wild-type strains ATCC 10231 and CAI4 were grown to late exponential phase at 30°C in YPD medium (2% glucose, 2% Bacto Peptone, 1% yeast extract). The sequences of primers used for the PCR amplification of the partial *Candida albicans RHO1* gene were 5′-GTIGGIGA(C/T)GGIGCITG(C/T)GG-3′ and 5′-(A/G)AAIAC IGTIGGIAC(A/G)TAIAC-3′. These primers were mixed with 0.044 μg of *C. albicans* DNA (IFO1060) in a buffer containing nucleotides and *Taq* polymerase and subjected to thermal cycling. Twenty-five amplification cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 30 s were performed. The amplified DNA fragment was subcloned into pT7Blue T-Vector (Novagen). The *C. albicans* genomic library was constructed by inserting DNA fragments of longer than 5 kb, obtained by partial *Sau*3AI digestion of *C. albicans* IFO1060 DNA, into the *Bam*HI site of pRS414. *Escherichia coli* JM109 was used for the plasmid amplification. *S. cerevisiae* YOC706 and plasmids pYO701, pYO702, and pYO714, which are described in reference 37, were used for the complementation of the *S. cerevisiae rho1* mutant. The sequence primers used for the PCR amplification for protein expression in insect cells and *E. coli* are shown in Fig. 2.

Southern and colony hybridizations. Genomic DNA was isolated as described by Philippsen et al. (36). Hybridization was performed on a nylon membrane (Hybond-N; Amersham) under the low-stringency conditions (42°C, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 20% formamide, 0.1% sodium dodecyl sulfate [SDS], and 50 mM NaP_i [pH 6.5]) recommended in reference 40. The probe was radiolabeled with [α-^{32}P]dCTP by PCR with 5′-GTGGGGGAT GGGGCGTGCGG-3′ and 5′-GAAGACGGTGGGGACGTAGAC-3′ as primers. Standard conditions were used for other procedures.

Sequence and dendrogram analyses. The DNA sequences of both strands of *Candida RHO1* were determined by a model 373A automated DNA sequencer with a Dye Terminator Cycle Sequencing Core kit (Applied Biosystems). The primers used for the sequence determination are shown in Fig. 2. The sequence comparisons and dendrogram were analyzed with the software GENETYX-MAC, version 8.0 (Software Development Co. Ltd.), and the PileUp program of the Genetics Computer Group package (12).

Baculovirus expression and partial purification of recombinant *Candida albicans* Rho1p. To produce *Candida albicans* Rho1p by means of the baculovirus expression system, the PCR-amplified *Candida albicans RHO1* gene was inserted into the baculovirus transfer vector pVL1393 (Invitrogen). Sf9 cells were coinfected with the recombinant vector and wild-type viral DNA (Pharmingen) according to Pharmingen's protocol. After purification and amplification of recombinant virus, Sf9 cells were exposed to the virus at a multiplicity of infection of 10 and harvested 85 h after exposure. Cells (approximately 5 g [wet weight]) were resuspended in 20 ml of homogenization buffer (10 mM Tris-Cl [pH 8.0], 1 mM dithiothreitol [DTT], 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) and sonicated for 30 s four times at 30-s intervals. The homogenate was centrifuged at 421,000 × *g* for 20 min, and the pellet was resuspended in 10 ml of extraction buffer (20 mM Tris-Cl [pH 8.0], 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM PMSF, 1% CHAPS {3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate}) and sonicated again for 30 s four times at 30-s intervals. The CHAPS extract was recovered by centrifugation at 421,000 × *g* for 20 min, applied to a Q-Sepharose column (1 by 10 cm) equilibrated with buffer A (20 mM Tris-Cl [pH 8.0], 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.6% CHAPS), and eluted with a linear gradient of NaCl (0 to 500 mM) in buffer A (200 ml for each concentration).

Preparation of anti-*Candida albicans* Rho1p antiserum. Plasmid pGEX4T-1 (Pharmacia) was used to produce the glutathione *S*-transferase [GST]–*Candida* Rho1p (from amino acids 130 to 190) fusion protein [GST-*Candida albicans* Rho1p (130–190)]. GST–*Candida albicans* Rho1p (130–190) was produced in *E. coli* JM109 with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h, and the recombinant protein was purified from cell lysates by glutathione Sepharose-4B affinity chromatography according to Pharmacia's protocol. Each of five mice (BALB/c) were immunized with 2.5 mg of purified GST-*Candida albicans* Rho1p (130–190) five times at 2-week intervals. Western blot (immunoblot) analysis was performed with serum diluted 1:2,000 on a polyvinylidene difluoride (PVDF) membrane (Millipore), goat anti-mouse immunoglobulin G antibody (H+L) AP-conjugate (Bio-Rad), and an AP-conjugate substrate kit (Bio-Rad).

Membrane preparation and Tergitol NP-40–NaCl treatment. *C. albicans* ATCC 10231 cells (100 g) were used for the membrane preparation as described in reference 16. Tergitol NP-40–NaCl treatment (30, 32) was done with some modifications. Tergitol NP-40 and NaCl were added to the membrane fraction (50 ml, 4 to 8 mg of protein/ml) at final concentrations of 2% and 2 M, respectively, and the membrane suspension was mixed at room temperature for 3 min. After centrifugation at 100,000 × *g* for 1 h, the membrane precipitate was resuspended in 50 ml of buffer B (50 mM Tris-Cl [pH 7.5], 1 mM EDTA, 33% glycerol, 1 mM β-mercaptoethanol) containing 2 M NaCl and 2% Tergitol NP-40. After centrifugation at 100,000 × *g* for 1 h, the membrane precipitate was washed three times with buffer B to remove the NaCl and Tergitol NP-40. The washed membrane fraction was resuspended in 20 ml of buffer B and stored at −80°C.

Partial purification of β-1,3-glucan synthase by product entrapment. Product entrapment was carried out as described before (31). Briefly, a 1/20 volume of 10% CHAPS and 2% cholesteryl hemisuccinate stock solution was added to 1 volume of *C. albicans* membrane fraction (4 to 8 mg of protein/ml, stored in buffer B) and mixed for 30 min on ice. Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was added at a final concentration of 5 μM. The membrane suspension was centrifuged at 100,000 × *g* for 1 h, and the supernatant was collected. The pellet was homogenized with buffer B containing 0.4% CHAPS, 0.08% cholesteryl hemisuccinate, and 5 μM GTPγS in half the volume of the original membrane suspension. This homogenate was centrifuged again at 100,000 × *g* for 1 h. After the addition of UDP-glucose at a final concentration of 5 mM, this combined detergent extract was left at room temperature for 1 h, during which time the β-1,3-glucan polymerized. The β-1,3-glucan polymer was collected by low-speed centrifugation (5,000 × *g*, 3 min); the loosely packed white pellet was suspended with buffer B containing 0.4% CHAPS, 0.08% cholesteryl hemisuccinate, and 5 mM UDP-glucose and centrifuged again (5,000 × *g*, 3 min). This step was repeated four times to remove contaminating proteins. After the last wash, the suspension was ultracentrifuged (100,000 × *g*, 3 min) and the tightly packed pellet was resuspended in a minimum amount of buffer B containing 0.4% CHAPS and 0.08% cholesteryl hemisuccinate but not 5 mM UDP-glucose. After a 10-min incubation on ice, the mixture was ultracentrifuged again (100,000 × *g*, 3 min) and the supernatant was collected. This extraction step was repeated a total of three times. When necessary, the entrapment step was repeated for the combined supernatant as described above.

