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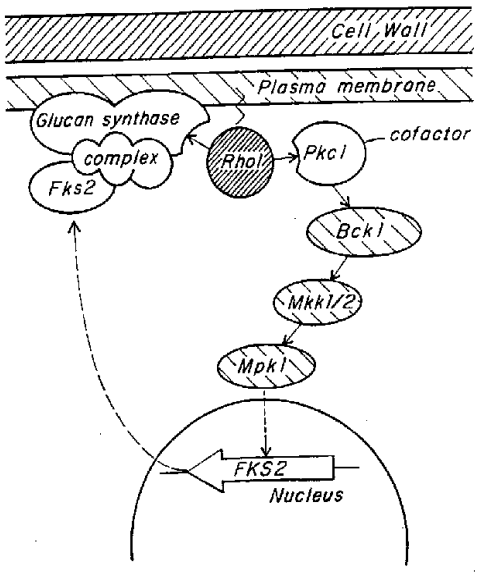
(54) Title: ASSAYS AND REAGENTS FOR IDENTIFYING ANTI-FUNGAL AGENTS, AND USES RELATED THERETO

(57) Abstract

The present invention relates to rapid, reliable and effective assays for screening and identifying pharmaceutically effective compounds that specifically inhibit the biological activity of fungal GTPase proteins, particularly GTPases involved in cell wall integrity, hyphael formation, and/or other cellular functions critical to pathogenesis. Another aspect of the present invention relates to novel *Candida* genes and gene products.



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Assays and Reagents for Identifying Anti-Fungal Agents, and Uses Related Thereto

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Background of the Invention

Fungal infections of humans range from superficial conditions, usually caused by dermatophytes or *Candida* species, that affect the skin (such as dermatophytoses) to deeply invasive and often lethal infections (such as *candidiasis* and *cryptococcosis*). Pathogenic fungi occur worldwide, although particular species may predominate in certain geographic areas.

In the past 20 years, fungal infections have increased dramatically - along with the numbers of potentially invasive species. Indeed, fungal infections, once dismissed as a nuisance, have begun to spread so widely that they are becoming a major concern in hospitals and health departments. Fungal infections occur more frequently in people whose immune system is suppressed (because of organ transplantation, cancer chemotherapy, or the human immunodeficiency virus), who have been treated with broad-spectrum antibacterial agents, or who have been subject to invasive procedures (catheters and prosthetic devices, for example). Fungal infections are now important causes of morbidity and mortality of hospitalized patients: the frequency of invasive candidiasis has increased tenfold to become the fourth most common blood culture isolate (Pannuti *et al.* (1992) *Cancer* 69:2653). Invasive pulmonary *aspergillosis* is a leading cause of mortality in bone-marrow transplant recipients (Pannuti *et al.*, *supra*), while *Pneumocystis carinii* pneumonia is the cause of death in many patients with acquired immunodeficiency syndrome in North America and Europe (Hughes (1991) *Pediatr Infect. Dis J.* 10:391). Many opportunistic fungal infections cannot be diagnosed by usual blood culture and must be treated empirically in severely immunocompromised patients (Walsh *et al.* (1991) *Rev. Infect. Dis.* 13:496).

The fungi responsible for life-threatening infections include *Candida* species (mainly *Candida albicans*, followed by *Candida tropicalis*), *Aspergillus* species, *Cryptococcus* neoforms, *Histoplasma capsulatum*, *Coccidioides immitis*, *Pneumocystis carinii* and some zygomycetes. Treatment of deeply invasive fungal infections has lagged behind bacterial chemotherapy.

There are numerous commentators who have speculated on this apparent neglect. See, for example, Georgopapadakou *et al.* (1994) *Science* 264:371. First, like mammalian cells, fungi are eukaryotes and thus agents that inhibit fungal protein, RNA, or DNA biosynthesis may do the same in the patient's own cells, producing toxic side effects. Second, life-threatening fungal infections were thought, until recently, to be too infrequent to warrant aggressive research by the pharmaceutical industry. Other factors have included:

(i) Lack of drugs. A drug known as Amphotericin B has become the mainstay of therapy for fungal infection despite side effects so severe that the drug is known as "amphoterrible" by patients. Only a few second-tier drugs exist.

(ii) Increasing resistance. Long-term treatment of oral candidiasis in AIDS patients has begun to breed species resistant to older anti-fungal drugs. Several other species of fungi have also begun to exhibit resistance.

(iii) A growing list of pathogens. Species of fungi that once posed no threat to humans are now being detected as a cause of disease in immune-deficient people. Even low-virulence baker's yeast, found in the human mouth, has been found to cause infection in susceptible burn patients.

(iv) Lagging research. Because pathogenic fungi are difficult to culture, and because many of them do not reproduce sexually, microbiological and genetic research into the disease-causing organisms has lagged far behind research into other organisms.

In the past decade, however, more antifungal drugs have become available. Nevertheless, there are still major weaknesses in their spectra, potency, safety, and pharmacokinetic properties, and accordingly it is desirable to improve the the panel of anti-fungal agents available to the practioner.

I. The fungal cell

The fungal cell wall is a structure that is both essential for the fungus and absent from mammalian cells, and consequently may be an ideal target for antifungal agents. Inhibitors of the biosynthesis of two important cell wall components, glucan and chitin, already exist. Polyoxins and the structurally related nikkomycins (both consist of a pyrimidine nucleoside linked to a peptide moiety) inhibit chitin synthase competitively, presumably acting as analogs of the substrate uridine diphosphate (UDP)-N-acetylglucosamine (chitin is an N-acetylglucosamine homopolymer), causing inhibition of septation and osmotic lysis. Unfortunately, the target of polyoxins and nikkomycins is in the inner leaflet of the plasma membrane; they are taken up by a dipeptide permease, and thus peptides in body fluids antagonize their transport.

In most fungi, glucans are the major components that strengthen the cell wall. The glucosyl units within these glucans are arranged as long coiling chains of β-(1,3)-linked residues, with occasional sidechains that involve β-(1,6) linages. Three β-(1,3) chains running in parallel can associate to form a triple helix, and the aggregation of helicies produces a network of water-insoluble fibrils. Even in the chitin-rich filamentous aspergilli, β-(1,3)-glucan is required to maintain the integrity and form of the cell wall (Kurtz *et al.* (1994) *Antimicrob Agents Chemother* 38:1408-1489), and, in *P. carinii*, it is important during the life cycle as a constituent of the cyst (ascus) wall (Nollstadt *et al.* (1994) *Antimicrob Agents Chemother* 38:2258-2265).

In a wide variety of fungi, β-(1,3)-glucan is produced by a synthase composed of at least two subunits (Tkacz, J.S. (1992) In: Emerging Targets in Antibacterial and Antifungal Chemotherapy Sutcliffe and Georgopapadakou, Eds., pp495-523, Chapman & Hall; and Kang *et al.* (1986) *PNAS* 83:5808-5812). One subunit is localized to the plasma membrane and is thought to be the catalytic subunit, while the second subunit binds GTP and associates with and activates the catalytic subunit (Mol *et al.* (1994) *J Biol Chem* 269:31267-31274).

Two groups of anticandidal antibiotics known in the art interfere with the formation of β-(1,3)-glucan: the papulacandins and the echinocandins (Hector *et al.* (1993) *Clin Microbiol Rev* 6:1-21). However, many of the papulacandins are not active against a variety of *Candida* species, or other pathogenic fungi including *aspergillus*. The *echinocandins*, in addition to suffering from narrow activity spectrum, are not in wide use because of lack of bioavilability and toxicity.

II. Protein Prenylation

Covalent modification by isoprenoid lipids (prenylation) contributes to membrane interactions and biological activities of a rapidly expnanding group of proteins (see, for example, Maltese (1990) *FASEB J* 4:3319; and Glomset *et al.* (1990) *Trends Biochem Sci* 15:139). Either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoids can be attached to specific proteins, with geranylgeranyl being the predominant isoprenoid found on proteins (Fransworth *et al.* (1990) *Science* 247:320).

Three enzymes have been described that catalyze protein prenylation: farnesyl-protein transferase (FPTase), geranylgeranyl-protein transferase type I (GGPTase-I), and geranylgeranyl-protein transferase type-II (GGPTase-II, also called Rab GGPTase). These enzymes are found in both yeast and mammalian cells (Schafer *et al.* (1992) *Annu. Rev. Genet.* 30:209-237). FPTase and GGPTase-I are α/β heterodimeric enzymes that share a common α subunit; the β subunits are distinct but share approximately 30% amino acid similarity (Brown *et al.* (1993). *Nature* 366:14-15; Zhang *et al.* (1994). *J. Biol. Chem.* 269:3175-3180). GGPTase II has different α and β subunits and complexes with a third

component (REP, Rab Escort Protein) that presents the protein substrate to the α/β catalytic subunits. Each of these enzymes selectively uses farnesyl diphosphate or geranylgeranyl diphosphate as the isoprenoid donor and selectively recognizes the protein substrate. FPTase farnesylates CaaX-containing proteins that end with Ser, Met, Cys, Gln or Ala. GGPTase-I geranylgeranylates CaaX-containing proteins that end with Leu or Phe. For FPTase and GGPTase-I, CaaX tetrapeptides comprise the minimum region required for interaction of the protein substrate with the enzyme. GGPTase-II modifies XXCC and XCXC proteins; the interaction between GGPTase-II and its protein substrates is more complex, requiting protein sequences in addition to the C-terminal amino acids for recognition. The enzymological characterization of these three enzymes has demonstrated that it is possible to selectively inhibit one with little inhibitory effect on the others (Moores *et al.* (1991) *J. Biol. Chem.* 266:17438).

GGPTase I transfers the prenyl group from geranylgeranyl diphosphate to the sulphur atom in the Cys residue within the CAAX sequence. *S. cerevisiae* proteins such as the Ras superfamily proteins Rho1, Rho2, Rsr1/Bud1 and Cdc42 appear to be GGPTase substrates (Madaule *et al.* (1987) *PNAS* 84:779-783; Bender *et al.* (1989) *PNAS* 86:9976-9980; and Johnson *et al.* (1990) *J Cell Biol* 111:143-152).

III. Protein Kinase C

Members of the family of phospholipid-dependent, serine/threonine-specific protein kinases known collectively as protein kinase C (PKC) respond to extracellular signals that act through receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (Hokin (1985) *Annu. Rev. Biochem.* 54, 205-235.). DAG serves as a second messenger to activate PKC (Takai *et al.* (1979) *Biochem. Biophys. Res. Commun.* 91:1218-1224; Kishimoto *et al.* (1980) *J. Biol. Chem.* 255:2273-2276; Nishizuka (1986) *Science* 233:305-312; and Nishizuka (1988) *Nature* 334:661-665), and IP_3 functions to mobilize Ca^{2+} from intracellular stores (Berridge *et al.* (1984) *Nature* 312:215-321). Twelve distinct subtypes of mammalian PKC have been reported to date (Nishizuka (1992) *Science* 258:607-614; Decker *et al.* (1994) *TIBS* 19:73-77). The four initially identified isozymes, α, βI, βII, and γ, are structurally closely related to each other and display similar catalytic properties.

Mammalian PKC is thought to play a pivotal role in the regulation of a host of cellular functions through its activation by growth factors and other agonists. These functions include cell growth and proliferation, release of various hormones, and control of ion conductance channels. Indirect evidence suggests that PKC induces the transcription of a wide array of genes, including the proto-oncogenes c-myc, c-fos, and c-sis, human collagenase, metallothionein II_A, and the SV40 early genes.

The PKC1 gene of budding yeast encodes a homolog of the α, β, and γ isoforms of mammalian Protein Kinase C that regulates a MAPK-activation pathway. Loss of PKC1 function results in a cell lysis defect that is due to a deficiency in cell wall construction.

Summary of the Invention

Accordingly, in a first aspect, the invention provides an assay for identifying compounds having potential anti-fungal activity, comprising:

(a) forming a reaction mixture including:

(i) a fungal geranylgeranyl transferase (GGPTase),

(ii) a GGPTase substrate, and

(iii) a test compound; and

(b) detecting interaction of the GGPTase substrate with the GGPTase,

wherein a statistically significant decrease in the interaction of the GGPTase substrate and GGPTase in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential anti-fungal activity for the test compound.

Accordingly, in a second aspect, the invention provides an assay for identifying compounds having potential anti-fungal activity, comprising:

(a) forming a cell-free reaction mixture including:

(i) a fungal geranylgeranyl transferase (GGPTase),

(ii) a target polypeptide comprising a fungal Rho-like GTPase, or a polypeptide portion thereof including a prenylation site,

(iii) an activated geranylgeranyl group,

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(iv) a divalent cation, and

(v) a test compound;

(b) detecting conjugation of the geranylgeranyl group of the target polypeptide in the reaction mixture,

wherein a statistically significant decrease in the prenylation of the target polypeptide and GGPTase in the presence of the test compound, relative to the level of prenylation in the absence of the test compound, indicates a potential anti-fungal activity for the test compound.

Accordingly, in a third aspect, the invention provides a method of identifying an agent which disrupts the ability of a geranylgeranyl protein transferase (GGPTase) to bind to a fungal GTPase, comprising:

(i) providing an interaction trap system including:

(a) a first fusion protein comprising at least a portion of a fungal GGPTase subunit,

(b) second fusion protein comprising at least a portion of a fungal GTPase, and

(c) a reporter gene, including a transcriptional regulatory sequence sensitive to interactions between the GGPTase portion of the first fusion protein and the GTPase portion of the second polypeptide;

(ii) contacting the interaction trap system with a candidate agent,

(iii) measuring the level of expression of a reporter gene in the presence of the candidate agent; and

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(iv) comparing the level of expression of the reporter gene in the presence of the candidate agent to a level of expression in the absence of the candidate agent,

wherein a decrease in the level of expression of the reporter gene in the presence of the candidate agent is indicative of an agent that inhibits interaction of the GGPTase and GTPase.

Accordingly, in a fourth aspect, the invention provides an assay for identifying compounds having potential anti-fungal activity, comprising:

(i) providing a first recombinant cell including a first prenylation substrate derived from a Rho-like GTPase which is a substrate for a geranylgeranyl transferase expressed by the cell,

(ii) providing a second recombinant cell including a second prenylation substrate identical to the first prenylation substrate except that it is mutated to be a substrate for a farnesyl transferase expressed by the recombinant cell,

(iii) contacting the first and second cells with a candidate agent; and

(iv) comparing the level of prenylation of the Rho-like GTPases in first and second cells,

wherein a statistically significant decrease in the prenylation of the first prenylation substrate, relative to the level of prenylation of the second prenylation substrate, is indicative of an agent that inhibits interaction of a GGPTase and GTPase.

Accordingly, in a fifth aspect, the invention provides an assay for screening test compounds to identify agents which modulate the interaction of a fungal geranylgeranyl transferase (GGPTase) with a fungal Rho-like GTPase, comprising:

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(i) providing a cell expressing a recombinant form of one or more of a fungal GGPTase and a fungal Rho-like GTPase;

(ii) contacting the cell with a test compound; and

(iii) detecting the level of interaction of the GGPTase and Rho-like GTPase,

wherein a statistically significant change in the level of interaction of the GGPTase and Rho-like GTPase is indicative of an agent that modulates the interaction of those two proteins.

Accordingly, in a sixth aspect, the invention provides an assay for identifying compounds having potential antifungal activity, comprising:

(i) forming a reaction mixture including a fungal Rho-like GTPase, a fungal protein kinase C (PKC), and a test compound; and

(ii) detecting interaction of the Rho-like GTPase and PKC, wherein a statistically significant decrease in the interaction of the Rho-like GTPase and PKC in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential antifungal activity for the test compound.

Accordingly, in a seventh aspect, the invention provides an assay for identifying compounds having potential antifungal activity, comprising:

(i) forming a reaction mixture including a fungal Rho-like GTPase, a fungal glucan synthase complex or subunit thereof (GS protein), and a test compound; and

(ii) detecting interaction of the Rho-like GTPase and GS protein, wherein a statistically significant decrease in the interaction of the Rho-like GTPase and GS protein in the presence of the test compound, relative to the level of interaction in the

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absence of the test compound, indicates a potential antifungal activity for the test compound.

Accordingly, in an eighth aspect, the invention provides a recombinant cell comprising: (i) exogenous nucleic acid encoding one or more subunits of a fungal geranylgeranyl protein transferase (GGPTase), and (ii) exogenous nucleic acid encoding a fungal Rho-like GTPase or a fragment thereof including at least one of: (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase.

Accordingly, in a ninth aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising:

(i) a recombinant fungal geranylgeranyl protein transferase (GGPTase), and (ii) a recombinant fungal Rho-like GTPase or a fragment thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase.