Determination of β-1,3-glucan synthesis activity. The reaction conditions were the same as described in reference 16, except that 0.18% phosphatidylcholine, 0.2% Tergitol NP-40, and 0.18% CHAPS were added to achieve higher enzyme activity. In contrast, the addition of bovine serum albumin to this reaction mixture gave minimum activation.

Ligand overlay and GTP binding assays. The ligand overlay and GTP binding assays were performed as previously described (17, 28) with the following modifications. The partially purified β-1,3-glucan synthase complex was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 5 to 20% gradient gels, and the separated proteins were transferred to a PVDF membrane (Millipore). The proteins on the membrane were denatured by agitating the membrane for 5 min in buffer C (25 mM MES [morpholineethanesulfonic acid]-NaOH [pH 6.5], 0.5 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 0.05% Triton X-100) containing 6 M guanidium hydrochloride, which was then diluted with an equal volume of buffer C. The denatured proteins on the membrane were renatured by agitating the membrane overnight in phosphate-buffered saline containing 0.1% bovine serum albumin, 0.5 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 0.1% Triton X-100, 5 mM DTT, 0.1% phosphatidylcholine, and 0.1% CHAPS. The membrane was washed three times with 25 mM Tris-Cl (pH 7.5) containing 1 mM $MgCl_2$, 0.1 M NaCl, 0.05% Tween 20, 5 mM DTT, and 2 mM EDTA. The membrane was then reacted at room temperature for 30 min and then at 4°C for 10 min with [^{35}S]GTPγS-labeled recombinant *Candida albicans* Rho1p, which had been prepared by mixing [^{35}S]GTPγS (1.25 pmol, 1,000 Ci/mmol; Dupont, NEN) with recombinant *Candida albicans* Rho1p, partially purified with Q-Sepharose in a buffer containing 8 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 6 mM phosphatidylcholine at 30°C for 30 min. The reacted membrane was briefly washed four times with 25 mM MES-NaOH (pH 6.5) containing 50 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100. The PVDF membrane was dried, exposed to an imaging plate, and analyzed with a Bio Imaging Analyzer, model BAS1000Mac (Fuji Photo Film Co. Ltd.).

Chemical cross-linking with *Candida albicans* Rho1p and *Candida albicans* Gsc1p. For the chemical cross-linking reaction, the β-1,3-glucan synthase was purified with a MOPS (morpholinepropanesulfonic acid)-NaOH buffer instead

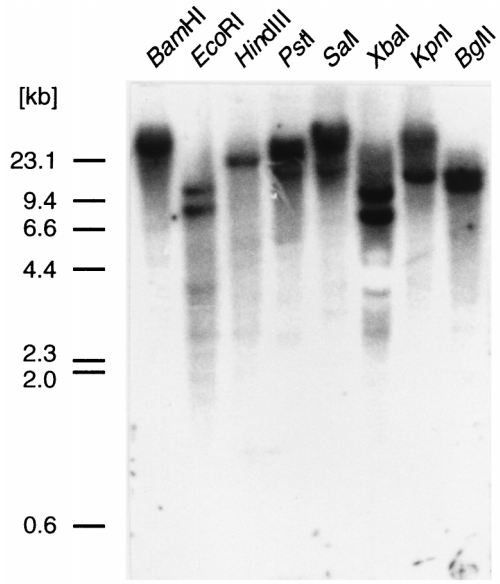

FIG. 1. Southern blot analysis of the *Candida albicans RHO1* gene. Genomic DNA prepared from ATCC 10231 was digested with restriction enzymes and probed with the ^{32}P-labeled 87-bp PCR product.

of the Tris-Cl buffer in buffer B for the product entrapment, because the Tris-Cl buffer hinders the reaction of the chemical cross-linking reagent. To the β-1,3-glucan synthase complex (approximately 2 mg of protein/ml, 40 μl), 4 μl of 10 mM EGS [ethylene glycol-bis(succinimidyl succinate); spacer arm, 16.1 Å; Pierce Chemical Co.] in 10% dimethyl sulfoxide was added, and the reaction mixture was incubated at room temperature for 10 min. The mixture (20 μl) was then subjected to SDS-PAGE on 5 to 20% gradient gels, and the separated proteins were transferred to a PVDF membrane (Millipore). Western blot analysis was performed with mouse anti-*Candida albicans* Rho1p antisera or anti-*Candida albicans* Gsc1p monoclonal antibody 2C2, goat anti-mouse immunoglobulin G antibody (H+L) AP-conjugate (Bio-Rad), and an AP-conjugate substrate kit (Bio-Rad).

Nucleotide sequence accession number. The sequence of the *C. albicans RHO1* gene has been submitted to GenBank as accession number D86430.

RESULTS

Cloning of the *RHO1* gene from *C. albicans*. To clone the *RHO1* homolog of *C. albicans*, we used the PCR method with several primers that were based on the sequence of the *S. cerevisiae RHO1* gene, with *C. albicans* ATCC 10231 genomic DNA as a template. An 87-bp PCR-amplified fragment was obtained with one of the primer combinations (see Materials and Methods), which was then subcloned into pT7Blue T-Vector, and the DNA sequences of several clones were determined. From the DNA sequence analysis of 10 clones, we found that this 87-bp fragment contained two kinds of sequences that were homologous to either *Saccharomyces cerevisiae RHO1* or *Saccharomyces cerevisiae RHO3*. The putative amino acid sequence of the protein encoded by the *Saccharomyces RHO1*-homologous fragment shared 28 of 29 residues with amino acids 16 to 44 of *Saccharomyces cerevisiae* Rho1p. The *Saccharomyces cerevisiae RHO3*-homologous protein shared 26 of 29 residues with *Saccharomyces cerevisiae* Rho3p and 23 of 29 residues with *Saccharomyces cerevisiae* Rho1p. With the *Saccharomyces cerevisiae RHO1*-homologous fragment as a probe, we performed a genomic Southern hybridization for *C. albicans* ATCC 10231 genomic DNA digested with restriction enzymes (Fig. 1). Although there are no *Eco*RI or *Xba*I sites in the 87-bp PCR product, two bands were hybridized in both *Eco*RI- and *Xba*I-digested DNA. When DNA from *C. albicans* CAI4 was examined under the same conditions, however, one of the two bands in each *Eco*RI and *Xba*I digestion disappeared (data not shown), suggesting that these two bands might have been derived from differences in the restriction sites of each allele. Using this 87-bp fragment as a probe, we screened the *C. albicans* genomic library by colony hybridization under the same stringency conditions. Of 20,000 colonies examined, one colony strongly hybridized with the probe. This clone contained a plasmid harboring an insert of at least 15 kb. To analyze the sequence of the *RHO1*-homologous gene within the insert, we first used primers corresponding to part of the 87-bp PCR fragment. The insert was found to have the sequence of the PCR-amplified 87-bp fragment within a 597-nucleotide open reading frame encoding a putative peptide of 22.0 kDa (Fig. 2). The deduced polypeptide was found to have 198 amino acids, 11 fewer than *Saccharomyces cerevisiae* Rho1p: 1 amino acid was deleted near the N-terminal portion and 10 were deleted near the C-terminal portion. Nevertheless, the putative amino acid sequence of this open reading frame contained all of the consensus sequences conserved among the Rho-type GTPases (Fig. 3A) (4, 41), was highly homologous to these GTPases, and was 82.9% identical to *Saccharomyces cerevisiae* Rho1p (Fig. 3B). Considering these facts, we named this gene *Candida albicans RHO1*, the *C. albicans* homolog of *Saccharomyces cerevisiae RHO1.*

```
-370                                                        TAGTAGTAAT
-360 AGTATTATCCAAATTAAATTGAATAACACATTACAATTTGTTTTTTATTATTTATTATTT
-300 TTTAGTCGCCTGAATTGATTCTTTTTTTTTTACTTCCCAGCCAAACACCAAAAACTTTTT
-240 TCTCTCTCACACTCTCAAAATTTCTTCCAACAACAAACCTTTTACTGAAAGAAAAAAAAA
-180 AATTTATTATAATTTAGTTCCCTCTTTCTCTTTCTCTCTCACTCTCTTTTTCTTTGATTC
-120 CATATATATTTTTAATCCCTTTATTTAATTACTTTCAACAACAACCACCCTACCTTCCCT
 -60 CCCCTCCCCTCTTCCCCTTTTAATAATACATCTATCAAATATAACATATAAACTTACATA

     (I)
   1 ATGGTTAACGGTCCAGCTGAACTTCGTAGAAAATTAGTCATTGTCGGTGATGGTGCTTGT
   1 M  V  N  G  P  A  E  L  R  R  K  L  V  I  V  G  D  G  A  C

  60 GGTAAGACTTGTTTATTAATTGTTTTTTCAAAAGGTACTTTCCCAGAAGTTTATGTCCCA
  20 G  K  T  C  L  L  I  V  F  S  K  G  T  F  P  E  V  Y  V  P

 120 ACAGTTTTTGAAAATTACGTTGCTGATGTTGAAGTTGATGGTAGAAAAGTTGAATTGGCA
  40 T  V  F  E  N  Y  V  A  D  V  E  V  D  G  R  K  V  E  L  A

 180 TTATGGGATACTGCTGGTCAAGAAGATTATGATAGATTAAGACCATTATCTTATCCAGAT
  60 L  W  D  T  A  G  Q  E  D  Y  D  R  L  R  P  L  S  Y  P  D

 240 TCTAATGTTATTTTGATTTGTTTTTCAGTTGATTCACCAGATTCTTTAGATAACGTTTTA
  80 S  N  V  I  L  I  C  F  S  V  D  S  P  D  S  L  D  N  V  L

 300 GAAAAATGGATTTCTGAAGTTTTACATTTCTGTCAAGGTGTTCCAATCATTTTAGTTGGT
 100 E  K  W  I  S  E  V  L  H  F  C  Q  G  V  P  I  I  L  V  G

                                                (II)
 360 TGTAAATCTGATTTAAGAGATGATCCTCATACTATTGAAGCCTTGAGACAACAACAACAA
 120 C  K  S  D  L  R  D  D  P  H  T  I  E  A  L  R  Q  Q  Q  Q

 420 CAACCAGTCTCAACTTCTGAAGGTCAACAAGTTGCTCAAAGAATTGGTGCTGCTGATTAC
 140 Q  P  V  S  T  S  E  G  Q  Q  V  A  Q  R  I  G  A  A  D  Y

 480 TTGGAATGTTCTGCTAAAACCGGTAGAGGTGTTAGAGAAGTGTTTGAAGCTGCTACTAGA
 160 L  E  C  S  A  K  T  G  R  G  V  R  E  V  F  E  A  A  T  R

        (II)                                    (I)
 540 GCTTCTTTAAGAGTTAAAGAAAAGAAGGAAAAAGAAGAAGAAATGTGTTGTCTTGTAAATG
 180 A  S  L  R  V  K  E  K  K  E  K  K  K  K  C  V  V  L  *

 600 TAACAACAACTAAAAGAACAAGAAGAAGAAGAAGAAGCATTAGCAATAGCAAAAGCTAAA
 660 AGAAAAAAAAAAGTCAAGCAAATACAACAAAAGGCAAAGTCAGAATAGAAAGAAACCTGAA
 720 GCCCTCTTATGAGTTGTGGTTTCCTTTCTTATTCTTTTTTTTTATTCATTTCA
```

FIG. 2. Nucleotide sequence and predicted amino acid sequence of *Candida albicans RHO1*. The sequence of the 87-bp PCR product is shown in italics. The primers for the sequencing analysis are underlined. Boxes indicate the primers used for production of intact *Candida albicans* Rho1p by the baculovirus expression system in insect cells (I) and for production of *Candida albicans* Rho1p in the GST-*Candida albicans* Rho1p (130–190) fusion protein in *E. coli* (II).

Complementation of the *S. cerevisiae rho1* null mutant by *Candida albicans RHO1*. To determine whether *Candida albicans RHO1* would be a functional homolog of *Saccharomyces cerevisiae RHO1*, we tested whether *Candida albicans RHO1* could rescue a *rho1* null mutant of *S. cerevisiae*. We constructed