Accordingly, in a tenth aspect, the invention provides a recombinant cell comprising (i) exogenous nucleic acid encoding a fungal Rho-like GTPase, and (ii) exogenous nucleic acid encoding a fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or one or more subunits of a fungal glucan synthase.

Accordingly, in an eleventh aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising (i) a recombinant fungal Rho-like GTPase, and (ii) a recombinant fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or a fungal glucan synthase.

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Accordingly, in a twelfth aspect, the invention provides a recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen.

Accordingly, in a thirteenth aspect, the invention provides a recombinant cell comprising exogenous nucleic acid encoding a Rho-like GTPase cloned from a human fungal pathogen.

Accordingly, in a fourteenth aspect, the invention provides a recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a glucan synthase cloned from a human fungal pathogen.

Accordingly, in a fifteenth aspect, the invention provides a recombinant cell comprising exogenous nucleic acid encoding a protein kinase C cloned from a human fungal pathogen.

Accordingly, in a sixteenth aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising one or more subunits of a recombinant geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen.

Accordingly, in a seventeenth aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising a recombinant Rho-like GTPase cloned from a human fungal pathogen

Accordingly, in an eighteenth aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising one or more recombinant subunits of a glucan synthase cloned from a human fungal pathogen.

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Accordingly, in a nineteenth aspect, the invention provides a reconstituted protein mixture or a cell lysate mixture comprising a recombinant protein kinase C cloned from a human fungal pathogen.

Accordingly, in a twentieth aspect, the invention provides a purified and/or recombinant polypeptide comprising a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1 or SEQ ID No. 5, the GTPase sequence (i) directing the binding of said polypeptide to a glucan synthase subunit, (ii) directing the binding of said polypeptide to PKC, (iii) serving as a substrate for prenylation by a GGPTase, or (iv) having a GTP hydrolytic activity, or a combination thereof.

Accordingly, in a twenty-first aspect, the invention provides a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 3, the RAM2 sequence (i) directing the binding of said polypeptide to a GGPTase or FPTase β subunit, or (ii) directing the binding of said polypeptide to a Rhol-like GTPase, or a combination thereof.

Accordingly, in a twenty-second aspect, the invention provides a purified protein complex comprising the polypeptide according to any one of the twentieth or twenty-first aspects.

Accordingly, in a twenty-third aspect, the invention provides an isolated nucleic acid comprising a coding sequence encoding the polypeptide according to any one of the twentieth or twenty-first aspects.

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Accordingly, in a twenty-fourth aspect, the invention provides an expression construct comprising the nucleic acid according to the twenty-third aspect.

Accordingly, in a twenty-fifth aspect, the invention provides a host cell transformed with the expression construct according to the twenty-fourth aspect.

Accordingly, in a twenty-sixth aspect, the invention provides a method for producing a recombinant protein comprising culturing the host cell according to the twenty-fifth aspect under conditions sufficient to produce a cell culture expressing said polypeptide, and isolating said polypeptide from said cell culture.

Accordingly, in a twenty-seventh aspect, the invention provides an isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 1 (sense or antisense) and selectively detects a *CaRho1* gene encoding a protein having GTP hydrolytic activity.

Accordingly, in a twenty-eighth aspect, the invention provides an isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 5 (sense or antisense) and selectively detects a *CaCdc42* gene encoding a protein having GTP hydrolytic activity.

Accordingly, in a twenty-ninth aspect, the invention provides an isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 3 (sense or antisense) and selectively detects a *CaRAM2* gene encoding a GGPTase or FPTase subunit.

Accordingly, in a thirtieth aspect, the invention provides a diagnostic test kit for detecting *Candida* cells comprising the nucleic acid according to the twenty-seventh, twenty-eight and twenty-ninth aspects.

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Accordingly, in a thirty-first aspect, the invention provides an isolated, recombinant and/or monoclonal antibody which is specifically cross-reactive with the GTPase sequence according to the twentieth aspect or the RAM2 sequence according to the twenty-first aspect.

Accordingly, in a thirty-second aspect, the invention provides a method for detecting the presence of *Candida* cells comprising detecting, in a sample, the presence of one or more of a (i) a nucleic acid encoding a *Candida* protein selected from the group consisting of *CaRho1, CaCdc42* and *CaRAM2*, and (ii) a *Candida* protein selected from the group consisting of *CaRho1, CaCdc42* and *CARAM2*.

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

The present invention provides drug screening assays for identifying pharmaceutically effective compounds that specifically inhibit the biological activity of fungal GTPase proteins, particularly GTPases involved in cell wall integrity, hyphael formation and other cell functions critical to pathogenesis. Briefly, as described in greater detail below, Applicants have discovered the critical involvement of Rho-like GTPase activities in cell wall integrity. For instance, the fungal Rho1 GTPase is required for glucan synthase activity, copurifies with 1,3-β-glucan synthase, and is found to associate with the Gsc1/Fks1 subunit of this complex *in vivo*. Rho1 is a regulatory subunit of 1,3-β-glucan synthase, and accordingly this interaction, and the resulting enzyme complex, are potential therapeutic targets for development of antifungal agents.

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Moreover, Rho1 is required for protein kinase C (PKC1) mediated MAPK activation, and confers upon PKC1 the ability to be stimulated by phosphatidylserine (PS), indicating that Rho1 controls signal transmission through PKC1. Loss of PKC1 activity results in cell lysis. Also, we demonstrate that prenylation of Rho1 by a geranylgeranyl transferase is a critical step to maintenance of cell wall integrity in yeast. As described in the appended examples, prenylation of Rho1 is required for sufficient glucan synthase activity. Loss of Rho1 prenylation results in cell lysis. In general, a salient feature of the subject assays is that each is generated to detect agents which are potentially cytotoxic to a fungal cell, rather than merely cytostatic. Moreover, given the uniqueness of the therapeutic fungal targets of the present assays, e.g., relative to homolgous proteins in mammalian cells, the therapeutic targeting of Rho-like GTPase(s) involvement in such interactions and complexes in yeast presents an opportunity to define antifungal agents which are highly selective for yeast cells relative to mammalian cells.

In one aspect, the present invention provides an assay for identifying potential antifungal agents by targeting the GGPTase/GTPase interaction. For instance, the assay can be run by forming a reaction mixture including (i) a fungal geranylgeranyl transferase (GGPTase), (ii) a substrate for the GGPTase, such as a target polypeptide comprising a fungal Rho-like GTPase such as Rho1, Rho2, Rsr1/Bud1 and Cdc42, or a polypeptide portion thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase, and (iii) a test compound. The interaction of the target

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polypeptide with the GGPTase can be detected. A statistically significant decrease in the interaction of the target

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polypeptide and GGPTase in the presence of the test compound, relative to the level of interaction in the absence of the test compound (or other control), indicates a potential anti-fungal activity for the test compound.

The reaction mixture can be a reconstituted protein mixture, a cell lysate or a whole cell. For instance, the reaction mixture can be a prenylation system including an activated geranylgeranyl group, and the step of detecting the interaction of the target polypeptide with the GGPTase includes detecting conjugation of the geranylgeranyl group to the target polypeptide. In preferred embodiments of such prenylation systems at least one of the geranylgeranyl group and the target polypeptide has a detectable label, and the level of geranylgeranyl group conjugated to the target polypeptide is quantified by detecting the label in at least one of the target polypeptide, free geranylgeranyl groups, and geranylgeranyl-conjugated target polypeptide. As illustrated below, the substrate target can incorporate a fluorescent (or other) label, the fluorescent characterization of which is altered by the level of prenylation of the substrate target, e.g., the substrate target can be a dansylated peptide substrate of the fungal GGPTase.

In other embodiments, the step of detecting the interaction of the target polypeptide with the GGPTase includes detecting the formation of protein-protein complexes including the target polypeptide with the GGPTase. For example, at least one of the GGPTase and the target polypeptide can include a detectable label, and the level of GGPTase/target polypeptide complexes formed in the reaction mixture is quantified by detecting the label in at least one of the target polypeptide, the GGPTase, and GGPTase/target polypeptide complexes. Exemplary labels for such embodiments, and for the prenylation assays above, include radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. For instance, the detectable label can be a protein having a measurable activity, and one of the PKC or GTPase is fusion protein including the detectable label. In other exemplary embodiments, conjugation of the geranylgeranyl group to the target polypeptide is detected by an immunoassay.

Where the reaction mixture is a whole cell, the cell will preferably include heterologous nucleic acid recombinantly expressing one or more of the fungal GGPTase subunits and target polypeptide. In certain preferred embodiments, the cell will also include a heterologous reporter gene construct having a reporter gene in operable linkage with a transcriptional regulatory sequence sensitive to intracellular signals transduced by interaction of the target polypeptide and GGPTase.

In one preferred embodiment, the assay includes forming a cell-free reaction mixture including: (i) a fungal GGPTase, (ii) a GGPTase substrate, e.g., a target polypeptide comprising a fungal Rho-like GTPase, or a polypeptide portion thereof including a prenylation site, (iii) an activated geranylgeranyl group, (iv) a divalent cation, and (v) a test

compound. The assay is derived to detect conjugation of the gernaylgernayl group of the target polypeptide in the reaction mixture, and a statistically significant decrease in the prenylation of the target polypeptide and GGPTase in the presence of the test compound, relative to an appropriate control, indicates a potential anti-fungal activity for the test compound.

In another preferred embodiment, the method utilizes an interaction trap system including (a) a first fusion protein comprising at least a portion of a fungal GGPTase subunit, (b) a second fusion protein comprising at least a portion of a fungal GTPase, and (c) a reporter gene, including a transcriptional regulatory sequence sensitive to interactions between the GGPTase portion of the first fusion protein and the GTPase portion of the second polypeptide. After contacting the interaction trap system with a candidate agentthe level of expression of a reporter gene is measured and compared to the level of expression in the absence of the candidate agent. A decrease in the level of expression of the reporter gene in the presence of the candidate agent is indicative of an agent that inhibits interaction of the GGPTase and GTPase.

In still another embodiment, the assay is derived from a a recombinant cell expressing a recombinant form of one or more of a fungal GGPTase and a fungal Rho-like GTPase. The cell is contacted with a test compound, and the level of interaction of the GGPTase and Rho-like GTPase is detected. A statistically significant change in the level of interaction of the GGPTase and Rho-like GTPase is indicative of an agent that modulates the interaction of those two proteins. In preferred embodiments, one or both of a GGPTase subunit or the Rho-like GTPase are fusion proteins, e.g., the fustion protein providing a detectable label and/or an affinity tag for purification. In a preferred embodiment, the Rho-like GTPase is a fusion protein further comprising a transcriptional regulatory protein, and level of prenylation of the Rho-like GTPase is detected by measuring the level of expression of a reporter gene construct which is sensitive to the transcriptional regulatory protein portion of the fusion protein, wherein inhibition of prenylation of the fusion protein results in loss of membrane partitioning of the fusion protein and increases expression of the reporter gene construct.

In other preferred embodiments, the level of interaction of the GGPTase and Rho-like GTPase is detected by detecting prenylation of the Rho-like GTPase.

In yet another preferred embodiment, the assay is generated from a set of cells in which prenylation of endogenous Rho-like GTPases by GGPTase I is made dispensible. According to this embodiment, the assay provides a first test cell in which one or more Rho-like GPTases are mutated to be a substrate for a farnesyl transferase expressed by the cell such that GGPTase I is dispensible for cell growth; and a second test cell identical to the first cell except that the Rho-like GTPases are substrates for GGPTase I and are

indispensible for cell growth. The first and second cells are contacted with a candidate agent, and the level of prenylation of the Rho-like GTPases in first and second test cells are compared. A statistically significant decrease in the prenylation of the GTPases in the second test cell, relative to the level of prenylation of the GTPase in the first cell, is indicative of an agent that inhibits interaction of a GGPTase and GTPase.

Yet another aspect of the present invention, the subject assays are derived for detecting agents which disrupt the formation of, or function of fungal protein complexes including Rho-like GTPases and PKC proteins. In one embodiment, the assay provides a reaction mixture including a fungal Rho-like GTPase, a fungal protein kinase C (PKC), and a test compound. Interaction of the Rho-like GTPase and PKC is detected in the reaction mixture, wherein a statistically significant decrease in the interaction of the Rho-like GTPase and PKC in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential antifungal activity for the test compound.

The reaction mixture can be a reconstituted protein mixture, a cell lysate or a whole cell. In preferred embodiments, the reaction mixture is a kinase system including ATP and a PKC substrate, and the step of detecting interaction of the GTPase and PKC includes detecting phosphorylation of the PKC substrate by a PKC/GTPase complex. Preferably, at least one of the PKC substrate and ATP includes a detectable label, and the level of phosphorylation of the PKC substrate is quantified by detecting the label in at least one of the phosphorylated PKC substrate or ATP. For instance, the PKC substrate may include a fluorescent (or other) label, the fluorescent characterization of which is altered by the level of phosphorylation of the PKC substrate.

In other preferred embodiments, the step of detecting the interaction of the GTPase with the PKC includes detecting the formation of protein-protein complexes including the GTPase and PKC. For instance, at least one of the PKC and GTPase includes a detectable label, and the level of PKC/GTPase complexes formed in the reaction mixture is quantified by detecting the label in at least one of the GTPase, the PKC, and PKC/GTPase complexes. For instance, phosphorylation of the PKC substrate is detected by immunoassay.

Cell-based assays are also provided, including cells comprising reporter gene constructs sensitive to PKC/GTPase complexes. In one embodiment, PKC/GTPases interaction trap assays are used for drug screening according to the present invention.

In still another aspect of the present invention, the subject assays are derived for detecting agents which disrupt the formation of, or function of fungal protein complexes including Rho-like GTPases and glucan synthase complexes or subunits thereof. In a preferred embodiment, the assay includes forming a reaction mixture including a fungal Rho-like GTPase, a fungal glucan synthase complex or subunit thereof (collectively "GS

protein"), and a test compound. The interaction of the Rho-like GTPase and GS protein can be detected in the reaction mixture. Similar to the assay embodiments set out above, a statistically significant decrease in the interaction of the Rho-like GTPase and GS protein in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential antifungal activity for the test compound.

The reaction mixture can be a reconstituted protein mixture, a cell lysate or a whole cell. In preferred embodiments, the reaction mixture is a glucan synthesis system including a GTP and a UDP-glucose, and the step of detecting interaction of the GTPase and GS protein includes detecting formation of glucan polymers in the reaction mixture, e.g., the UDP-glucose can include a detectable label, and the level of glucan polymer formation is quantified by detecting the labeled glucan polymers.

In other embodiments, the step of detecting the interaction of the GTPase with the GS protein includes detecting the formation of protein-protein complexes including the GTPase and GS protein. As above, at least one of the GS protein and GTPase can include a detectable label, and the level of GS protein/GTPase complexes formed in the reaction mixture is quantified by detecting the label in at least one of the GTPase, the GS protein, and GS protein/GTPase complexes. Alternatively, the formation of protein-protein complexes including the GTPase and GS protein is detected by an immunoassay.

As above, cell-based assays are also provided, including cells comprising reporter gene constructs sensitive to GS/GTPase complexes. Permeabilization of cells due to disruption of GS activity by the test compound can also be detected by loss of cytoplasmic localization or cytoplasmic exclusion (depending on the embodiment) of a detectable label.

For each of the assay embodiments set out above, the assay is preferably repeated for a variegated library of at least 100 different test compounds, though preferably libraries of at least 10^3, 10^5, 10^7, and 10^9 compunds are tested. The test compound can be, for example, small organic molecules, and/or natural product extracts.

Also, in preferred embodiments of the subject assay, one or more of the GTPase of other proteins which interacting with the GTPase (e.g., GGPTase subunits, PKC and glucan synthase subunits) are derived from a human pathogen which is implicated in mycotic infection.

The subject assay also preferably includes a further step of preparing a pharmaceutical preparation of one or more compounds identified as having potential antifungal activity.

Still another aspect of the invention concerns various compositions and reagents for performing the subject drug screening assays. For instance, the present invention provides a variety of recombinant cells expressing one or more different fungal proteins implicated as

targets in the subject screening assays. In a preferred embodiment, the recombinant cell includes exogenous nucleic acid (e.g., expression vectors) encoding a fungal Rho-like GTPase. In a more preferred embodiment, the recombinant cell includes (i) exogenous nucleic acid(s) encoding one or more subunits of a fungal geranylgeranyl protein transferase (GGPTase), and (ii) exogenous nucleic acid encoding a fungal Rho-like GTPase or a fragment thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase. In still other preferred embodiments, the cell inlcudes (i) exogenous nucleic acid encoding a fungal Rho-like GTPase, and (ii) exogenous nucleic acid encoding a fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or one or more subunits of a fungal glucan synthase.