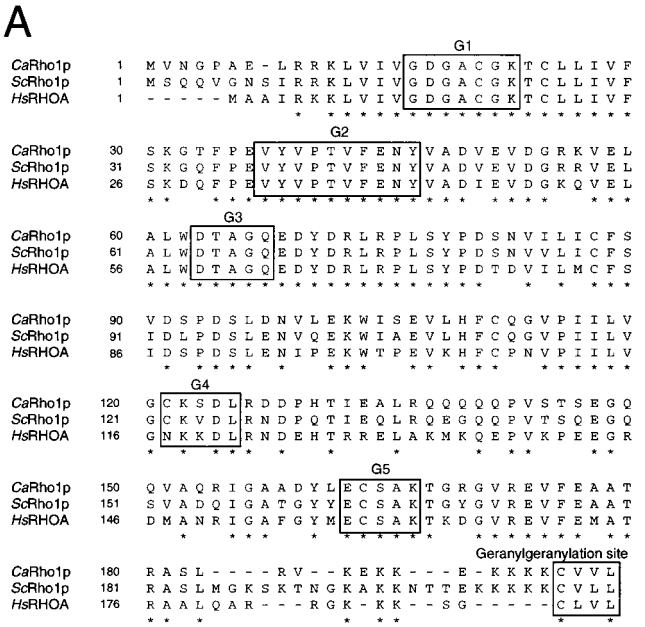

FIG. 3. (A) Amino acid sequence alignment of *Candida albicans* Rho1p, *Saccharomyces cerevisiae* Rho1p, and *H. sapiens* RhoA. Identical amino acids among the three proteins are denoted by asterisks. Boxes indicate the conserved domains among Rho-type GTPases (G1, G2, G3, G4, G5, and the geranylgeranylation site [4, 41]). (B) Dendrogram analysis of *Candida albicans* Rho1 with other GTPases by the PileUp program of the Genetics Computer Group package (12). Percentages of identity with *Candida albicans* Rho1p are shown. *Ca*, *C. albicans*; *Sc*, *S. cerevisiae*; *Sp*, *Schizosaccharomyces pombe*; *Hs*, *H. sapiens*; *Cf*, *Canis familiaris*; *Dm*, *D. melanogaster*; *Ce*, *Caenorhabditis elegans*; *Ac*, *A. californica*; *Do*; *Discopyge ommata.*

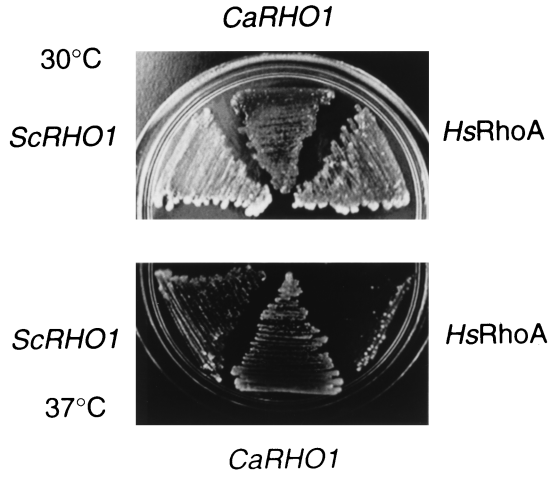

FIG. 4. Complementation of a *rho1* null mutant of *S. cerevisiae* by production of *Candida albicans* Rho1p. *S. cerevisiae* YOC706 was transformed with pYO-*Ca*RHO1, pYO702 (*Saccharomyces cerevisiae RHO1*), and pYO714 (*H. sapiens rhoA*). Growth of the transformants was tested on YPD plates at 30°C (upper panel) and 37°C (lower panel). *Sc*, *S. cerevisiae*; *Hs*, *H. sapiens*; *Ca*, *C. albicans.*

a plasmid that expressed the *Candida albicans RHO1* gene under the control of the *Saccharomyces cerevisiae RHO1* promoter and its terminator (pYO-*CaRHO1*) by inserting the PCR-amplified *Candida albicans RHO1* gene into plasmid pYO701, which also contains the *TRP1* gene as a marker. *S. cerevisiae* YOC706, which has a *rho1* deletion and harbors a plasmid containing both *Saccharomyces cerevisiae RHO1* and *URA3*, was transformed with pYO-*CaRHO1*. Several *TRP1*$^+$ transformants were transferred onto agar plates containing 1 mg of 5-fluoroorotic acid per ml to eliminate the plasmids containing only the *Saccharomyces cerevisiae RHO1* gene. Figure 4 shows that the 5-fluoroorotic acid-treated transformants could grow at both 30 and 37°C, as could the control strain with the wild-type *Saccharomyces cerevisiae RHO1* gene in place of the *Candida albicans RHO1* gene. *Homo sapiens RhoA*, the human homolog of *Saccharomyces cerevisiae RHO1*, could complement only at 30°C, as reported previously (37). This result showed that *Candida albicans RHO1* could rescue the *rho1* null mutant of *S. cerevisiae*, suggesting that *Candida albicans RHO1* has the same function as *Saccharomyces cerevisiae RHO1* in vivo.

Regulation of β-1,3-glucan synthesis by *Candida albicans* Rho1p. Recently Qadota et al. (38), Drgonova et al. (10), and Mazur and Baginsky (30) reported that *Saccharomyces cerevisiae* Rho1p is the regulatory protein of β-1,3-glucan synthase. We tested whether *Candida albicans* Rho1p could regulate β-1,3-glucan synthase in the same manner as *S. cerevisiae* Rho1p does. In *S. cerevisiae*, treatment with Tergitol NP-40 and NaCl depletes Rho1p from the membrane fraction; when the supernatant and treated membrane fraction are then mixed together, the β-1,3-glucan synthase activity is reconstituted (30, 32). We first tested whether recombinant *Candida albicans* Rho1p could recover the β-1,3-glucan synthetase activity of the Tergitol NP-40–NaCl-treated membrane. The recombinant *Candida albicans* Rho1p, produced in Sf9 insect cells by using the baculovirus expression system, was solubilized with CHAPS and partially purified by Q-Sepharose column chromatography as described in Materials and Methods. The identity of *Candida albicans* Rho1p was confirmed by Western blot analysis (Fig. 5B, lane 4). The *C. albicans* membrane treated with Tergitol NP-40–NaCl showed almost no β-1,3-glucan synthetase activity, but that activity could be reconstituted in the washed membrane by the addition of recombinant *Candida albicans* Rho1p in the presence of GTPγS but not in its absence (Fig. 6). Similarly, we found that *Candida albicans* Rho1p could also reconstitute the enzyme activity of the treated *S. cerevisiae* membrane (Fig. 6). These results indicated that the product of the gene we cloned can function as a regulatory protein of β-1,3-glucan synthetase (Rho1p) both in vivo and in vitro.

To make an antibody for immunological characterization of *Candida albicans* Rho1p, we first tried to produce recombinant *Candida albicans* Rho1p in *E. coli*. We tried to produce GST-

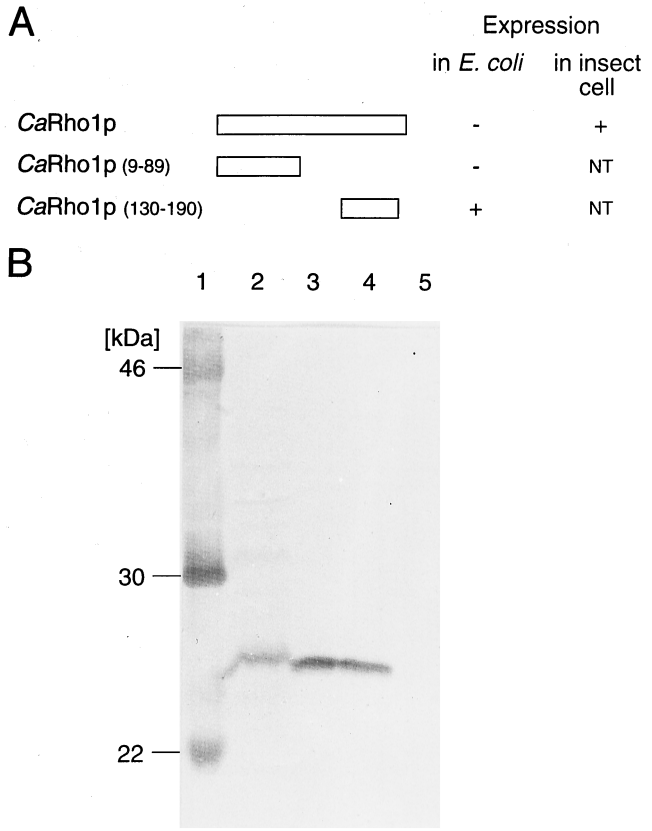

FIG. 5. (A) Production of recombinant *Candida albicans* Rho1p in *E. coli* and in insect cells. A plus sign indicates that the protein could be produced, and a minus sign indicates that it could not. NT indicates that expression was not tested. *Ca*, *C. albicans*. (B) Western blot analysis of *Candida albicans* Rho1p. Marker proteins (lane 1), the *C. albicans* membrane fraction (lane 2), the partially purified β-1,3-glucan synthase obtained by product entrapment (lane 3), fractions from baculovirus-infected cells (lane 4), and uninfected cells (lane 5) were separated by SDS-PAGE on 12.5% gels, and then Western blot analysis was performed.

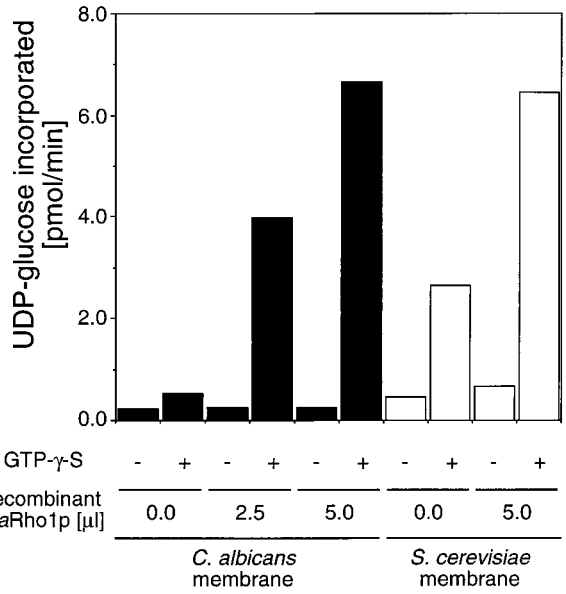

FIG. 6. Reconstitution of β-1,3-glucan synthesis activity by recombinant *Candida albicans* Rho1p. β-1,3-Glucan synthesis activity was measured with Tergitol NP-40–NaCl-treated membranes from *C. albicans* or from *S. cerevisiae* with or without GTPγS. The Q-Sepharose fraction of *Candida albicans* Rho1p was added as recombinant *Candida albicans* Rho1p. *Ca*, *C. albicans.*

Candida albicans Rho1p, GST-*Candida albicans* Rho1p (9–89), and GST-*Candida albicans* Rho1p (130–190) in *E. coli* but succeeded in producing only the C-terminal part of *Candida albicans* Rho1p, GST-*Candida albicans* Rho1p (130–190) (Fig. 5A), suggesting that the N terminus of *Candida albicans* Rho1p might be toxic in *E. coli*. We used the purified GST-*Candida albicans* Rho1p (130–190) as an antigen to prepare anti-*Candida albicans* Rho1p antisera from five mice. Rho GTPases are thought to be anchored in the cytosolic side of the plasma membrane through their C-terminal regions after the post-translational geranylgeranylation at the cysteine residue (23, 41). Western blot analysis of the anti-*Candida albicans* Rho1p antiserum (Fig. 5B, lane 1) detected *Candida albicans* Rho1p in the membrane fraction, suggesting that *Candida albicans* Rho1p is localized at the membrane, presumably by interaction with the geranylgeranyl residue. We also examined if *Candida albicans* Rho1 could be copurified with *Candida albicans* Gsc1p by product entrapment, as has been observed in *S. cerevisiae* (38). Product entrapment is a kind of affinity purification method, and all of the components necessary for β-1,3-glucan synthetase activity in vitro are enriched in the sample prepared by this procedure. Generally, one cycle of product entrapment increases the specific activity of an enzyme by more than 300-fold. As shown in Fig. 5B, the partially purified β-1,3-glucan synthase complex contained *Candida albicans* Rho1p (lane 2). This result indicates that *Candida albicans* Rho1p is one of the components of β-1,3-glucan synthase in *C. albicans.*

Ligand overlay and chemical cross-linking. The direct interaction of *Candida albicans* Rho1p and *Candida albicans* Gsc1p was examined by ligand overlay assay. This assay is a powerful tool for identifying the function and ligand binding of proteins separated by SDS-PAGE (17, 28). Recombinant *Candida albicans* Rho1p was radiolabeled with [^{35}S]GTPγS and reacted with the PVDF membrane on which partially purified *Candida albicans* Gsc1p had been immobilized following the SDS-PAGE separation. Recombinant *Candida albicans* Rho1p binding was observed at the position of *Candida albicans* Gsc1p (210 kDa) but not at the positions of the marker proteins (Fig. 7, right panel). No signal was detected when an uninfected insect cell fraction was used in the same way as recombinant *Candida albicans* Rho1p (Fig. 7, left panel). In these experiments, the uninfected Sf9 cell membrane control and recombinant *Candida albicans* Rho1p were treated in exactly the same way.

We also confirmed the direct interaction between *Candida albicans* Rho1p and *Candida albicans* Gsc1p by chemical cross-linking. As shown in Fig. 8, cross-linker EGS, which has a spacer arm length of 16.1 Å, cross-linked *Candida albicans* Rho1p with *Candida albicans* Gsc1p so that *Candida albicans* Gsc1p became reactive with anti-*Candida albicans* Rho1p antisera, although the difference in the migrations of *Candida albicans* Gsc1p cross-linked with *Candida albicans* Rho1p (210 plus 22 kDa) and *Candida albicans* Gsc1p alone (210 kDa) was not detectable in the 5 to 20% gradient gels.

Both of these results clearly demonstrate that *Candida albicans* Rho1p directly interacts with a β-1,3-glucan synthase putative catalytic subunit, *Candida albicans* Gsc1p, and regulates the activity of β-1,3-glucan synthase in the same manner as has been shown to occur in *S. cerevisiae* (10, 30, 38).