The nucleic acids encoding the GGPTase, GTPase, PKC and/or glucan synthase are preferably derived from a human pathogen which is implicated in mycotic infection. For instance, the recombinant genes can be derived from fungus involved in such mycotic infections as selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis. To further illustrate, the expression vectors can be generated from genes cloned from human pathogen selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*. Another source for recombinant genes is the human pathogen is *Pneumocystis carinii*.

In preferred embodiments, the cell is a recombinantly manipulated yeast cell selected from the group consisting of such genuses as *Kluyverei*, *Schizosaccharomyces*, *Ustilaqo* and *Saccharomyces*, though a prefered host cell is the *Schizosaccharomyces cerivisae* cell. Moreover, the host cell can be constitutively or inducibly defective for an endogenous activity corresponding to one or more of the GGPTase and GTPase encoded by the exogenous nucleic acids.

In similar fashion, another aspect of the present invention concerns reconstituted protein mixtures or cell lysate mixtures including a recombinant fungal Rho-like GTPase, e.g, or a fragment thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase, along with one or more of a recombinant fungal glucan synthase, a recombinant fungal GGPTase, and/or a recombinant fungal PKC. As above, the

fungal target proteins are preferably derived from a human pathogen which is implicated in mycotic infection.

Another aspect of the present invention relates to the discovery and isolation of genes encoding novel regulatory proteins from the human fungal pathogen *Candida*, namely the αsubunit of a GGPTase I enzyme and a Rho-like GTPase. The present invention specifically contemplates a purified and/or recombinant polypeptide including a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1, a *Candida CaRho1* gene, or to SEQ ID No. 5, a *Candida CaCdc42* gene, the GTPase sequence (i) directing the binding of the polypeptide to a glucan synthase subunit, (ii) directing the binding of the polypeptide to PKC, (iii) serving as a substrate for prenylation by a GGPTase, or (iv) having a GTP hydrolytic activity, or a combination thereof. In other embodiments, there is provided a purified and/or recombinant polypeptide including a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 3, a *Candida CaRAM2* gene, the RAM2 sequence (i) directing the binding of the polypeptide to a GGPTase or FPTase β subunit, or (ii) directing the binding of the polypeptide to a Rho1-like GTPase, or a combination thereof.

In preferred embodiments of the above polypeptides, the GTPase sequence or the RAM2 sequence is at least 80% identical, more preferably 90% identical, and even more preferably identical to one of the polypeptides represented by SEQ ID Nos. 2, 4 or 6.

The subject polypeptides can be derived from, e.g., encoded by, an endogenous gene from *Candida spp.* Exemplary *Candida* organisms include *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii* and *Candida rugosa*.

In some embodiments, the polypeptide is a fusion protein. For instance, the fusion protein can include, in addition to the RAM2 sequence, a RAM1 or cdc43 sequence, the fusion protein possessing prenylation activity. In other embodiments, the fusion protein can include, in addition to the GTPase or RAM2 sequence, as appropriate, a second polypeptide portion selected from the group consisting of a DNA binding domain and a transciptional activitation domain, the fusion protein being functional in a two-hybrid assay.

Still another aspect of the present invention relates to purified protein complexes including the GTPase or RAM2 polypeptide described herein. For instance, in the case of the complexes including *CaRho1* or *CaCdc42*, the protein complex can also include a glucan synthase subunit, a PKC, a GGPTase, or a combination thereof. Exemplary complexes including the subject *CaRAM2* polypeptide include a GGPTase β subunit, an FPTase β subunit, a Rho1-like GTPase, or a combination thereof.

Yet another aspect of the present invention relates to isolated nucleic acids including a coding sequence encoding one of the subject *CaRho1*, *CaCdc42* or *CaRAM2* polypeptides. The present invention also provides isolated nucleic acids which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 1, 3 or 5 (sense or antisense) and which selectively detect either a *CaRho1* or *CaCdc42* gene (e.g., encoding a protein having GTP hydrolytic activity) or a *CaRAM2* gene (e.g., encoding a GGPTase or FPTase subunit).

Such nucleic acids can be provided as part of a diagnostic test kit for detecting *Candida* cells. For instance, the nucleic acid can be an antisense oligonucleotide which hybridizes to *CaRho1*, *CaCdc42* or *CaRam2* gene, as appropriate. In preferred embodiments, the nucleic acid is at least 25 nucleotides in length, though more preferably at least 50 nucleotides in length.

In many embodiments of the kit, the nucleic acid will be labelled with a detectable label. Exemplary detectable labels include enzymes, enzyme substrates, coenzymes, enzyme inhibitors, fluorescent markers, chromophores, luminescent markers, and radioisotopes.

In preferred embodiments, the kit, by selection of the nucleic acid, is desigened to detect the presence of nucleic acid from a *Candida* cell selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii* and *Candida rugosa*.

The present invention also provides expression constructs encoding the subject *CaRho1*, *CaCdc42* or *CaRAM2* proteins, as well as host cells transformed with such expression constructs. Furthermore, the present invention provides a method for producing a recombinant *CaRho1*, *CaCdc42* or *CaRAM2* polypeptide by culturing such host cells under conditions sufficient to produce a cell culture expressing the polypeptide, and isolating the polypeptide from the cell culture.

Still another aspect of the present invention provides an isolated, recombinant and/or monoclonal antibody which is specifically cross-reactive with the subject *CaRho1*, *CaCdc42* or *CaRAM2* proteins. The antibody can be labelled with a detectable label, such as enumerated above.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning: a Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And*

Translation (B. D. Hames & S. J. Higgins eds. 1984); *Culture of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *a Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description of the Drawings

Figure 1. Overexpression of PKC1 suppresses the cell lysis defect of a rho1^{ts} mutant. (a) the rho1-5 allele lyses at restrictive temperature. Yeast strains patched on a YPD plate were incubated at 23°C for 3 days, then shifted overnight to 37°C. The patches were assayed in situ for release of alkaline phosphatase as an indication of cell lysis. 1, wild-type; 2, rho1-3; 3, rho1-5; 4, pkc1^{ts} (stt1-1; SYT11-12A). (B) An episomal plasmid (YEp352) with or without PKC1 was transformed into the rho1^{ts} mutants (rho1-3 and rho1-5). Transformants were streaked onto a YPD plate and incubated at 37°C for 3 days.

Figure 2. RHO1 is required for Mpk1 activation in response to heat shock. (Top panel) Phosphorylation of myelin basic protein (MBP) by Mpk1^{HA} immunoprecipitated from extracts of cells shifted from growth at 23°C to 39°C for 30 min. This treatment did not affect the viability of the mutant strains (data not shown). Mpk1 activity in rho1-5 (lanes 4 and 5) and rho1-3 (lanes 6 and 7) relative to wild-type (RHO1; lane 1-3) maintained at 23°C (lane 1) is indicated. (Bottom panel) Immunoblot of immunoprecipitated Mpk1^{HA}.

Figure 3. PKC1 associates with Rho1 *in vivo* and *in vitro*. (a) ^{HA}Rho1 was immunoprecipitated from extracts of cells growing at 23°C (lane 4), or shifted from 23°C to 39°C for 30 min (lane 6). ^{HA}Rho1 immunoprecipitates (left) and whole-cell extracts (100 μg protein; right) were analyzed by immunoblot with anti-PKC1 antibodies (top panels), or with anti-HA (to detect ^{HA}Rho1; bottom panels). Untagged Rho1 was used as a negative control (lanes 1, 2, and 7). Band indicated by * is derived from immunoprecipitating antibodies. (B) Recombinant GST-Rho1 (1 μg), purified from Sf9 insect cells and bound to glutathione agarose beads, was preloaded with the indicated guanine nucleotide (lanes 2-5). Soluble yeast cell extract (400 μg protein) containing PKC1^{HA} was incubated with the beads (lanes 1, 3, and 5), and bound PKC1^{HA} was detected by immunoblot analysis. A control in which naked glutathione agarose beads were used (lane 1) demonstrates dependence of PKC1^{HA} binding on GST-Rho1.

Figure 4. Rho1 allows cofactors to activate PKC1. (a) Phosphorylation of synthetic Bck1 peptide by $PKC1^{HA}$ immunoprecipitated from 50 µg of soluble yeast cell extract protein. Recombinant GST-Rho1 or GST-Cdc42 (1 µg) was preloaded with the indicated guanine nucleotide. Cofactors (80 µg/ml PS, 8 µg/ml DAG, and 100 µM $CaCl_2$) were added to the reaction where indicated. Lanes 1 and 2 are control reactions with no GTPase. Mean and standard error for three experiments is shown. (B) PS alone is sufficient to stimulate PKC1 fully in the presence of Rho1. Phosphorylation of Bck1 peptide by $PKC1^{HA}$ in the presence of GTPγS-bound GST-Rho1 and the indicated cofactors. Conditions were as in a, except for PMA (16 ng/ml). Concentrations of PS as low as 8 µg/ml fully activated PKC1 (data not shown).

Figure 5. Model for the dual role of Rho1 in the maintenance of cell integrity.

Figure 6. GS activity from *rho1* mutants (See reference of Example 3). (a) GS activity is thermolabile in $rho1^{ts}$ mutants. Crude extracts were made from cells growing at room temperature, and assayed for GS activity at the indicated temperatures in the presence of 50 µM GTPγS. (B) Reconstitution of GS activity in *rho1-3* membranes with recombinant Rho1. GS activity in *rho1-3* membrane fractions was measured at 37°C in the presence of 1 µg of the indicated recombinant GTPase and 50 µM GTPγS (19). (C) Reconstituted GS activity requires GTP. GS activity in wild-type membranes or *rho1-3* membranes complemented with 1 µg of GST-Rho1 was measured at 37°C in the presence of the indicated guanine nucleotide (20 µM). Results are expressed as percent activity relative to GTPγS.

Figure 7. GS activity in a constitutively active *RHO1* mutant is GTP independent. Cultures of *rho1-3* cells harboring plasmids with either *RHO1* or *RHO1-Q68H* (Boguski *et al.* (1992) *New Biol.* 4:408) under the control of the inducible *GAL1* promoter were grown at room temperature in medium containing 2% raffinose (repressing conditions). Galactose was added (to 2%) to half of each culture, and cells were cultured for an additional 4h to induce expression of *RHO1*. GS activity in membrane fractions was assayed at 37°C in the presence or absence of GTPγS.

Figure 8. Rho1 and Gsc1/Fks1 are enriched during purification of GS. GS was purified from a wild-type strain (A451; 3). (a) Immunoblot analysis of Rho1 (upper) and Gsc1/Fks1 (lower) through purification (See reference 20 of Example 2). (B) GS specific activity through purification. Purification steps were: lane 1, membrane fraction; lane 2, detergent extract; lane 3, first product entrapment; lane 4, second product entrapment.

Figure 9. (a) Coimmunoprecipitation of Rho1 with Gsc1/Fks1 (21). Partially purified GS was incubated with anti-Gsc1/Fks1 monoclonal antibodies, 1A6 (lane 1) and 1F4 (lane 2), and anti-human endothelin B type receptor (lane 3) (3). Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting. (B) Colocalization of

Gsc1/Fks1 and Rho1 at sites of cell wall remodeling (See reference 22 of Example 2). Indirect immunofluorescence microscopy was used to visualize Gsc1/Fks1 and ^{HA}Rho1 in double-stained cells.

Figure 10. Alignments of the β-subunits of GGPTase-Is showing *cal1/cdc43* mutations. Positions of the *cal1/cdc43* mutations are shown under the box representing the *CAL1/CDC43* coding region. The closed box represents the homologous region among the β-subunits of the protein isoprenyltransferase. Cluster was used to align Cal1p, and the β-subunits of the *S. pombe*, rat and human GGPTase-Is near the *cal1/cdc43* mutation points.

Figure 11. Overproduction of *CDC42* is toxic in *cal1-1* cells. *cal1-1* (1), *cdc43-2* (2), *cdc43-3* (3) *cdc43-4* (4) *cdc43-5* (5), *cdc43-6* (6), *cdc43-7* (7) and wild-type strain (Boguski *et al.* (1992) *supra*) harboring *pGAL-CDC42* were streaked on the plate containing glucose (a) or galactose (B), and incubated at 23°C for 1 week.

Figure 12. Fractionation of Rho1p and Cdc42p in wild-type and mutant strains. Yeast strains were grown to midlog phase at the permissive temperature (23°C), shifted to the restrictive temperature, collected after 2 hr (37°C), and the cell lysates were prepared. Rho1p was detected by Western blotting analysis with guinea pig polyclonal antibody against Rho1p. In order to express HA-tagged version of Cdc42p, yeast strains transformed with pYO920 were incubated at 23°C in 2% galactose-containing medium for 6 hr before the temperature shift. HA-tagged version of Cdc42p was detected by Western blotting analysis with 12CA5. WT, YPH500; *cal1-1*, YOT159-3C; *cdc43-5*, YOT435-1A.

Figure 13. Reduced GS activity in the membrane fractions of GGPTase I-deficient cells. Cultures of wild-type (YPH500), *cal1-1* (YOT159-3C), *cdc43-5* (YOT435-1A) cells were grown at room temperature in YPD medium. GS activity in membrane fractions was assayed at 30°C according to Inoue *et al.* (1995) *Eur. J. Biochem.* 231: 845. Reconstitution of GS activity in *cal1-1* membrane was performed with recombinant mutant Rho1 (G19V) which is constitutively active for its activity.

Figure 14. Thin section electron micrograph of Pkc1-depleted cells demonstrating cell lysis.

Figure 15. Genomic sequences flanking the CaRAM2 gene (the CaRAM2 gene is in lowercase letters).

Figure 16. Genomic sequences flanking the CaRHO1 gene (the CaRHO1 gene is in lowercase letters).

Detailed Description of the Invention

The use of, and need for anti-fungal agents is widespread and ranges from the treatment of mycotic infections in animals; to additives in feed for livestock to promote weight gain; to disinfectant formulations. In general, a salient feature of effective anti-fungal agents is that the agent is cytotoxic to a fungal cell rather than only cytostatic. The mere knowledge that a particular protein is critical to cell growth is accordingly not sufficient to render that protein a suitable target for generation of anti-fungal agents. Rather, assays which are useful for identifying potential anti-fungal agents should target a fungal bioactivity which, when altered in a particular manner, results in cell death rather than quiescence or sporulation. For example, as is illustrated in Figure 14, cell lysis is a preferred outcome to treatment with the potential antifungal agent in order to ensure destruction of the pathogen. Moreover, at least for anti-fungal agents which are to be administered to humans and other animals, the therapeutic index is preferably such that toxicity to the host is several orders of magnitude less than it is for the targeted fungus.

The present invention relates to rapid, reliable and effective assays for screening and identifying pharmaceutically effective compounds that specifically inhibit the biological activity of fungal GTPase proteins, particularly GTPases involved in cell wall integrity, hyphael formation, and other cellular functions critical to pathogenesis.

The cell wall of many fungus, as set out above, is required to maintain cell shape and integrity. The main structural component responsible for the rigidity of the yeast cell wall is 1,3-β-linked glucan polymers with some branches through 1,6-β-linkages. The biochemistry of the yeast enzyme catalyzing the synthesis of 1,3-β-glucan chains has been studied extensively, but little was previously known at the molecular level about the genes encoding subunits of this enzyme. Only a pair of closely related proteins (Gsc1/Fks1 and Gsc2/Fks2) had previously been described as subunits of the 1,3-β-glucan synthase (GS) (Inoue *et al.* (1995) *supra*; and Douglas *et al.* (1994) *PNAS* 91:12907). GS activity in many fungal species, including *S. cerevisiae*, requires GTP or a non-hydrolyzable analog (e.g. GTPγS) as a cofactor, suggesting that a GTP-binding protein stimulates this enzyme (Mol *et al.* (1994) *J. Biol. Chem.* 269:31267).

As described in the appended examples, we demonstrate that the Rho1 GTPase activity is required for glucan synthase activity, copurifies with 1,3-β-glucan synthase, and is found to associate with the Gsc1/Fks1 subunit of this complex *in vivo*. Both proteins were also found to reside predominantly at sites of cell wall remodeling. Therefore, Rho1 is an regulatory subunit of 1,3-β-glucan synthase, and accordingly this interaction, and the resulting enzyme complex, are potential therapeutic targets for development of antifungal agents. Moreover, given the uniqueness of the yeast glucan cell wall relative to mammalian cells, the therapeutic targeting of Rho-like GTPase(s) involvement in glucan synthase

complexes in yeast presents an opportunity to define antifungal agents which are highly selective for yeast cells relative to mammalian cells.