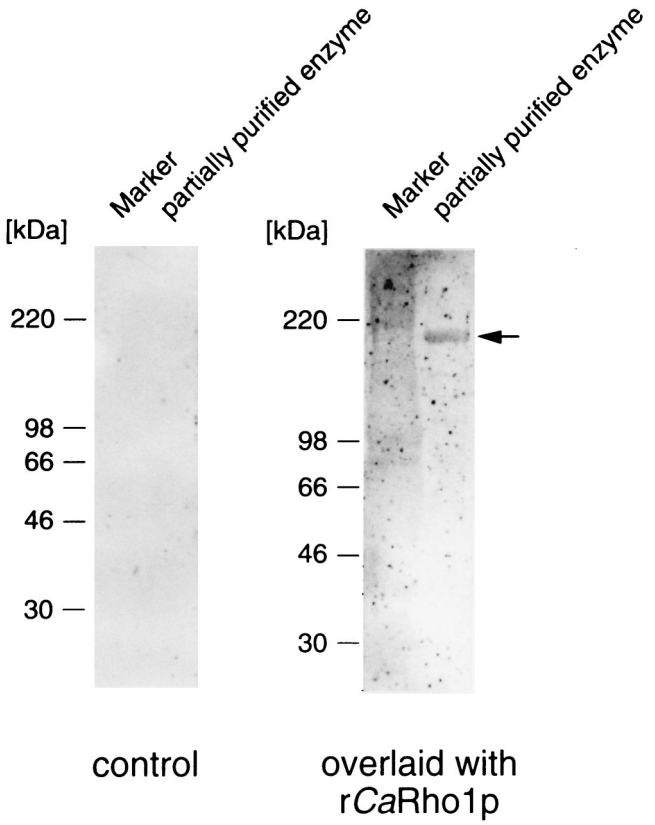

FIG. 7. Ligand overlay assay. Marker proteins and *Candida albicans* Gsc1p partially purified by product entrapment were analyzed by the ligand overlay assay described in Materials and Methods. Uninfected insect cells were used for the control (left panel). Filter images were detected with an Imaging Analyzer, model BAS1000Mac. The arrow denotes the binding of recombinant *Candida albicans* Rho1p (r*Ca*Rho1p) to *Candida albicans* Gsc1p.

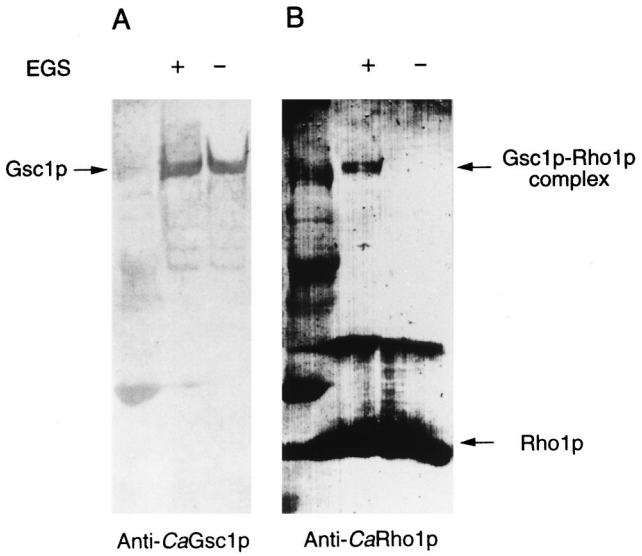

FIG. 8. Cross-linking with *Candida albicans* Gsc1p and *Candida albicans* Rho1p. Marker proteins (left lane) and *Candida albicans* Gsc1p partially purified by product entrapment were treated with or without the chemical cross-linker EGS. Western blot analysis was performed with mouse anti-*Candida albicans* Gsc1p monoclonal antibody 2C2 (A) and mouse anti-*Candida albicans* Rho1 antisera (B). *Ca*, *C. albicans.*

DISCUSSION

It has been suggested that one function of Rho1p in eukaryotic cells is to regulate morphological changes in the cells such as the reorganization of the cortical cytoskeleton and to control processes such as cell morphogenesis, motility, and cytokinesis (13, 43). Until recently, many studies have been focused on the roles of Rho1p and other Rho proteins in regulating cytoskeletal rearrangement during the cell cycle and in response to outside stimuli (42). *S. cerevisiae* and other fungi have cell walls in addition to the plasma membranes, and cell wall biosynthesis should also be in harmony with any changes in the cytoplasm so that fungi can grow and change their morphologies in response to the outside stimuli. Recently, we and other groups have identified Rho1p as the regulatory component of β-1,3-glucan synthase, the enzyme that synthesizes the cell wall β-1,3-glucan in *S. cerevisiae* (10, 30, 38). This finding links the morphological changes taking place inside the cell with changes in the envelope of the fungal cell.

We cloned the *Candida albicans RHO1* gene, the *C. albicans* homolog of *Saccharomyces cerevisiae RHO1*, which encodes a small Rho-type GTPase. Rho-type GTPases have been identified in several organisms, such as *S. cerevisiae* (27), *Schizosaccharomyces pombe* (33), *H. sapiens* (45), *Caenorhabditis elegans* (7), *A. californica* (26), *Drosophila melanogaster* (14) *Discopyge ommata* (34), and *Canis familiaris* (6). These Rho-type GTPases share the six domains shown in Fig. 3A (4, 41). The putative amino acid sequence coded for by *Candida albicans RHO1* possesses all of these domains and exhibits more than 66.8% identity to all of these GTPases, and especially to *S. cerevisiae* Rho1p (82.9%). Though in *S. cerevisiae*, four other Rho-type GTPases in addition to Rho1p have been identified, i.e., Rho2p, Rho3p, Rho4p, and Cdc42p, each of them exhibits less than 55.5% identity to *Candida albicans* Rho1p (Fig. 3B). Considering the sequence homology of its encoding protein, we would expect *Candida albicans RHO1* to be a homolog of *Saccharomyces cerevisiae RHO1* but not of the genes encoding the other GTPases isolated from *S. cerevisiae*. *Candida albicans* Rho1p is 11 amino acids smaller than *Saccharomyces cerevisiae* Rho1p, which is in agreement with its size detected by SDS-PAGE after [^{32}P]ADP ribosylation with exoenzyme C3 (30).