We have also discovered other interactions with Rho1-like GTPase which are consequential to cell integrity in yeast. As described in the appended examples, we find that Rho1 is required for protein kinase C (PKC1) mediated MAPK activation. Moreover, PKC1 co-immunoprecipitates with Rho1 in yeast extracts, and recombinant Rho1 associates with PKC1 *in vitro* in a GTP-dependent manner. Moreover, the data provided herein demonstrates that recombinant Rho1 confers upon PKC1 the ability to be stimulated by phosphatidylserine (PS), indicating that Rho1 controls signal transmission through PKC1. This applications provides the first example of a PKC isoform whose stimulation by cofactors is dependent on a GTPase, and provides the basis for yet other drug screening assays that target the interaction of a PKC and GTPase, or the catalytic activity of the resulting complex. Furthermore, no mammalian PKC activities have been reported to require a G-protein co-factor, suggesting that the fungal Rho/PKC complex represents a specific target for developing antiproliferative agents selective for yeast cells.

Finally, we have demonstrated that prenylation of Rho1 by a geranylgeranyl transferase is a critical step to maintenance of cell wall integrity in yeast. As described in the appended examples, prenylation of Rho1 is required for sufficient glucan synthase activity. Taken together with the results respecting Rho1's participation as a GS subunit, we demonstrate that not only is the prenylatin of Rho1 by GGPTase I critical to cell growth, but inhibition of the prenylation reaction is a potential target for developing a cytotoxic agent for killing various fungi. Moreover, the relatively high divergence between fungal and human GGPTase subunits suggests that selectivity for the fungal GGPTase activity may be obtained to provide antifungal agents having desirable therapeutic indices.

In one embodiment, the subject assay comprises a prenylation reaction system that includes a fungal geranylgeranyl protein transferase (GGPTase), a fungal GTPase protein, or a portion thereof, which serves as a prenylation target substrate, and an activated geranylgeranyl moiety which can be covalent attached to the prenylation substrate by the GGPTase. The level of prenylation of the target substrate brought about by the system is measured in the presence and absence of a candidate agent, and a statistically significant decrease in the level prenylation is indicative of a potential anti-fungal activity for the candidate agent.

As described below, the level of prenylation of the GTPase target protein can be measured by determining the actual concentration of substrate:geranylgeranyl conjugates formed; or inferred by detecting some other quality of the target substrate affected by prenylation, including membrane localization of the target. In certain embodiments, the present assay comprises an *in vivo* prenylation system, such as a cell able to conduct the

target substrate through at least a portion of a geranylgeranyl conjugation pathway. In other embodiments, the present assay comprises an *in vitro* prenylation system in which at least the ability to transfer isoprenoids to the GTPase target protein is constituted. Still other embodiments provide assay format which detect protein-protein interaction between the GGPTase and a target protein, rather than enzymatic activity *per se*.

With respect to the interaction of the fungal GTPase with other cellular components, and the significance of those interactions to cell wall integrity, another aspect of the present invention relates to assays which seek to identify agents which alter protein-protein interactions involving a fungal GTPase and PKC or glucan synthase subunits, or which inhibit the catalytic activity of a protein complex resulting from such interactions. For instance, as described in more detail below, one therapeutic target of interest are glucan synthase complexes which include a Rho1-like GTPase. In another embodiment, the therapeutic target is a protein kinase C complex including a GTPase. The particular assay format selected will reflect the desire to identify compounds which disrupt protein-protein interactions and thereby alter the enzyme complex, or which disrupt the interaction with, and chemical alteration of a given substrate by the enzyme complex. For instance, the interaction with, and chemical alteration of a given substrate by the enzyme complex. For instance, the interaction of Rho1 with the glucan synthase subunit Gce1 can be the screening target in some embodiments, while the synthase activity of the resulting complex can be the screening target in other embodiments. Likewise, screening assays targeting PKC1/Rho1 complex can provide agents which disrupt the formation of the complex, or target the complex's interaction with substrate proteins.

As described herein, inhibitors of a fungal GTPase bioactivity refer generally to those agents which may act anywhere along the prenylation pathway, e.g., from the reaction steps leading up to and including conjugation of an isoprenoid to the GTPase target, to the interaction of the GTPase protein with other cellular proteins, such as glucan synthase subunits and/or PKC. A subset of this class of inhibitors comprises the prenylation inhibitors, which include those agents that act at the level of preventing conjugation of geranylgeranyl moieties to the target GTPase, rather than at the steps of protein-protein interactions involving the prenylated GTPase, e.g., as part of enzymatic complexes. Moreover, as will be clear from the following description, particular embodiments of the present assay can be chosen so as to discriminate between prenylation inhibitors and inhibitors of prenylated-GTPase complexes.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, "recombinant cells" include any cells that have been modified by the introduction of heterologous DNA. Control cells include cells that are substantially

identical to the recombinant cells, but do not express one or more of the proteins encoded by the heterologous DNA, e.g., do not include or express a recombinant Rho1-like GTPase, a recombinant GGPTase, a recombinant glucan synthase and/or a recombinant PKC1.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

As used herein, "heterologous DNA" or "heterologous nucleic acid" include DNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differs from that in which it occurs in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such DNA encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, isolated DNA that encodes a Rho1-like GTPase, a GGPTase, a glucan synthase and/or a PKC1.

"Inactivation", with respect to genes of the host cell, means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved by deletion of the gene, mutation of the promoter so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive (constitutively or inducibly). Inactivation may be partial or total.

"Complementation", with respect to genes of the host cell, means that at least partial function of inactivated gene of the host cell is supplied by an exogenous nucleic acid. For instance, yeast cells can be "mammalianized", and even "humanized", by complementation of Rho1 with mammalian homologs such as RhoA.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to a transcriptional regulatory sequences. Transcription of the reporter gene is controlled by these sequences. The transcriptional regulatory sequences include the promoter and other regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or regulatory sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or regulatory sequences are recognized by effector molecules.

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence, giving rise to a homology in conformation and thus to similar biological activity. The term is not intended to imply a common evolution of the sequences.

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein.

As used here, the terms "geranylgeranyl protein transferase" and "GGPTase" are recognized and refer to the enzyme complexes responsible for the covalent modification of proteins with geranylgeranyl moieties. Particular reference to fungal GGPTases sub-types such as GGPTase-I, or the subunits of a fungal GGPTase, such as cdc43 and RAM2 (unless otherwise evident from the contest) is intended to refer generically to the analogous GGPTase complex and/or subunits in any fungal cell. Accordingly, reference to the subunit cdc43 (also referred to as CAL1 and DPR1) refers to the *S. cerevisiae* subunit as well as homologous proteins in that cell or other fungi which form a GGPTase I enzyme complex.

Likewise, the terms "Rho-like GTPase" and "fungal GTPase" will refer generally to GTPases related structurally to the yeast GTPases Rho1, Rho2, cdc42, and/or Rsr1/Bud1, whether the enzyme is isolated from *S. cerevisiae* or other fungi.

In similar fashion, the term "glucan synthase" refers generically to fungal enzymes involved in synthesis of a β-(1,3)-glucan and comprised of subunits including Gsc1 (also called Fks1) homologs and Rho-like GTPases. As above, reference to a "Gsc1 subunit" refers to the *S. cerevisiae* protein as well as structurally and functionally related homologs from other fungi.

The terms "PKC" and "PKC1" are also used generically to refer to protein kinase C homologs in fungi, and other fungal homologs of the PKC1 protein of *S. cerevisiae*, respectively.

The terms "fungi" and "yeast" are used interchangeably herein and refer to the art recognized group of eukaryotic protists known as fungi. That is, unless clear from the context, "yeast" as used herein can encompass the two basic morphologic forms of yeast and mold and dimorphisms thereof.

The present invention provides a systematic and practical approach for the identification of candidate agents able to inhibit one or more of the cellular functions of fungal GTPase proteins. In a general sense, the assays of the present invention evaluate the ability of a compound to modulate binding between a GTPase protein and another protein, whether the GTPase is acting as a subunit of a multiprotein complex or as a substrate for modification. The assays may be formatted to evaluate the ability of a compound to modulate (*i*) protein complexes which include a GTPase protein; (*ii*) the enzymatic activity

of such multiprotein complexes; or (*iii*) the enzymatic activity which produces a prenylated GTPase.

Exemplary compounds which can be screened for activity against fungal GTPase activity include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries, such as isolated from animals, plants, fungus and/or microbes.

Cell-free Assay Formats

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins or cell-lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements. Accordingly, in an exemplary screening assay of the present invention, a reaction mixture is generated to include a fungal GTPase polypeptide, compound(s) of interest, and a "target polypeptide", e.g., a protein, which interacts with the GTPase polypeptide, whether as a prenylating activity, or by some other protein-protein interaction. Exemplary target polypeptides include GGPTase activities such as GGPTase I, PKC homologs such as PKC1, and glucan synthase subunits such as Gsc1. Detection and quantification of the enzymatic conversion of the fungal GTPase, or the formation of complexes containing the fungal GTPase protein, provide a means for determining a compound's efficacy at inhibiting (or potentiating) complex the bioactivity of the GTPase. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison.

In one embodiment, the subject drug screening assay comprises a prenylation system, e.g. a reaction mixture which enzymatically conjugates isoprenoids to a target protein, which is arranged to detect inhibitors of the prenylation of a Rho-like GTPase with a geranylgeranyl group. For instance, in one embodiment of a cell-free prenylation system, one or more cell lysates including a fungal GGPTase, a fungal Rho-like GTPase (or substrate analog thereof), and an activated geranylgeranyl group are incubated with the test compound and the level of prenylation of the Rho-like GTPase substrate is detected. Lysates can be derived from cells expressing one or more of the relevant proteins, and

mixed appropriately (or spilled) where no single lysate contains all the components necessary for generating the prenylation system. In preferred embodiments, one or more of the components, especially the substrate target, are recombinantly produced in a cell used to generate a lysate, or added by spiking a lysate mixture with a purified or semi-purified preparation of the substrate. These embodiments have several advantages including: the ability to use a labeled substrate, e.g. a dansylated peptide, or fusion protein for facilitating purification e.g. a Rho1-GST fusion protein; the ability to carefully control reaction conditions with respect to concentrations of reactants; and where targets are derived from fungal pathogens, the ability to work in a non-pathogenic system by recombinantly or synthetically producing by components from the pathogen for constituting the prenylation system.

The prenylates can be derived from any number of cell types, ranging from bacterial cells to yeast cells to cells from metazoan organisms including insects and mammalian cells. To illustrate, a fungal prenylation system can be reconstituted by mixing cell lysates derived from insect cells expressing fungal GGPTase subunits cloned into baculoviral expression vectors. For example, the exemplary GGPTase-I expression vectors described below in the section *Reagents* can be recloned into baculoviral vectors (e.g. pVL vectors), and recombinant GGPTase-I produced in transfected *spodoptera fungiperda*-cells. The cells can than be lysed, and if the RAM2 and CDC43 subunits are produced by different sets of cells, cell lysates can be accordingly mixed to produce an active fungal GGPTase. The level of activity can be assessed by enzymatic activity, or by quantitating the level of expression by detecting, e.g., an exogenous tag added to the recombinant protein. Substrate and activated geranylgeranyl diphosphate can be added to the lysate mixtures. As appropriate, the transfected cells can be cells which lack an endogenous GGPTase activity, or the substrate can be chosen to be particularly sensitive to prenylation by the exogenous fungal GGTPase relative to any endogenous activity of the cells.

In other cell-free embodiments of the present assay, the prenylation system comprises a reconstituted protein mixture of at least semi-purified proteins. By semi-purified, it is meant that the proteins utilized in the reconstituted mixture have been previously separated from other cellular proteins. For instance, in contrast to cell lysates, the proteins involved in conjugation of geranylgeranyl moieties to a target protein, together with the target protein, are present in the mixture to at least 50% purity relative to all other proteins in the mixture, and more preferably are present at 90-95% purity. In certain embodiments of the subject method, the reconstituted protein mixture is derived by mixing highly purified proteins such that the reconstituted mixture substantially lacks other proteins which might interfere with or otherwise alter the ability to measure specific prenylation rates of the target GTPase substrate.

Each of the protein components utilized to generate the reconstituted prenylation system are preferably isolated from, or otherwise substantially free of, other proteins normally associated with the proteins in a cell or cell lysate. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") is defined as encompassing individual preparations of each of the component proteins comprising less than 20% (by dry weight) contaminating protein, and preferably comprises less than 5% contaminating protein. Functional forms of each of the component proteins can be prepared as purified preparations by using a cloned gene as described below and known in the art. By "purified", it is meant, when referring to the component protein preparations used to generate the reconstituted protein mixture, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins (particularly other proteins which may substantially mask, diminish, confuse or alter the characteristics of the component proteins either as purified preparations or in their function in the subject reconstituted mixture). The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either protein in its native state (e.g. as a part of a cell), or as part of a cell lysate, or that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins) substances or solutions. The term isolated as used herein also refers to a component protein that is substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

In the subject method, prenylation systems derived from purified proteins may have certain advantages over cell lysate based assays. Unlike the reconstituted protein system, the prenylation activity of a cell-lysate may not be readily controlled. Measuring kinetic parameters is made tedious by the fact that cell lysates may be inconsistent from batch to batch, with potentially significant variation between preparations. *In vitro* evidence indicates that prenyltransferases have the ability to cross-prenylate CAAX-related sequences, so that farnesyl transferase present in a lysate may provide an unwanted kinetic parameter. Moreover, cycling of prenylated proteins by guanine nucleotide dissociation inhibitor (GDI)-like proteins in the lysate could further complicate kinetics of the reaction mixture. Evaluation of a potential inhibitor using a lysate system is also complicated in those circumstances where the lysate is charged with mRNA encoding the GTPase substrate polypeptide or GGPTase activity, as such lysates may continue to synthesize proteins active in the assay during the development period of the assay, and can do so at unpredictable

rates. Knowledge of the concentration of each component of the prenylation system can be required for each lysate batch, along with the overall kinetic data, in order to determine the necessary time course and calculate the sensitivity of experiments performed from one lysate preparation to the next. The use of reconstituted protein mixtures can allow more careful control of the reaction conditions in the prenylation reaction.

The purified protein mixture includes a purified preparation of the substrate polypeptide and a geranylgeranyl isoprenoid (or analog thereof) under conditions which drive the conjugation of the two molecules. For instance, the mixture can include a fungal GGPTase I complex including *RAM2* and *CDC43* subunits, a geranylgeranyl diphosphate, a divalent cation, and a substrate polypeptide, such as may be derived from Rho1.

Furthermore, the reconstituted mixture can also be generated to include at least one auxiliary substrate recognition protein, such as a Rab escort protein where GGPTase II is the prenylase employed in the reaction mixture.

Prenylation of the target regulatory protein via an *in vitro* prenylation system, in the presence and absence of a candidate inhibitor, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In such embodiments, a wide range of detection means can be practiced to score for the presence of the prenylated protein.

In one embodiment of the present assay, the products of a prenylation system are separated by gel electrophoresis, and the level of prenylated substrate polypeptide assessed, using standard electrophoresis protocols, by measuring an increase in molecular weight of the target substrate that corresponds to the addition of one or more geranylgeranyl moieties. For example, one or both of the target substrate and geranylgeranyl group can be labeled with a radioisotope such as ^{35}S, ^{14}C, or ^{3}H, and the isotopically labeled protein bands quantified by autoradiographic techniques. Standardization of the assay samples can be accomplished, for instance, by adding known quantities of labeled proteins which are not themselves subject to prenylation or degradation under the conditions which the assay is performed. Similarly, other means of detecting electrophoretically separated proteins can be employed to quantify the level of prenylation of the target substrate, including immunoblot analysis using antibodies specific for either the target substrate or geranylgeranyl epitopes.

As described below, the antibody can be replaced with another molecule able to bind one of either the target substrate or The isoprenoid. By way of illustration, one embodiment of the present assay comprises the use of a biotinylated target substrate in the conjugating system. Indeed, biotinylated GGPTase substrates have been described in the art (c.f. Yokoyama *et al.* (1995) *Biochemistry* 34:1344-1354). The biotin label is detected in a gel during a subsequent detection step by contacting the electrophoretic products (or a blot thereof) with a streptavidin-conjugated label, such as a streptavidin linked fluorochrome or

enzyme, which can be readily detected by conventional techniques. Moreover, where a reconstituted protein mixture is used (rather than a lysate) as the conjugating system, it may be possible to simply detect the target substrate and geranylgeranyl conjugates in the gel by standard staining protocols, including coomassie blue and silver staining.

In a similar fashion, prenylated and unprenylated substrate can be separated by other chromatographic techniques, and the relative quantities of each determined. For example, HPLC can be used to quantitate prenylated and unprenylated substrate (Pickett *et al.* (1995) *Analytical Biochem* 225:60-63), and the effect of a test compound on that ratio determined.

In another embodiment, an immunoassay or similar binding assay, is used to detect and quantify the level of prenylated target substrate produced in the prenylation system. Many different immunoassay techniques are amenable for such use and can be employed to detect and quantitate the conjugates. For example, the wells of a microtitre plate (or other suitable solid phase) can be coated with an antibody which specifically binds one of either the target substrate or geranylgeranyl groups. After incubation of the prenylation system with and without the candidate agent, the products are contacted with the matrix bound antibody, unbound material removed by washing, and prenylated conjugates of the target substrate specifically detected. To illustrate, if an antibody which binds the target substrate is used to sequester the protein on the matrix, then a detectable anti-geranylgeranyl antibody can be used to score for the presence of prenylated target substrate on the matrix.

Still a variety of other formats exist which are amenable to high through put analysis on microtitre plates or the like. The prenylation substrate can be immobilized throughout the reaction, such as by cross-linking to activated polymer, or sequestered to the well walls after the development of the prenylation reaction. In one illustrative embodiment, a Rho-like GTPase, e.g. a fungal Rho1, Rho2, Cdc42 or Rsr1/Bud1, is cross-linked to the polymeric support of the well, the prenylation system set up in that well, and after completion, the well washed and the amount of geranylgeranyl sidechains attached to the immobilized GTPase detected. In another illustrative embodiment, wells of a microtitre plate are coated with streptavidin and contacted with the developed prenylation system under conditions wherein a biotinylated substrate binds to and is sequestered in the wells. Unbound material is washed from the wells, and the level of prenylated target substrate is detected in each well. There are, as evidenced by this specification, a variety of techniques for detecting the level of prenylation of the immobilized substrate. For example, by the use of dansylated (described *infra*) or radiolabelled geranylgeranyl diphosphaste in the reaction mixture, addition of appropriate scintillant to the wells will permit detection of the label directly in the microtitre wells. Alternatively, the substrate can be released and detected, for example, by any of those means described above, e.g. by radiolabel, gel electrophoresis, etc. Reversibly bound substrate, such as the biotin-conjugated substrate set out above, is

particularly amenable to the latter approach. In other embodiments, only the geranylgeranyl moiety is released for detection. For instance, the thioether linkage of the isoprenoid with the substrate peptide sequence can be cleaved by treatment with methyl iodide. The released geranylgeranyl products can be detected, e.g., by radioactivity, HPLC, or other convenient format.

Other geranylgeranyl derivatives include detectable labels which do not interfere greatly with the conjugation of that group to the target substrate. For example, in an illustrative embodiment, the assay format provides fluorescence assay which relies on a change in fluorescent activity of a group associated with a GGPTase substrate to assess test compounds against a fungal GGPTase. To illustrate, GGPTase-I activity can be measured by a modified version of the continuous fluorescence assay described for farnesyl transferases (Cassidy *et al.*, (1985) *Methods Enzymol.* 250: 30-43; Pickett *et al.* (1995) *Analytical Biochem* 225:60-63; and Stirtan *et al.* (1995) *Arch Biochem Biophys* 321:182-190). In an illustrative embodiment, dansyl-Gly-Cys-Ile-Ile-Leu (d-GCIIL) and the geranylgeranyl diphosphate are added to assay buffer, along with the test agent or control. This mixture is preincubated at 30°C for a few minutes before the reaction is initiated with the addition of GGPTase enzyme. The sample is vigorously mixed, and an aliquot of the reaction mixture immediately transferred to a prewarmed cuvette, and the fluorescence intensity measured for 5 minutes. Useful excitation and emission wavelengths are 340 and 486 nm, respectively, with a bandpass of 5.1 nm for both excitation and emission monochromators. Generally, fluorescence data are collected with a selected time increment, and the inhibitory activity of the test agent is determined by detecting a decrease in the initial velocity of the reaction relative to samples which lack a test agent.

In yet another embodiment, the geranylgeranyl transferase activity against a particular substrate can be detected in the subject assay by using a phosphocellulose paper absorption system (Roskoski *et al.* (1994) *Analytical Biochem* 222:275-280), or the like. To effect binding of a peptidyl substrate to phosphocellulose at low pH, several basic residues can be added, preferably to the amino-terminal side of the CAAX target sequence of the peptide, to produce a peptide with a minimal minimum charge of +2 or +3 at pH less than 2. This follows the strategy used for the phosphocellulose absorption assay for protein kinases. In an illustrative embodiment; the transfer of the [H^3] geranylgeranyl group from [H^3]-geranylgeranyl pyrophosphate to KLKCAIL or other acceptor peptides can be measured under conditions similar to the farnesyl transferase reactions described by Reiss *et al.* (Reiss *et al.*, (1990) *Cell* 62: 81-88) In an illustrative embodiment, reaction mixtures can be generated to contain 50 mM Tris-HCL (pH 7.5), 50μM $ZnCl_2$, 20 mM KCl, 1 mM dithiothreitol, 250 μM KLKCAIL, 0.4 μM [H^3] geranylgeranyl pyrophosphate, and 10-1000 μg/ml of purified fungal GGPTase protein. After incubation, e.g., for 30 minutes at 37 °C, samples are applied to Whatman P81 phosphocellulose paper strips. After the liquid

permeates the paper (a few seconds), the strips are washed in ethanol/phosphoric acid (prepared by mixing equal volumes of 95% ethanol and 75 mM phosphoric acid) to remove unbound isoprenoids. The samples are air dried, and radioactivity can be measured by liquid scintillation spectrometry. Background values are obtained by using reaction mixture with buffer in place of enzyme.

An added feature of this strategy is that it produces hydrophilic peptides that are more readily dissolved in water. Moreover, the procedure outlined above works equally well for protein substrates (most proteins bind to phosphocellulose at acidic pH), so should be useful where full length protein, e.g., Rho1 or Cdc42, are utilized as the GGPTase substrate.

Likewise, a variety of techniques are known in the art for accessing the activity of a glucan synthase and can be adapted for generating drug screening assays designed to detect inhibitors of a fungal glucan synthase complex which includes a Rho-like GTPase. As above, the cell-free glucan synthesis systems can be utilized in the subject assay, and include reconstituted protein mixtures and/or cell lysates/membrane preparations. Accordingly, in preferred embodiments, the glucan synthesis system is derived from purified protein preparations (preferably reconstituted in a lipid formulation) or membrane preparations derived from a reagent cells, e.g., a cell expressing a recombinant RhoI/Gsc1 complex. To illustrate, membrane extracts are prepared from selected cells, homogenized with glass beads, and unbroken cells and debris are removed by centrifugation. The supernatant fluids are centrifuged at high speed, and the resulting pellets are washed with buffer containing 0.05M potassium phosphate (pH 7.5), 0.5 mM DTT, and 1.0 mM PMSF. The washed pellet is resuspended in the same buffer containing 5% glycerol. This protein extract serves as the source for β(1-3)-glucan synthase in the enzymatic assays.

The β(1-3)-glucan synthase reactions can be performed similar to those described in the art (e.g., Cabib *et al.* (1987) *Methods Enzymol.* 138:637-642) and the appended examples. Briefly, a reaction mixture is generated containing Tris (or other suitable buffer), dithiothretol, KF, glycerol, PMSF, UDP-glucose, guanosine 5'-(γ-S)-triphosphate (GTPγS), UDP-[^{3}H]glucose (Amersham) plus a sample of membrane protein extract. Optionally, α-amylase can be added to reaction mixtures to eliminate the contribution of [3H]glucose incorporation into glycogen. The reactions are performed in the presence or absence of the test compound. Following incubation for a selected time, the [^{3}H]-glucose incorporated into trichloroacetic acid-insoluble material is collected onto glass fiber filters and measured using a liquid scintillation counter.

In still other embodiments of the subject assay, cell-free mixtures can be utilized to identify agents which inhibit the enzymatic activity of a fungal PKC/GTPase complex such as the PKC1/Rho1 complex. In an exemplary embodiment, the kinase activity of a

PKC1/GTPase complex can assessed by such methods as described in Watanabe *et al.* (1994) *J Biol Chem* 269:16829-16836. For instance, phosphorylation reactions can be initiated by adding reaction cocktail (40 mM MOPS pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 μM [γ-^{32}P]ATP [6 μCi/reaction], a substrate peptide and the PKC/GTPase complex, and incubated for the reaction to develop. Reactions can be terminated by adding 4X Laemmli's sample buffer, and the samples boiled and subjected to SDS/PAGE. After electrophoresis, gels are fixed in 12.5% trichloroacetic acid for 10 min, washed in 10% methanol/10% acetic acid to reduce background, dried, and subjected to autoradiography. Likewise, capillary zone electrophoresis (CZE) techniques can be used to separate and quantitate phosphorylated and unphosphorylated PKC substrate, especially peptide substrates, following such protocols as described by Dawson *et al.* (1994) *Analytical Biochem* 220: 340-345. Alternatively, reactions can be terminated by spotting onto P81 paper (Whatman). The paper washed three times with 75 mM H_3PO_4 and subjected to scintillation counting.

In another embodiment, the assay is started with the addition of enzyme and stopped after a set time by the addition of 25% trichloroacetic acid (TCA) and 1.0 mg/ml bovine serum albumin (BSA). The radioactive product is retained and washed on glass fiber filters that allow the unreacted ^{32}P-ATP to pass through. As above, the amount of phosphorylation is determined by the radioactivity measured in a scintillation counter.

In still another embodiment, the kinase substrate can be separated by affinity tags. For instance, a biotinylated peptide substrate of the PKC/Rho I complex can be provided in the kinase reaction mixture with [α^{32}P]ATP, the ^{32}P label incorporated into the peptide substrate can be detected by standard scintillation methods. An advantage to the biotin-capture system is that it tends to be more quantitative with respect to peptide sequestration relative to, for example, phosocellulose paper.

The artificial substrate used can be a synthetic peptide resembling the pseudosubstrate site of PKC1p. All known isoforms of PKC possess a sequence within their regulatory domains that is related to PKC phosphorylation sites, except for an alanine in place of the target serine or threonine of a substrate. These sequences, known as pseudosubstrate sites, have been proposed to act as autoinhibitors of PKC activity. Autoinhibition is thought to be relieved upon binding of activating cofactors to the regulatory domain. Peptides resembling pseudosubstrate sites, except with a serine or threonine in place of alanine, are known to be excellent substrates for PKC (House *et al.* (1987) *Science* 238:1726-1728). Therefore, one substrate that may be used to test fungal PKC1 complexes is the 15-amino acid peptide, GGLHRHGTIINRKEE, corresponding to residues 394-408 of PKC1p of *S. cerevisae* (the putative pseudosubstrate site), with a threonine in place of alanine at position 401.

Yet another technique which can be used to follow the kinase activity of a PKC/GTPase complex in the presence of a test agent involves a spectrophotometric assay relying on an ADP produced by the kinase-mediated phosphorylation reaction. Briefly, the formation of ADP in the kinase reaction can be coupled to the pyruvate kinase reaction to produce pyruvate which is, in turn, coupled to the lactate dehydrogenase reaction with the concomitant oxidation of DPNH to DPN+. The decrease in absorbance of 340 nm is used to determine the reaction rate. See, for example, Roskosi (1983) *Methods Enzymol*, 99:3-6

. In addition to the prenylation and other enzymatic reaction-based assays, it is contemplated that any of the novel protein-protein interactions described herein could be directly be the target of a drug screening assay. For example, in one embodiment, the interaction between a GTPase and a catalytic subunit of a fungal glucan synthase, such as Gsc1/Fsk1 homologs, can be detected in the presence and the absence of a test compound. In another embodiment, the ability of a compound to inhibit the binding of a GTPase protein with a fungal PKC-like protein, such as PKC1, can be assessed in the subject assay. A variety of assay formats for detecting non-enzymatic protein interactions will suffice and, in light of the present invention, will be comprehended by a skilled artisan.

Complex formation between the GTPase polypeptide and a "target polypeptide" (e.g., a PKC polypeptide, a GS subunit, or a GGPTase) may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled GTPase polypeptides, by immunoassay, by chromatographic detection, or by detecting the intrinsic activity of either the GTPase or target polypeptide.

Typically, it will be desirable to immobilize either the GTPase or the target polypeptide to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a GTPase polypeptide to the target polypeptide, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/GTPase (GST/GTPase) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with a preparation of a target polypeptide, e.g. a labeled target polypeptide, along with the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and labeled target polypeptide retained on the matrix determined directly, or in the supernatant after the complexes are subsequently dissociated. Alternatively, the

complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of target polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either the GTPase or target polypeptide can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated GTPase molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with GTPase, but which do not interfere with the interaction between the GTPase and target polypeptide, can be derivatized to the wells of the plate, and GTPase trapped in the wells by antibody conjugation. As above, preparations of a target polypeptide and a test compound are incubated in the GTPase-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Other exemplary methods for detecting such complexes, in addition to those described above, include detection of a radiolabel or fluorescent label; immunodetection of complexes using antibodies reactive with the target polypeptide, or which are reactive with GTPase protein and compete with the target polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target polypeptide, e.g., either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the target polypeptide. To illustrate, the target polypeptide can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diamino-benzadine terahydrochloride or 4-chloro-1-napthol. Likewise, a fusion protein comprising the target polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig *et al* (1974) *J Biol Chem* 249:7130). Alternatively, using such substrates as described above, an intrinsic activity of the target polypeptide can be used to facilitate detection.

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the target protein or GTPase protein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison *et al.* (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well

as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein a system (Pharamacia, NJ).

Cell-based Assay Formats

In yet further embodiments, the drug screening assay is derived to include a whole cell expressing a fungal GTPase protein, along with one or more of a GGPTase, a PKC or a glucan synthase catalytic subunit. In preferred embodiments, the reagent cell is a non-pathogenic cell which has been engineered to express one or more of these proteins from recombinant genes cloned from a pathogenic fungus. For example, non-pathogenic fungal cells, such as *S. cerevisae*, can be derived to express a Rho-like GTPase from a fungal pathogen such as *Candida albicans*. Furthermore, the reagent cell can be manipulated, particularly if it is a yeast cell, such that the recombinant gene(s) complement a loss-of-function mutation to the homologous gene in the reagent cell. In an exemplary embodiment, a non-pathogenic yeast cell is engineered to express a Rho-like GTPase, e.g. Rho1, and at least one of the subunits of a GGPTase, e.g. *RAM2* and/or *Cdc43*, derived from a fungal protein. One salient feature to such reagent cells is the ability of the practitioner to work with a non-pathogenic strain rather than the pathogen itself. Another advantage derives from the level of knowledge, and available strains, when working with such reagent cells as *S. cerevisae*.

The ability of a test agent to alter the activity of the GTPase protein can be detected by analysis of the cell or products produced by the cell. For example, agonists and antagonists of the GTPase biological activity can be detected by scoring for alterations in growth or viability of the cell. Other embodiments will permit inference of the level of GTPase activity based on, for example, detecting expression of a reporter, the induction of which is directly or indirectly dependent on the activity of a Rho-like GTPase. General techniques for detecting each are well known, and will vary with respect to the source of the particular reagent cell utilized in any given assay.

For example, quantification of proliferation of cells in the presence and absence of a candidate agent can be measured with a number of techniques well known in the art, including simple measurement of population growth curves. For instance, where the assay involves proliferation in a liquid medium, turbidimetric techniques (i.e. absorbence/ transmittance of light of a given wavelength through the sample) can be utilized. For example, in the instance where the reagent cell is a yeast cell, measurement of absorbence of light at a wavelength between 540 and 600 nm can provide a conveniently fast measure of cell growth. Likewise, ability to form colonies in solid medium (e.g. agar) can be used to readily score for proliferation. In other embodiments, a GTPase substrate protein, such as a histone, can be provided as a fusion protein which permits the substrate to be isolated from

cell lysates and the degree of acetylation detected. Each of these techniques are suitable for high through-put analysis necessary for rapid screening of large numbers of candidate agents.

Additionally, visual inspection of the morphology of the reagent cell can be used to determine whether the biological activity of the targeted GTPase protein has been affected by the added agent. To illustrate, the ability of an agent to create a lytic phenotype which is mediated in some way by a recombinant GTPase protein can be assessed by visual microscopy.

The nature of the effect of test agent on reagent cell can be assessed by measuring levels of expression of specific genes, e.g., by reverse transcription-PCR. Another method of scoring for effect on GTPase activity is by detecting cell-type specific marker expression through immunofluorescent staining. Many such markers are known in the art, and antibodies are readily available.