We further characterized *Candida albicans* Rho1p by focusing on the regulation of β-1,3-glucan synthesis. M. S. Kang and E. Cabib showed that the β-1,3-glucan synthase of membranes from *Hansenula anomala* and *Neurospora crassa* could be dissociated into a soluble fraction and a membrane fraction by Tergitol NP-40–NaCl treatment and that the active component in the soluble fraction could bind GTP or GTPγS. Furthermore, the Tergitol NP-40–NaCl-treated membrane fraction from *Hansenula anomala* or *N. crassa* could be reactivated by the soluble fractions from several other fungi, including *S. cerevisiae* (20). Recently Mazur and Baginsky showed that the GTP-binding protein in the soluble fraction is Rho1p in *S. cerevisiae* (30). We showed that the *C. albicans* membrane, when treated with Tergitol NP-40–NaCl in the same way, showed almost no β-1,3-glucan synthase activity and that recombinant *Candida albicans* Rho1p could reactivate the membrane activity in the presence of GTPγS (Fig. 6), indicating that *Candida albicans* Rho1p is the regulator of β-1,3-glucan synthase. Moreover, *Candida albicans* Rho1p could also reactivate the activity of the *S. cerevisiae* membrane treated in the same way. Thus, *Candida albicans* Rho1p could act in the same manner as *Saccharomyces cerevisiae* Rho1p on β-1,3-glucan synthesis both in vivo and in vitro. These observations suggested that β-1,3-glucan syntheses of other fungi, such as *Han-*

senula anomala and *N. crassa*, might be regulated by Rho1p in a way similar to the way they are regulated in *S. cerevisiae* and *C. albicans.*

The regulation of β-1,3-glucan synthesis by *Candida albicans* Rho1p was confirmed by immunoblot analysis of this protein. *Candida albicans* Rho1p was copurified with *Candida albicans* Gsc1p, the putative catalytic subunit of β-1,3-glucan synthase in *C. albicans*, by product entrapment (Fig. 5B, lane 3), suggesting that *Candida albicans* Rho1p is one of the components of the active complex of β-1,3-glucan synthase of *C. albicans*. *Candida albicans* Rho1p was also shown to be localized in the membrane fraction by immunoblot analysis (Fig. 5B, lane 2). Rho GTPases were shown to be posttranslationally geranylgeranylated at their C-terminal regions, and this modification facilitates the association of the Rho GTPases with the membrane (23, 41). The consensus sequence C-α-α-L, where α is usually an aliphatic amino acid, is conserved among these GTPases and is also found at the C terminus of *Candida albicans* Rho1p (Fig. 3A). These observations indicate that *Candida albicans* Rho1p is also localized at the plasma membrane.

In addition to β-1,3-glucan synthase, several other proteins were identified as potential targets of Rho1p in *S. cerevisiae*: Pkc1p, a homolog of mammalian protein kinase C (19, 35); Bni1p, a protein thought to be involved in cytokinesis (24); and Rom7p/Bem4p, related to bud emergence (15). We showed here that the *Candida albicans RHO1* gene could complement the *S. cerevisiae rho1* null mutant even at 37°C, suggesting the possibilities that *Candida albicans* Rho1p takes over all of the cellular functions of *Saccharomyces cerevisiae* Rho1p in *S. cerevisiae* and that, in *C. albicans*, the homologs of these effector proteins function in the rearrangement of the actin cytoskeleton during cell growth and in response to outside stimuli. *C. albicans* has two vegetative growth forms, budding and hyphal growth; the hyphal growth is induced by several stimuli, such as high temperature, alkaline pH, and the supply of serum. The functions of *Candida albicans* Rho1p in this dimorphic change with respect to β-1,3-glucan synthase regulation and actin cytoskeletal rearrangement remain to be elucidated.

In this study, direct interaction between *Candida albicans* Rho1p and the β-1,3-glucan synthase putative catalytic subunit was clearly shown by the ligand overlay and chemical crosslinking experiments (Fig. 7 and 8). The same *Candida albicans* Rho1p is thought to regulate actin cytoskeletal rearrangement through the interactions with Pkc1p, Bni1p, and Rom7p/Bem4p homologs in *C. albicans.*

C. albicans is a major pathogenic fungus in deep mycosis and differs from *S. cerevisiae* in such characteristics as pathogenicity, asexual dymorphism, and sensitivity to the well-known inhibitor of β-1,3-glucan synthase Echinocandin B (3, 25). Furthermore, *Hansenula murakii* toxin HM-1, an inhibitor of β-1,3-glucan synthase in *S. cerevisiae*, has no effect on the enzyme in *C. albicans*. To understand the differences in these phenomena, it is important to fully characterize β-1,3-glucan synthase at the molecular level by purifying the enzyme and cloning its regulatory component. Work to determine the interaction domain between the putative catalytic subunit *Candida albicans* Gsc1p and the regulatory subunit *Candida albicans* Rho1p and the inhibition mechanisms of Echinocandin B and HM-1 remains for further studies.

Moreover, the conditional complementation of the *S. cerevisiae rho1* null mutant by the human homolog *RhoA* and complete complementation by *Candida albicans RHO1* (Fig. 4) (37) suggest a functional difference between the two, although *Candida albicans* Rho1p is 71.5% identical to *H. sapiens* RhoA. In light of this difference, *Candida albicans* Rho1p might be a good target for antifungal drugs.

ACKNOWLEDGMENTS

We thank H. Qadota (Nara Institute of Science and Technology, Nara, Japan) for his helpful advice and kind gifts of the primers, *S. cerevisiae* YOC706, and plasmids pYO701, pYO702, and pYO714. We also thank H. Yamada-Okabe (Nippon Roche Research Center, Kanagawa, Japan) for the *C. albicans* genomic library and W. Fonzi (Georgetown University, Washington, D.C.) for his kind gift of *C. albicans* CAI4.

REFERENCES

1. **Adams, A. E., D. I. Johnson, R. M. Longnecker, B. F. Sloat, and J. R. Pringle.** 1990. *CDC42* and *CDC43*, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. **111:**131–142.
2. **Arellano, M., A. Duran, and P. Perez.** 1996. Rho1 GTPase activates the (1-3)β-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. EMBO J. **15:**4584–4591.
3. **Balkovec, J. M.** 1994. Lipopeptide antifungal agents. Exp. Opin. Invest. Drugs **3:**65–82.
4. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117–127.
5. **Castro, C., J. C. Ribas, M. H. Valdivieso, R. Varona, F. D. Rey, and A. Duran.** 1995. Papulacandin B resistance in budding and fission yeast: isolation and characterization of a gene involved in (1,3)β-D-glucan synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **177:**5732–5739.
6. **Chavrier, P., M. Vingron, C. Sander, K. Simons, and M. Zerial.** 1990. Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. Mol. Cell. Biol. **10:**6578–6585.
7. **Chen, W., and L. Lim.** 1994. The *Caenorhabditis elegans* small GTP-binding protein RhoA is enriched in the nerve ring and sensory neurons during larval development. J. Biol. Chem. **269:**32394–32404.
8. **Cid, V. J., A. Duran, F. del Rey, M. P. Snyder, C. Nombela, and M. Sanchez.** 1995. Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **59:**345–386.
9. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz.** 1994. The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:**12907–12911.
10. **Drgonova, J., T. Drgon, K. Tanaka, R. Kollar, G. Chen, R. A. Ford, C. S. M. Chan, Y. Takai, and E. Cabib.** 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science **272:**277–279.
11. **Eng, W., L. Faucette, M. M. McLaughlin, R. Cafferkey, Y. Koltin, R. A. Morris, P. R. Young, R. K. Johnson, and G. P. Livi.** 1994. The yeast *FKS1* gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin-dependent growth. Gene **151:**61–71.
12. **Feng, D. F., and R. F. Doolittle.** 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. **25:**351–360.
13. **Hall, A.** 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol. **10:**31–54.
14. **Hariharan, I. K., K. Hu, H. Asha, A. Quintanilla, R. M. Ezzell, and J. Settleman.** 1995. Characterization of rho GTPase family homologues in *Drosophila melanogaster*: overexpressing *Rho1* in retinal cells causes a late developmental defect. EMBO J. **14:**292–302.
15. **Hirano, H., K. Tanaka, K. Ozaki, H. Imamura, H. Kohno, T. Hihara, T. Kameyama, K. Hotta, M. Arisawa, T. Watanabe, H. Qadota, Y. Ohya, and Y. Takai.** 1996. *ROM7*/*BEM4* encodes a novel protein that interacts with the Rho1p small GTP-binding protein in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**4396–4403.
16. **Inoue, S. B., N. Takewaki, Y. Takasuka, T. Mio, M. Adachi, Y. Fujii, C. Miyamoto, M. Arisawa, Y. Furuichi, and T. Watanabe.** 1995. Characterization and gene cloning of 1,3-β-D-glucan synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem. **231:**845–854.
17. **Ishizaki, T., M. Maekawa, K. Fujisawa, K. Okawa, A. Iwamatsu, A. Fujita, N. Watanabe, Y. Saito, A. Kakizuka, N. Morii, and S. Narumiya.** 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. **15:**1885–1893.
18. **Johnson, D. I., and J. R. Pringle.** 1990. Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J. Cell Biol. **111:**143–152.
19. **Kamada, Y., H. Qadota, C. P. Python, Y. Anraku, Y. Ohya, and D. E. Levin.** 1996. Activation of yeast protein kinase C by Rho1 GTPase. J. Biol. Chem. **271:**9193–9196.
20. **Kang, M. S., and E. Cabib.** 1986. Regulation of fungal cell wall: guanine