In yet another embodiment, in order to enhance detection of cell lysis, the target cell can be provided with a cytoplasmic reporter which is readily detectable, either because it has "leaked" outside the cell, or substrate has "leaked" into the cell, by perturbations in the cell wall. Preferred reporters are proteins which can be recombinantly expressed by the target cell, do not interfere with cell wall integrity, and which have an enzymatic activity for which chromogenic or fluorogenic substrates are available. In one example, a fungal cell can be constructed to recombinantly express the β-galactosidase gene from a construct (optionally) including an inducible promoter. At some time prior to contacting the cell with a test agent, expression of the reporter protein is induced. Agents which inhibit prenylation of a Rho-like GTPase in the cell, or the subsequent involvement of a Rho-like GTPase in cell wall integrity, can be detected by an increase in the reporter protein activity in the culture supernatant or from permeation of a substrate in the cell. This, for example, β-galactosidase activity can be scored using such colorimetric substrates as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or fluorescent substrates such as methylumbelliferyl-β-D-galactopyranoside. Permeation of the substrate into the cell, or leakage of the reporter into the culture media, is thus readily detectable.

In yet another embodiment, the alteration of expression of a reporter gene construct provided in the reagent cell provides a means of detecting the effect on GTPase activity. For example, reporter gene constructs derived using the transcriptional regulatory sequences, e.g. the promoters, for genes regulated by signal transduction processes downstream of the target Rho-like GTPase can be used to drive the expression of a detectable marker, such as a luciferase gene or the like. In an illustrative embodiment, the construct is derived using the promoter sequence from a gene expressed in PKC1-dependent heat shock response.

In still another embodiment, the membrane localization resulting from prenylation of the fungal GTPase can be exploited to generate the cell-based assay. For instance, the subject assay can be derived with a reagent cell having: (*i*) a reporter gene construct including a transcriptional regulatory element which can induce expression of the reporter upon interaction of the transcriptional regulatory protein portion of the above fusion protein. For example, a gal4 protein can be fused with a Rho1 polypeptide sequence which includes the CAAX prenylation target. Absent inhibitors of GGPTase activity in the reagent cell, prenylation of the fusion protein will result in partitioning of the fusion protein at the cell surface membrane. This provides a basal level of expression of the reporter gene construct. When contacted with an agent that inhibits prenylation of the fusion protein, partitioning is lost and, with the concomitant increase in nuclear concentration of the protein, expression from the reporter construct is increased.

In a preferred embodiment, the cell is engineered such that inhibition of the GGPTase activity does not result in cell lysis. For example, as described in Ohya *et al.* (1993) *Mol Cell Biol* 4:1017-1025, mutation of the C-terminus of Rho1 and cdc42 can provide proteins which are targets of farsenyl transferase rather than geranylgeranyl transferase. As Ohya et al. describe, such mutants can be used to render the GGPTase I activity dispensable. Accordingly, providing a reporter gene construct and an expression vector for the GGPTase substrate/transcription factor fusion protein in such cells as YOT35953 cells (Ohya et al., *supra)* generates a cell whose viability *vis-à-vis* the GGPTase activity is determined by the reporter construct, if at all, rather than by prenylation of an endogenous Rho-like GTPase by the GGPTase. of course, the reporter gene product can be derived to have no effect on cell viability, providing for example another type of detectable marker (described, *infra*). Such cells can be engineered to express an exogenous GGPTase activity in place of an endogenous activity, or can rely on the endogenous activity. To further illustrate, the Cal1 mutant YOT35953 cell can be further manipulated to express a Cal1 homolog from, e.g., a fungal pathogen or a mammalian cell.

Alternatively, where inhibition of a GGPTase activity causes cell lysis and reporter gene expression, the leakage assay provided above can be utilized to detect expression of the reporter protein. For instance, the reporter gene can encode β-galactosidase, and inhibition of the GGPTases activity scored for by the presence of cells which take up substrate due to loss of cell wall integrity, and convert substrate due to the expression of the reporter gene.

In preferred embodiments, the reporter gene is a gene whose expression causes a phenotypic change which is screenable or selectable. If the change is selectable, the phenotypic change creates a difference in the growth or survival rate between cells which express the reporter gene and those which do not. If the change is screenable, the

phenotype change creates a difference in some detectable characteristic of the cells, by which the cells which express the marker may be distinguished from those which do not.

The marker gene is coupled to GTPase-dependent activity, be it membrane association, or a downstream signaling pathway induced by a GTPase complex, so that expression of the marker gene is dependent on the activity of the GTPase. This coupling may be achieved by operably linking the marker gene to a promoter responsive to the therapeutically targeted event. The term "GTPase-responsive promoter" indicates a promoter which is regulated by some product or activity of the fungal GTPase. By this manner, the activity of a GGPTase can be detected by its effects on prenylation of GTPase and, accordingly, the downstream targets of the prenylated protein. Thus, transcriptional regulatory sequences responsive to signals generated by PKC/GTPase, GS/GTPase and/or other GTPase complexes, or to signals by other proteins in such complexes which are interupted by GTPase binding, can be used to detect function of Rho-like GTPases such as Rho1 and cdc42.

In the case of yeast, suitable positively selectable (beneficial) genes include the following: *URA3, LYS2, HIS3, LEU2, TRP1; ADE1,2,3,4,5,7,8; ARGl, 3, 4, 5, 6, 8; HIS1, 4, 5; ILV1, 2, 5; THR1, 4; TRP2, 3, 4, 5; LEU1, 4; MET2,3,4,8,9,14,16,19; URA1,2,4,5,10; H0M3,6; ASP3; CHO1; ARO 2,7; CYS3; OLE1; IN01,2,4; PR01,3.* Countless other genes are potential selective markers. The above are involved in well-characterized biosynthetic pathways. The imidazoleglycerol phosphate dehydratase (IGP dehydratase) gene (*HIS3*) is preferred because it is both quite sensitive and can be selected over a broad range of expression levels. In the simplest case, the cell is auxotrophic for histidine (requires histidine for growth) in the absence of activation. Activation of the gene leads to synthesis of the enzyme and the cell becomes prototrophic for histidine (does not require histidine). Thus the selection is for growth in the absence of histidine. Since only a few molecules per cell of IGP dehydratase are required for histidine prototrophy, the assay is very sensitive.

The marker gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include beta-galactosidase (Xgal, C_{12}FDG, Salmon-gal, Magenta-Gal (latter two from Biosynth Ag)), alkaline phosphatase, horseradish peroxidase, exo-glucanase (product of yeast exbl gene; nonessential, secreted); luciferase; bacterial green fluorescent protein; (human placental) secreted alkaline phosphatase (SEAP); and chloramphenicol transferase (CAT). Some of the above can be engineered so that they are secreted (although not β-galactosidase). a preferred screenable marker gene is β-galactosidase; yeast cells expressing the enzyme convert the colorless substrate Xgal into a blue pigment.

In another embodiment, the present invention provides a cell-based assay which is based on our finding that the Cal1-1 mutant (see Example 3), e.g., a mutant of the GGPTase subunit cdc43, results in supersensitivity to echinocandin. This observation suggests to us that GGPTase I inhibitors can enhance sensitivity to GS inhibitors, a phenotype which can be easily detected. In an exemplary embodiment, a fungal cell can be contacted with a test agent, and a GS inhibitor such as echinocandin B (other congeners of the echinocandin class of agents, such as cilofungin, certain pneumocandins, and WF11899A, B and C). The amount of cell lysis is determined and compared to the amount of cell lysis is the absence of the GS inhibitor. Synergism, e.g., a statistically significant increase in lysis of the GS inhibitor treated cell relative to the cell contacted only with the test agent, suggests that the test agent is likely to be a cytotoxic agent which targets prenylation of Rho-like GTPases, or the association of prenylated Rho-like GTPases with proteins critical to cell wall integrity. The fungal cell can be a wild-type or recombinant cell, e.g., such as an *S. cerevisiae* cell engineered to express Candida proteins.

It has also been observed in the art that mutations to Gsc1 (Fks1) confer hypersensitivity to the immunosuppressants FK506 and cyclosporin a (Douglas *et al.* (1994) *PNAS* 91:12907). The mechanism of action of such agents is understood to involve inhibition of expression of the Fks2 gene (Mazur *et al.* (1995) *Mol Cell Biol* 15:5671). Similar to the echinocandin-sensitivity assay embodiments provided above, another assay format provides a cell in which Fks2 activity is compromised. Synergism of the Fks2 impairment with a test compound can be used to identify inhibitors of, for example, the glucan synthase subunit Gsc1. For instance, FK506 or cyclosporin a can be used to impair Fks2 activity, as can mutations to calcineurin or to the Fks2 gene.

These observations also suggest that Cal1-1 cells or the like, e.g., impaired for certain GGPTase activities, are suitable for use in assay to detect GS inhibitors, as such cells are more sensitive to the effects of GS inhibitors. The benefits to enhanced sensitivity include speedier development of assay readouts, and the further prejudicing of the assay towards GS inhibitors rather than other targets which may not provide cytotoxicity. The latter can provide the ability to identify potential hits which may not themselves be potent GS inhibitors, but which can be manipulated, e.g., by combinatorial chemistry approaches, to provide potent and specific GS inhibitors.

In yet another embodiment, fungal proteins involved in the various interactions set out as targets above can be used to generate an interaction trap assay (see also, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J Biol Chem* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696), for subsequently detecting agents which disrupt binding of the proteins to one and other.

In particular, the method makes use of chimeric genes which express hybrid proteins. To illustrate, a first hybrid gene comprises the coding sequence for a DNA-binding domain of a transcriptional activator fused in frame to the coding sequence for a "bait" protein, e.g., a fungal Rho1. The second hybrid protein encodes a transcriptional activation domain fused in frame to a gene encoding a "fish" protein which interacts with the Rho1 protein, e.g. a Gsc1 protein. If the bait and fish proteins are able to interact, e.g., form a Rho1/Gsc1 complex, they bring into close proximity the two domains of the transcriptional activator. This proximity is sufficient to cause transcription of a reporter gene which is operably linked to a transcriptional regulatory site responsive to the transcriptional activator, and expression of the reporter gene can be detected and used to score for the interaction of the bait and fish proteins.

In accordance with the present invention, the method includes providing a host cell, preferably a yeast cell, e.g., *Kluyverei lactis, Schizosaccharomyces pombe, Ustilaqo maydis, Saccharomyces cerevisiae, Neurospora crassa, Aspergillus niger, Aspergillus nidulans, Pichia pastoris, Candida tropicalis, and Hansenula polymorpha*, though most preferably *S cerevisiae* or *S. pombe*. The host cell contains a reporter gene having a binding site for the DNA-binding domain of a transcriptional activator, such that the reporter gene expresses a detectable gene product when the gene is transcriptionally activated. Such activation occurs when the activation domain of the transcriptional activator is brought into sufficient proximity to the DNA-binding domain of a transcriptional activator bound to the regulatory element of the reporter gene. The first chimeric gene may be present in a chromosome of the host cell, or as part of an expression vector.

A first chimeric gene is provided which is capable of being expressed in the host cell. The gene encodes a chimeric protein which comprises (i) a DNA-binding domain that recognizes the responsive element on the reporter gene in the host cell, and (ii) bait protein, such as Rho1.

A second chimeric gene is provided which is capable of being expressed in the host cell. In one embodiment, both the first and the second chimeric genes are introduced into the host cell in the form of plasmids. Preferably, however, the first chimeric gene is present in a chromosome of the host cell and the second chimeric gene is introduced into the host cell as part of a plasmid. The second chimeric gene includes a DNA sequence that encodes a second hybrid protein comprising a transcriptional activation domain fused to a fish protein, or a fragment thereof, which is to be tested for interaction with the bait protein. The fish protein can be a subunit of a GGPTase which interacts with Rho1, or a subunit of a glucan synthase which interacts with Rho1, or Pkc1.

Preferably, the DNA-binding domain of the first hybrid protein and the transcriptional activation domain of the second hybrid protein are derived from

transcriptional activators having separable DNA-binding and transcriptional activation domains. For instance, these separate DNA-binding and transcriptional activation domains are known to be found in the yeast GAL4 protein, and are known to be found in the yeast GCN4 and ADR1 proteins. Many other proteins involved in transcription also have separable binding and transcriptional activation domains which make them useful for the present invention, and include, for example, the LexA and VP16 proteins. It will be understood that other (substantially) transcriptionally -inert DNA-binding domains may be used in the subject constructs; such as domains of ACE1, λcI, lac repressor, jun or fos. In another embodiment, the DNA-binding domain and the transcriptional activation domain may be from different proteins. The use of a LexA DNA binding domain provides certain advantages. For example, in yeast, the LexA moiety contains no activation function and has no known effect on transcription of yeast genes. In addition, use of LexA allows control over the sensitivity of the assay to the level of interaction (see, for example, the Brent *et al.* PCT publication WO94/10300).

In preferred embodiments, any enzymatic activity associated with the bait or fish proteins is inactivated, e.g., dominant negative mutants of Rho1 and the like can be used. Where the interacting proteins are of the enzyme-substrate relationship, mutation of one or more catalytic residues of the enzyme can provide a mutant protein which retains the ability to bind the substrate but not catalytically convert it to product.

Continuing with the illustrated example, the Rho1/Gsc1-mediated interaction, if any, between the first second fusion proteins in the host cell, therefore, causes the activation domain to activate transcription of the reporter gene. The method is carried out by introducing the first chimeric gene and the second chimeric gene into the host cell, and subjecting that cell to conditions under which the first hybrid protein and the second hybrid protein are expressed in sufficient quantity for the reporter gene to be activated. The formation of a Rho1/Gsc1 complex results in a detectable signal produced by the expression of the reporter gene. Accordingly, the formation of a complex in the presence of a test compound to the level of Rho1/GSC1 complex in the absence of the test compound can be evaluated by detecting the level of expression of the reporter gene in each case.

In an illustrative embodiment, *Saccharomyces cerevisiae* YPB2 cells are transformed simultaneously with a plasmid encoding a GAL4db-Rho1 fusion and with a plasmid encoding the GAL4ad domain fused to a fungal Gsc1 gene. Moreover, the strain is transformed such that the GAL4-responsive promoter drives expression of a phenotypic marker. For example, the ability to grow in the absence of histidine can depend on the expression of the LacZ gene. When the LacZ gene is placed under the control of a GAL4-responsive promoter, the yeast cell will turn blue in the presence of β-gal if a functional GAL4 activator has been reconstituted through the interaction of Rho1 and Gsc1. Thus, a

convenient readout method is provided. Other reporter constructs will be apparent, and include, for example, reporter genes which produce such detectable signals as selected from the group consisting of an enzymatic signal, a fluorescent signal, a phosphorescent signal and drug resistance.

A similar method modifies the interaction trap system by providing a "relay gene" which is regulated by the transcriptional complex formed by the interacting bait and fish proteins. The gene product of the relay gene, in turn, regulates expression of a reporter gene, the expression of the latter being what is scored in the modified ITS assay. Fundamentally, the relay gene can be seen as a signal inverter.

As set out above, in the standard ITS, interaction of the fish and bait fusion proteins results in expression of a reporter gene. However, where inhibitors of the interaction are sought, a *positive* readout from the reporter gene nevertheless requires detecting inhibition (or lack of expression) of the reporter gene.

In the inverted ITS system, the fish and bait proteins positively regulate expression of the relay gene. The relay gene product is in turn a repressor of expression of the reporter gene. Inhibition of expression of the relay gene product by inhibiting the interaction of the fish and bait proteins results in concomitant relief of the inhibition of the reporter gene, e.g., the reporter gene is expressed. For example, the relay gene can be the repressor gene under control of a promoter sensitive to the Rho1/Gsc1 complex described above. The reporter gene can accordingly be a positive signal, such as providing for growth (e.g., drug selection or auxotrophic relief), and is under the control of a promoter which is constitutively active, but can be suppressed by the repressor protein. In the absence of an agent which inhibits the interaction of the fish and bait protein, the repressor protein is expressed. In turn, that protein represses expression of the reporter gene. However, an agent which disrupts binding of the Rho1 and Gsc1 proteins results in a decrease in repressor expression, and consequently an increase in expression of the reporter gene as repression is relieved. Hence, the signal is inverted.

Returning to the teachings of Ohya *et al.* (1993) *supra*, it is noted that there are only two essential targets of GGPTase in *S. cerevisae*, the Rho-like GTPases Rho1 and cdc42. With such observations in mind, yet another embodiment of the subject assay utilizes a side-by-side comparison of the effect of a test agent on (i) a cell which prenylates a Rho-like GTPase by adding geranylgeranyl moieties, and (ii) a cell which prenylates an equivalent Rho-like GTPase by adding farnesyl moieties. In particular, the assay makes use of the ability to suppress GGPTase I defects in yeast by altering the C-terminal tail of Rho1 and cdc42 to become substrate targets of farnesyl transferase (see Ohya et al., *supra*). According to the present embodiment, the assay is arranged by providing a yeast cell in which the target Rho-like GTPases is prenylated by a GGPTase activity of the cell. Both

the GGPTase and GTPase can be endogenous to the "test" cell, or one or both can be recombinantly expressed in the cell. The level of prenylation of the GTPase is detected, e.g., cell lysis or other means described above. The ability of the test compound to inhibit the addition of geranylgeranyl groups to the GTPase in the first cell is compared against the ability of test compound to inhibit the farnesylation of the GTPase in a control cell. The "control" cell is preferably identical to the test cell, with the exception that the targeted GTPase(s) are mutated at their CAAX sequence to become substrates for FPTases rather than GGPTases. Agents which inhibit prenylation in the test cell but not the control cell are selected as potential antifungal agents. Such differential screens can be exquisitely sensitive to inhibitors of GGPTase I prenylation of Rho-like GTPases. In a preferred embodiment, the test cell is derived from the *S. cerivisae* cell YOT35953 (Ohya et al., *supra*) or the like which is defective in GGPTase subunit cdc43. The cell is then engineered with a cdc43 subunit from a fungal pathogen such as *Candida albicans* to generate the test cell, and additionally with the mutated Rho-like GTPases to generate the control cell.

Differential Screening Formats

In a preferred embodiment, assays can be used to identify compounds that have therapeutic indexes more favorable than such antifungal as, for example, papulacandins or *echinocandins* or the like. For instance, antifungal agents can be identified by the present assays which inhibit proliferation of yeast cells or other lower eukaryotes, but which have a substantially reduced effect on mammalian cells, thereby improving therapeutic index of the drug as an anti-mycotic agent.

In one embodiment, the identification of such compounds is made possible by the use of differential screening assays which detect and compare the ability of the test compound to inhibit an activity associated with a fungal GTPase, relative to its ability to inhibit an analogous activity of a human GTPase. To illustrate, the assay can be designed for side-by-side comparison of the effect of a test compound on the prenylation activity or protein interactions of fungal and human GGPTase and GTPase proteins. Given the apparent diversity of GGPTase proteins, it is probable that the fungal and human GGPTases differ both in substrate specificity and mechanistic action which can be exploited in the subject assay. Running the fungal and human prenylation systems side-by-side permits the detection of agents which have a greater inhibitory effect (e.g. statistically significant) on the prenylation reaction mediated by the fungal GGPTase than the human enzyme.

Accordingly, differential screening assays can be used to exploit the difference in protein interactions and/or catalytic mechanism of mammalian and fungal GGPTases in order to identify agents which display a statistically significant increase in specificity for inhibiting the fungal prenylation reaction relative to the mammalian prenylation reaction.

Thus, lead compounds which act specifically on the prenylation reaction in pathogens, such as fungus involved in mycotic infections, can be developed. By way of illustration, the present assays can be used to screen for agents which may ultimately be useful for inhibiting the growth of at least one fungus implicated in such mycosis as *candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, coccidioidomycosis, conidiosporosis, histoplasmosis, maduromycosis, rhinosporidosis, nocaidiosis, para-actinomycosis, penicilliosis, monoliasis, or sporotrichosis.* For example, if the mycotic infection to which treatment is desired is candidiasis, the present assay can comprise comparing the relative effectiveness of a test compound on inhibiting the prenylation of a mammalian GTPase protein with its effectiveness towards inhibiting the prenylation of a GTPase from a yeast selected from the group consisting of *Candida albicans, Candida stellatoidea, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida pseudotropicalis, Candida quillermondii*, or *Candida rugosa*. Likewise, the present assay can be used to identify anti-fungal agents which may have therapeutic value in the treatment of *aspergillosis* by selectively targeting, relative to human cells, GTPase homologs from yeast such as *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans*, or *Aspergillus terreus*. Where the mycotic infection is mucormycosis, the GTPase system to be screened can be derived from yeast such as *Rhizopus arrhizus, Rhizopus oryzae, Absidia corymbifera, Absidia ramosa*, or *Mucor pusillus*. Sources of other assay reagents for includes the pathogen *Pneumocystis carinii*.

Thus, it is also deemed to be within the scope of this invention that the recombinant GTPase cells of the present assay can be generated so as to comprise heterologous GTPase proteins from metazoan sources such as humans (i.e. cross-species expression). For example, GTPase proteins from humans can be expressed in the reagent cells under conditions wherein the heterologous protein is able to rescue loss-of-function mutations in the host cell. For example, the reagent cell can be a yeast cell in which a human GTPase protein (e.g. exogenously expressed) is to be a counter-screen for identifying agents which selectively inhibit yeast GTPase activities. To illustrate, the YOC706 strain, described by Qadota *et al.* (1994) *Genetics* 91:9317-9321, lacks a functional endogenous Rho1 gene, and can be transfected with an expression plasmid including a human GTPase gene such as RHoA in order to complement the Rho1 loss-of-function. For example, the coding sequence for RHoA can be cloned into a pRS integrative plasmid containing a selectable marker (Sikorski *et al.* (1989) *Genetics* 122:19-27), and resulting construct used to transform the YOC706 strain. The resulting cells should produce a human RHoA protein which is capable of performing at least some of the functions of the yeast Rho1 protein. The GTPase transformed yeast cells can be easier to manipulate than mammalian cells, and can also provide access to certain assay formats, such as turbidity detection, which may not be obtainable with mammalian cells.

Reagents

If yeast cells are used, the yeast may be of any species which are cultivable and, preferably, in which an exogenous Rho1-like protein can be made to engage the appropriate prenylation enzyme and/or participate in protein complexes such as with glucan synthesase subunits or PKC homologs of the host cell. Suitable species include *Kluyverei lactis, Schizosaccharomyces pombe*, and *Ustilaqo maydis; Saccharomyces cerevisiae* is preferred. Other yeast which can be used in practicing the present invention. The term "yeast", as used herein, includes not only yeast in a strictly taxonomic sense, i.e., unicellular organisms, but also yeast-like multicellular fungi or filamentous fungi.

The choice of appropriate host cell can be influenced by the choice of detection signal. For instance, reporter constructs, as described below, can provide a selectable or screenable trait upon transcriptional activation (or inactivation) in response to a signal provided by the GTPase target. Suitable genes and promoters can be dependent on the reagent cell. Likewise, ease of complementation, genetic manipulation, etc., may also affect the choice of reagent cell.

With respect to sources for constituting recombinant proteins of the subject assays, various GGPTases, GTPases, glucan synthase subunits, and PKC homologs have been identified from a variety of fungal species, and in a significant number of instances, have been cloned so that recombinant sources exist.

For example, identification of enzymes involved in the prenylation pathway from different sources have facilitated the cloning of corresponding genes. For instance, genes GGPTase enzymes, PKC homologs and GTPase homologs have been cloned from various fungal organisms, and are generally described in the literature and available on GenBank or other such databases. Complementation of defects in yeast cells such as *S. cereviae* also constitute a standard protocol for isolating genes encoding fungal and mammalian homologs (as appropriate) of such target proteins as GGPTase subunits, Rho-like GTPases, PKC homologs and glucan synthase subunits.

The proteins provided in the subject assay can be derived by purification from a cell in which it is endogenously expressed, or from a recombinant source of the protein. In each instance where a recombinant source of a protein is used in the subject assay, the manipulation of the gene encoding the protein and the subsequent expression of the protein can be carried out by standard molecular biological techniques. Ligating the polynucleotide sequence encoding the recombinant protein into a gene construct, such as an expression vector, and transforming or transfecting into host cells, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in

producing other well-known proteins, including the *S. cerevisae* proteins PKC1, GGPTase, RhoI and the like. Similar procedures, or obvious modifications thereof, can be employed to prepare and purify recombinant proteins of the prenylation system from other sources.

The recombinant protein can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vehicles for production of recombinant proteins include plasmids and other vectors. For instance, suitable vectors for the expression of these proteins include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

In general, it will be desirable that the gene construct be capable of replication in the host cell. It may be a DNA which is integrated into the host genome, and thereafter is replicated as a part of the chromosomal DNA, or it may be DNA which replicates autonomously, as in the case of a plasmid. In the latter case, the vector will include an origin of replication which is functional in the host. In the case of an integrating vector, the vector may include sequences which facilitate integration, e.g., sequences homologous to host sequences, or encoding integrases.

Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are known in the art, and are described in, for example, Powels *et al.* (*Cloning Vectors: a Laboratory Manual*, Elsevier, New York, 1985). Mammalian expression vectors may comprise non-transcribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a poly-adenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as

well as general recombinant procedures, see *Molecular Cloning: a Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17, as well as the pRS vectors, e.g., pRS303, pRS304, pRS305, pRS306, etc., are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) In: *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

Moreover, when yeast are used as the reagent cell, it will be understood that the expression of a gene in a yeast cell requires a promoter which is functional in yeast. Suitable promoters include the promoters for gal1, metallothionein, 3-phosphoglycerate kinase (Hitzeman *et al.*(1980) *J. Biol. Chem.* 255:2073 or other glycolytic enzymes (Hess *et al.* (1968) *J. Adv. Enzyme Req.* 7:149; and Holland *et al.* (1978) *Biochemistry* 17:4900), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexòkinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman *et al.*, EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the afore-mentioned metallothionein and glyceraldehyde -3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. Finally, promoters that are active in only one of the two haploid mating types may be appropriate in certain circumstances. Among these haploid-specific promoters, the pheromone promoters MFal and MFα1 are of particular interest.

In some instances, it may be desirable to derive the host cell using insect cells. In such embodiments, recombinant polypeptides can be expressed by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

Furthermore, the recombinant protein can be encoded by a fusion gene created to have additional sequences coding for a polypeptide portion of a fusion protein which would facilitate its purification. For instance, a fusion gene coding for a purification leader

sequence comprising a poly-(His)/enterokinase cleavage site sequence can be engineered at the a terminus of the protein, thereby enabling purification of the expressed fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli *et al.* (1987) *J. Chromatography* 411:177; and Janknecht *et al. PNAS* 88:8972).

Exemplary construction of the expression plasmid for recombinant GGPTase-I.

Polymerase chain reaction (PCR) can be carried out to isolate the *CDC43* coding sequence from *S cerevisiae*. Using a sense strand primer (5'-CCATCGATCATATG-TGTCAAGCTAGGAAT-3') can introduce a unique ClaI restriction site upstream of the *CDC43* start codon and an NdeI site that overlaps the ATG initiation codon. An antisense strand PCR primer (5'-GCGGGTACCCTGCAGTCAAAAACAGCACCTTTT-3') introduces unique PstI and KpnI restriction sites downstream of the *CDC43* stop codon. The PCR product is ligated into a convenient vector, such as bluescript II SK-(+) using ClaI and KpnI. An XbaI-ClaI fragment containing *RAM2* (Mayer *et al.*, (1993) *Gene* 132:41-47) can be cloned into the *CDC43* containing vector, upstream of the *CDC43* sequence, to produce a bicistronic construct. The *RAM2* and *CDC43* orfs are then coupled by deletion mutagenesis with the antisense strand primer (5'-GGTAGCTTGAVACATCAAAACTCCTCCTG-CAGATTTATTTTG-3'), which overlaps the *RAM2* translation termination codon with the *CDC43* initiation codon. The *RAM2-CDC43* cassette can then be cloned into an appropriate expression vector and used to transform *E coli*.

Recombinant GGPTase-I can be purified from the resulting cultures as described for recombinant yeast FPTase (Mayer *et al.*, *supra*), with minor modifications (Stirtan *et al.* (1995) *Arch Biochem Biophys* 321:182-190). Wet cell paste is resuspended in 16 ml of lysis buffer (50 mM Tris-HCl, pH 7.0, 10 mM BME, 10 mM $MgCl_2$, 50 μM $ZnCl_2$, 1 mM PMSF) and disrupted by sonication. The cell-free homogenate is clarified by centrifugation and chromatographed on DE52 ion-exchange resin (1.5 x 14 cm) at 4°C, preequilibrated with low-salt buffer (50 mM Tris-Hcl, pH 7.0, 10 mM $MgCl_2$, 50 μM $ZnCl_2$, 10 mM BME). Protein is eluted with a stepwise gradient of 0 to 800 mM NaCl in low-salt buffer. Recombinant PGGPTase-I is expected to elute at 200 mM NaCl. The DE52-purified material is dialyzed at 4°C against low-salt buffer, diluted to ~1mg/ml with the same buffer, and loaded onto an anti-α-tubulin immunoaffinity column (Mayer *et al.*, *supra*) preequilibrated with binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM BME, 50 mM NaCl). The column is washed with binding buffer (~25 ml) and then eluted with binding buffer containing 5 mM Asp-Phe. Fractions containing GGPTase-I

activity are combined. Recombinant GGPTase-I has been demonstrated to be stable for several months at -80°C and for several days at 0°C.

Preparation of dansyl-Gly-Cys-Ile-Ile-Leu.

Dansyl-Gly-Cys-Ile-Ile-Leu is prepared essentially as described previously for dansyl-Gly-Cys-Val-Ile-Ala (Cassidy *et al.*, (1985) *Methods Enzymol.* 250: 30-43), the farnesylated substrate corresponding to Cys-Val-Ile-Ala. Dansyl-Gly-Cys-Ile-Ile-Leu can be purified by preparative HPLC on a Vydac protein and peptide C18 reversed-phase column (22 mm x 25 cm) by elution with a gradient of 85-92% CH_3CN/0.1% TFA in H_2O/0.1% TFA over 20 min, followed by a gradient of 92-100% CH_3CN/0.1% TFA over 5 min, and finally with 100% CH_3CN/0.1% TFA for 10 min. Organic materials are removed by rotary evaporation, and the resulting aqueous suspension is lyophilized to afford dansyl-Gly-Cys-Ile-Ile-Leu

Pharmaceutical Preparations of Identified Agents

After identifying certain test compounds as potential antifungal agents, the practioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

The subject compounds selected in the subject, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical

formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the compound can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

Novel Fungal Pathogen Genes

Another aspect of the present invention relates to the discovery and isolation of genes encoding novel regulatory proteins from the human fungal pathogen *Candida*, namely the αsubunit of a GGPTase I enzyme and two Rho-like GTPases. The present invention specifically contemplates the use of the subject *Candida* proteins in drug screening assays which detect agents that disrupt the activity of one or more of the subject regulatory proteins, such as by disruption of binding to other cellular proteins or, as applicable, by inhibition of activity of the protein. Exemplary drug screening assays are described above.

Another benefit provided by the present invention derives from the use of the subject proteins, antibodies and nucleic acids as reagents for diagnositic assays. Conventional diagnosis, as indicated above, often involves time-consuming steps for determining the presence of infection. Such delays can be unacceptable where prompt treatment must be accorded in order to provide positive prognosis. The subject diagnostic assays, particularly PCR-based procedures, can provide diagnostically relevant information in shorter time periods.

In particular, we have isolated *Candida* genes which encode an apparent α subunit ("*CaRAM2*") of a *Candida* FPTase and/or GGPTase I complex, and two Rho-like GTPase homologs ("*CaRho1*" and "*CaCdc42*"). With respect to the *CaRho1* gene, while sharing some degree of homology with genes of other eukaryotes, the *CaRho1* gene product is less than about 80 percent identical overall with the *S. pombe* and *S. cerevisiae* Rho1 proteins (GenBank dpeosits D38180 and M15189, respectively), and typically less than 50 percent identical with other known GTPase gene products. Likewise, the *CaCdc42* gene encodes a GTPase less than about 90 percent identical with any GTPase of *S. pombe* and *S. cerevisiae*. The *CaRAM2* gene encodes a protein sharing less than 50% identity with any

other known protein. For convenience, a guide to the relevant Sequence Listing entries is set forth below with the nucleic acid and amino acid sequences for the each of the subject regulatory genes.