nucleotide-binding, proteinaceous component required for activity of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **83:**5808–5812.
21. **Kelly, R., E. Register, M. Hsu, M. Kurtz, and J. Nielsen.** 1996. Isolation of a gene involved in 1,3-β-glucan synthesis in *Aspergillus nidulans* and purification of the corresponding protein. J. Bacteriol. **178:**4381–4391.
22. **Klis, F. M.** 1994. Review: cell wall assembly in yeast. Yeast **10:**851–869.
23. **Kohl, N. E., R. E. Diehl, M. D. Schaber, E. Rands, D. D. Soderman, B. He, S. L. Moores, D. L. Pompliano, S. Ferro-Novick, S. Powers, K. A. Thomas, and J. B. Gibbs.** 1991. Structural homology among mammalian and *Saccharomyces cerevisiae* isoprenyl-protein transferases. J. Biol. Chem. **266:**18884–18888.
24. **Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura, T. Fujiwara, Y. Fujita, K. Hotta, H. Qadota, T. Watanabe, Y. Ohya, and Y. Takai.** 1996. Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. EMBO J. **15:**6060–6068.
25. **Kurtz, M. B., and C. M. Douglas.** 1996. Lipopeptide inhibitors of fungal glucan synthase. J. Med. Vet. Mycol. **35:**79–86.
26. **Madaule, P., and R. Axel.** 1985. A novel *ras*-related gene family. Cell **41:**31–40.
27. **Madaule, P., R. Axel, and A. M. Myer.** 1987. Characterization of two members of *rho* gene family from yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **84:**779–783.
28. **Manser, E., T. Leung, H. Salihuddin, Z. Zhao, and L. Lim.** 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature **367:**40–46.
29. **Matsui, Y., and A. Toh-E.** 1992. Yeast *RHO3* and *RHO4 ras* superfamily genes are necessary for bud growth, and their defect is supressed by a high dose of bud formation genes *CDC42* and *BEM1*. Mol. Cell. Biol. **12:**5690–5699.
30. **Mazur, P., and W. Baginsky.** 1996. *In vitro* activity of 1,3-β-D-glucan synthase requires the GTP-binding protein Rho1. J. Biol. Chem. **271:**14604–14609.
31. **Mio, T., M. Adachi-Shimizu, Y. Tachibana, H. Tabuchi, S. B. Inoue, T. Yabe, M. Arisawa, T. Watanabe, and H. Yamada-Okabe.** 1997. Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae GSC1*/*FKS1* and its involvement in β-1,3-glucan synthesis. J. Bacteriol. **179:**4096–4105.
32. **Mol, P. C., H. Park, J. T. Mullins, and E. Cabib.** 1994. A GTP-binding protein regulates the activity of (1-3)-β-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. J. Biol. Chem. **269:**31267–31274.
33. **Nakano, K., and I. Mabuchi.** 1995. Isolation and sequencing of two cDNA clones encoding Rho proteins from the fission yeast *Schizosaccharomyces pombe*. Gene **155:**119–122.
34. **Ngsee, J. K., L. A. Elferink, and R. H. Scheller.** 1991. A family of ras-like GTP-binding proteins expressed in electromotor neurons. J. Biol. Chem. **266:**2675–2680.
35. **Nonaka, H., K. Tanaka, H. Hirano, T. Fujiwara, H. Kohno, M. Umikawa, A. Mino, and Y. Takai.** 1995. A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. EMBO J. **14:**5931–5938.
36. **Philippsen, P., A. Stotz, and C. Scherf.** 1991. DNA of *Saccharomyces cerevisiae*. Methods Enzymol. **194:**169–182.
37. **Qadota, H., Y. Anraku, D. Botstein, and Y. Ohya.** 1994. Conditional lethality of yeast strain expressing human *RHOA* in place of *RHO1*. Proc. Natl. Acad. Sci. USA **91:**9317–9321.
38. **Qadota, H., C. P. Python, S. B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D. E. Levin, and Y. Ohya.** 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science **272:**279–281.
39. **Ram, A. F. J., S. S. C. Brekelmans, L. J. M. W. Oehlen, and F. M. Klis.** 1995. Identification of two cell cycle regulated genes affecting the β 1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. FEBS Lett. **358:**165–170.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Seabra, M. C., Y. Reiss, P. J. Casey, M. S. Brown, and J. L. Goldstein.** 1991. Protein farnesyltransferase and geranylgeranyltransferase share a common α subunit. Cell **65:**429–434.
42. **Symons, M.** 1996. Rho family GTPases: the cytoskeleton and beyond. Trends Biochem. Sci. **21:**178–180.
43. **Takai, Y., T. Sasaki, K. Tanaka, and H. Nakanishi.** 1995. Rho as a regulator of the cytoskeleton. Trends Biochem. Sci. **20:**227–231.
44. **Yamochi, W., K. Tanaka, H. Nonaka, A. Maeda, T. Musha, and Y. Takai.** 1994. Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. J. Cell Biol. **125:**1077–1093.
45. **Yaramian, P., P. Chardin, P. Madaule, and A. Tavitian.** 1987. Nucleotide sequence of human rho cDNA clone 12. Nucleic Acids Res. **15:**1869.