Sequence Listing Guide

clone	nucleic acid sequence	amino acid sequence
CaRho1	SEQ ID No. 1	SEQ ID No. 2
CaRAM2	SEQ ID No. 3	SEQ ID No. 4
CaCdc42	SEQ ID No. 5	SEQ ID No. 6

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, which may optionally include intron sequences which are either derived from a chromosomal DNA. Exemplary recombinant genes encoding the subject regulatory proteins are represented in SEQ ID Nos: 1, 3 or 5.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which

they are operably linked. In preferred embodiments, transcription of a recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the protein.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding one of the subject polypeptides with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of the first polypeptide. a chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, isolated nucleic acids encoding the subject polypeptides preferably include no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks that gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or polypeptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As described below, one aspect of this invention pertains to an isolated nucleic acid comprising the nucleotide sequence encoding one of the subject *Candida* proteins, biologically active fragments thereof, and/or equivalents of such nucleic acids. The term "nucleic acid" as used herein is intended to include such fragments and equivalents. Moreover, the term "nucleic acid encoding a *CaRho1 GTPase*" is understood to include nucleotide sequences encoding homologous proteins functionally equivalent to the *C. albicans CaRho1* protein set forth in SEQ ID No. 2, or functionally equivalent polypeptides which, for example, retain a GTPase activity, and which may additionally retain other activities of a *CaRho1* protein, e.g., the ability to bind to a PKC or a glucan synthase subunit. Likewise, the term "nucleic acid encoding a *CaRho1 GTPase*" is understood to include nucleotide sequences encoding homologous proteins functionally equivalent to the *C. albicans CaCdc42* protein set forth in SEQ ID No. 6, or functionally equivalent

polypeptides which, for example, retain a GTPase activity, and which may additionally retain other activities of a *CaCdc42* protein. The term "nucleic acid encoding a *CaRAM2* protein" is understood to include nucleotide sequences encoding homologous proteins functionally equivalent to the *C. albicans CaRAM2* protein of SEQ. ID NO. 4, or functionally equivalent polypeptides which, for example, retain the ability to form a functional GGPTase and/or FTase enzyme, e.g., which can preylate a polypeptide/peptide substrate such as *CaRho1*. In certain embodiments, the present invention contemplates that the subject nucleic acid will encode a *CaRho1*, *CaCdc42* or *CaRAM2* protein from another species of *Candida*, e.g., *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. pseudotropicalis*, *C. quillermondii*, *C. glabrata*, *C. lusianiae*, or *C. rugosa*, or will hydribize to a *CaRho1*, *CaCdc42* or *CaRAM2* gene thereof.

Moreover, it will be understood that such equivalent polypeptides as described above may mimic (agonize) the actions of the authentic form of one of the subject regulatory proteins. However, it is expressly provided that such equivalents include polypeptides which function to antagonize the normal function of the wild-type protein. For instance, dominant negative mutants of the subject proteins may competitively inhibit the enzymatic function of an authentic form of the protein or a complex thereof by binding to substrate without catalytically acting upon it. Mutants of either of the subject proteins which produce non-productive complexes with other regulatory proteins can likewise be antagonistic homologs. Accordingly, the term "biological activity", with respect to homologs of the proteins enumerated in the Sequence Listing, refers to both agonism and antagonism of the ordinary function of the wild-type form of that protein.

Thus, equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as intragenus variants; and will also include sequences that differ from the nucleotide sequence encoding the portion of the a protein represented in one of SEQ ID Nos. 1, 3 or 5 due to the degeneracy of the genetic code. Equivalent nucleic acids will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to a nucleotide sequence of a *Candida* gene represented in one of SEQ ID Nos. 1, 3 or 5.

Preferred nucleic acids encode polypeptides comprising an amino acid sequence which is at least 70% homologous, more preferably 80% homologous and most preferably 85% homologous with an amino acid sequence shown in one of SEQ ID Nos. 2, 4 or 6. Nucleic acids encoding polypeptides, particularly polypeptides retaining an activity of one of the subject regulatory proteins, and comprising an amino acid sequence which is at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99%

homologous with a sequence shown in one of SEQ ID Nos. 2, 4 or 6 are also within the scope of the invention.

In yet a further embodiment, the recombinant regulatory genes may further include, in addition to the nucleic acid sequences shown in SEQ ID Nos. 1, 3 or 5, additional nucleotide sequences. For instance, the recombinant gene can include nucleotide sequences of a PCR fragment generated by amplifying the gene from a genomic DNA library, e.g., 5' and 3' non-coding sequences of either of the subject genes.

Another aspect of the invention provides nucleic acid that hybridizes under high or low stringency conditions to nucleic acid which encodes a polypeptide identical or homologous with an amino acid sequence represented in one of SEQ ID Nos. 2, 4 or 6. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Isolated nucleic acids encoding a *Candida* regulatory protein of the present invention, yet which differ from the nucleotide sequences shown in SEQ ID Nos. 1, 3 or 5 due to degeneracy in the genetic code, are also within the scope of the invention. Such nucleic acids are understood to be capable of encoding functionally equivalent polypeptides (i.e., a polypeptide having at least a portion of the biological activity of a protein encoded by the enumerated sequences). For instance, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the protein will exist even within the same species. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-4% of the nucleotides) of a gene encoding a protein may exist among individual cells of a given species, e.g., amongst a population of *C. albicans* cells, due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

Fragments of the nucleic acid encoding portions of the subject *Candida* proteins, such as a catalytic domain of the *CaRho1* or *CaCdc42* GTPases, are also within the scope of the invention. As used herein, such fragments refer to nucleotide sequences having fewer nucleotides than the coding sequence of the gene, yet still include enough of the coding

sequence so as to encode a polypeptide with at least some of the activity of the full-length protein activity.

Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of the recombinant polypeptides.

As indicated by the examples set out below, a nucleic acid encoding one of the subject proteins may be obtained from mRNA present in the cells of a pathogen from the genus *Candida*. It will also be possible to obtain nucleic acids encoding the subject proteins from genomic DNA obtained from such cells. For example, a gene encoding one of the pathogen regulatory proteins can be cloned from either a cDNA or a genomic library from other *Candida* species in accordance with protocols described herein, as well as those generally known in the art. For instance, a cDNA encoding a *CaRAM2* of *CaRho1* protein can be obtained by isolating total mRNA from a culture of *Candida* cells, generating double stranded cDNAs from the total mRNA, cloning the cDNA into a suitable plasmid or bacteriophage vector, and isolating clones expressing the subject protein using any one of a number of known techniques, e.g., oligonucleotide probes, western blot analysis, or complementation. Genes encoding related proteins can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA.

Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridizes (e.g. binds) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one of the subject regulatory proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes one of the regulatory proteins. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell, causes inhibition of expression by hybridizing with the complementary mRNA and/or genomic sequences. In any event, it will be generally desirable to choose an antisense molecule which uniquely

hybridizes to the *Candida* gene, e.g. does not hybridize under physiological conditions to DNA or RNA from a mammalian cell, especially a human cell. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775, as well as the peptide nucleic acids known in the art). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by van der Krol *et al.* (1988) *Biotechniques* 6:958-976; and Stein *et al.* (1988) *Cancer Res* 48:2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of modes of administration, including systemic and topical or other localized administration. Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA.

Moreover, the nucleotide sequence determined from the cloning of the subject regulatory proteins will permit the generation of probes designed for use in identifying the presence of a *Candida* infection, such as an infection involving *C.albicans* or other fungicemia. In particular, because of the significant difference in sequence between the subject *Candida* nucleic acids and apparent orthologs of other eukaryotes, even other single cell eukaryotes, the probe/primer of the present invention will permit diagnostic assays which can rapidly distinguish *Candida* infection from other causative agents of , e.g., fungicemia. For instance, the present invention provides a probe/primer comprising a substantially purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 10, more preferably 25, 50, or 100 consecutive nucleotides of sense or anti-sense sequence of one of SEQ ID Nos: 1, 3 or 5, or naturally occurring mutants thereof. In preferred embodiments, the probe/primer further comprises a label group attached thereto and able to be detected, e.g. the label group is selected from the group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. Such probes can be used as a part of a diagnostic test kit for identifying and phenotyping particular mycotic infections, such as in a sample of cells from a patient, or in a foodstuff, or on equipment.

This invention also provides expression vectors which include a nucleotide sequence encoding one of the subject polypeptides and operably linked to at least one regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence.

Regulatory sequences are art-recognized. Accordingly, the term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences-sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding the *Candida* proteins of this invention. Such useful expression control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

This invention also pertains to a host cell transfected with a recombinant gene in order that it may express a recombinant protein of the present invention. The host cell may be any prokaryotic or eukaryotic cell. For example, a *Candida* protein of the present invention may be expressed in bacterial cells, such as *E. coli*, insect cells, yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art. Exemplary cells genetically engineered to produce a recombinant protein of the present invention are the *Kluyverei lactis, Schizosaccharomyces pombe, Ustilaqo maydis, Saccharomyces cerevisiae, Neurospora crassa, Aspergillus niger, Aspergillus nidulans, Pichia pastoris, Candida tropicalis, and Hansenula polymorpha*, though most preferably *S cerevisiae* or *S. pombe*.

Another aspect of the present invention concerns recombinant forms of the subject *Candida* proteins. The term "recombinant protein" refers to a protein of the present invention which is produced by recombinant DNA techniques, wherein generally DNA encoding the protein is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene encoding one of the subject proteins, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of the native (or "authentic") form of the pathogen protein, or an amino acid

sequence similar thereto, which is generated by mutation so as to include substitutions and/or deletions relative to a naturally occurring form of the protein. To illustrate, recombinant proteins preferred by the present invention, in addition to those having an amino acid sequence of the native proteins, are those recombinant proteins having amino acid sequences which are at least 70% homologous, more preferably 80% homologous and most preferably 90% homologous with an amino acid sequence shown in one of SEQ ID Nos: 2, 4 or 6. A polypeptide which having an amino acid sequence that is at least about 95%, more preferably at least about 98%, and most preferably identical to one of the sequences shown in SEQ ID Nos: 2, 4 or 6 are also within the scope of the invention. Thus, the present invention pertains to recombinant proteins which are derived from *Candida* genes and which have amino acid sequences evolutionarily related to a protein represented by any one of SEQ ID Nos: 2, 4 or 6, wherein "evolutionarily related to" refers to polypeptides having amino acid sequences which have arisen naturally (e.g. by allelic variance), as well as mutational variants of the regulatory proteins which are derived, for example, by combinatorial mutagenesis.

The present invention further pertains to methods of producing the subject polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding one of the subject *Candida* proteins can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be secreted and isolated from a mixture of cells and medium containing the recombinant protein, e.g., by including a secretion signal sequence fused in frame to *Candida* protein. Alternatively, the polypeptide may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A "cell culture" includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and/or immunoaffinity purification. In a preferred embodiment, the protein is a fusion protein containing a domain which facilitates its purification, such as a GST or poly-histidine fusion protein.

Thus, a nucleotide sequence derived from the cloning of one of the subject proteins, encoding all or a selected portion of the protein, can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known intracellular proteins. Similar procedures, or modifications thereof, can be employed to prepare recombinant forms of the subject proteins, or portions thereof, by microbial means or tissue-

culture technology in accord with the subject invention. Exemplary expression vectors are described above.

The coding sequences for the subject polypeptides can be incorporated as a part of fusion genes so as to be covalently linked in-frame with a second nucleotide sequence encoding a different polypeptide. This type of expression system can be useful, for instance, where it is desirable to produce an immunogenic fragment of the protein. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of the *CaRAM2*, *CaCdc42* or *CaRho1* polypeptides, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of the protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of the protein as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of a subject protein and the poliovirus capsid protein can be created to enhance immunogenicity of the set of polypeptide antigens (see, for example, EP Publication No. 0259149; and Evans *et al.* (1989) *Nature* 339:385; Huang *et al.* (1988) *J. Virol.* 62:3855; and Schlienger *et al.* (1992) *J. Virol.* 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can also be utilized, wherein a desired portion of a one of the subject proteins is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett *et al.* (1988) *JBC* 263:1719 and Nardelli *et al.* (1992) *J. Immunol.* 148:914). Antigenic determinants of the subject proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins. For example, recombinant forms of each of the subject pathogen proteins can be generated as glutathione-S-transferase (GST) fusion proteins. Such GST fusion proteins can be used to simplify purification of the protein, such as through the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, Ausabel *et al.*, Eds. John Wiley & Sons, N.Y., 1991). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can facilitate purification of the fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli *et al.* (1987) *J. Chromatography* 411:177; and Janknecht *et al. PNAS* 88:8972).

In still other embodiments, the heterologous polypeptide sequence(s) can have some other activity, such as in forming a catalytically active complex (e.g. a cdc43/RAM2 fusion protein), or a transcriptional activation complex (e.g. for use in an ITS), etc. For instance, a *CaRAM2* fusion protein can be generated with, e.g., a *RAM1* polypeptide sequence to form an FPTase or a cdc43 polypeptide to form a GGPTase. The source of the cdc43 or *RAM1* polypeptide can be, for instance, another *Candida* gene, or a yeast gene such as *S. pombe* or *S. cerevisiae* genes, or that of a higher eukaryote.

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausabel *et al.* John Wiley & Sons: 1992).

The present invention also makes available purified, or otherwise isolated forms of the subject fungal proteins, which are isolated from, or otherwise substantially free of, other intracellular proteins which may be normally associated. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") is defined as encompassing, for example, protein preparations comprising less than 20% (by dry weight) contaminating protein, and preferably comprises less than 5% contaminating protein. Purified forms of the subject polypeptides can be prepared as purified preparations, for example, by using the cloned genes as described herein. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions. The isolated protein can include, for example, nucleosides, metalias, or other non-protein co-factors required for biological activity. In certain embodiments it will be desirable to include a divalent cation (Zn^{+2} or Mg^{+2}) with the *CaRAM2* protein.

Another aspect of the present invention pertains to isolated/purified complexes of proteins including the subject *Candida* proteins. As set out in more detail herein, each of the *CaRho1*, *CaCdc42* and *CaRAM2* proteins are understood to participate in oligomeric complexes. For instance, the present invention contemplates purified protein complexes including a *CaRho1* or *Cdc42* polypeptide and one or more of *(i)* a glucan synthase subunit(s), *(ii)* PKC, and/or *(iii)* a GGPTase subunit(s). Other exemplary complexes include a *CaRAM2* polypeptide and/or *(i)* a cdc43 protein, *(ii)* a RAM1 protein, and/or *(iii)* a Rho-like GTPase such as *CaRho1* or *CaCdc42*. In preferred embodiments, the isolated complex has a molecular weight of less 2000Kd, more preferably less than 100Kd. As above, by "purified" or "isolated" protein complex, the present application intends to include, e.g., complexes of teh subject *Candida* proteins which substantially lack contaminating proteins, e.i., which do not specifically bind to the protein complex.

Another aspect of the invention related to polypeptides derived from the full-length forms of the subject proteins. Isolated peptidyl portions can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, *CaRho1* can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of, for example, GGPTase I prenylation (see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/ 02502). An exemplary technique for refining binding domains in protein fragments is described by Román et al. (1994) *Eur J Biochem* 222:65-73 (attached as Exhibit D). Román et al. describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins ranging is size from 16-28kd; e.g., the technique of Román et al. were applied to identify binding domains in proteins of the same approximate size range as the subject *Candida* proteins.

Moreover, there are several forms of mutagenesis generally applicable, in addition to a general combinatorial mutagenesis approach. For example, homologs of the subject proteins (both agonist and antagonist forms) can be generated and screened using, for example, alanine scanning mutagenesis and the like (Ruf *et al.* (1994) *Biochemistry* 33:1565-1572; Wang *et al.* (1994) *J Biol Chem* 269:3095-3099; Balint *et al.* (1993) *Gene* 137:109-118; Grodberg *et al.* (1993) *Eur J Biochem* 218:597-601; Nagashima *et al.* (1993) *J Biol Chem* 268:2888-2892; Lowman *et al.* (1991) *Biochemistry* 30:10832-10838; and Cunningham *et al.* (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin *et al.* (1993) *Virology* 193:653-660; Brown *et al.* (1992) *Mol Cell Biol* 12:2644-2652; McKnight *et al.* (1982) *Science* 232:316); or by saturation mutagenesis (Meyers *et al.*

(1986) *Science* 232:613). Such techniques will be generally understood to provides for reduction of the subject regulatory proteins to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a naturally-occurring form of a protein of the present invention with other cell-cycle regulatory proteins of the pathogen from which it was derived.

Thus, such mutagenic techniques as described above are particularly useful to map the determinants of the subject proteins which participate in protein-protein interactions. To illustrate, the critical residues of a *CaRho1* or *CaCdc42* protein which are involved in molecular recognition as a substrate for GGPTase prenylation, can be determined and used to generate peptidomimetics which competitively inhibit binding of the native GTPase with the GGPTase enzyme (see, for example, "Peptide inhibitors of human papillomavirus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A). By employing, for example, scanning mutagenesis to map the amino acid residues of one of the subject GTPases involved in binding as a substrate to GGPTase I, peptidomimetic compounds (e.g. diazepine or isoquinoline derivatives) can be generated which mimic those residues, and which therefore can inhibit binding of authentic GTPase to GGPTase I. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted γ-lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71). In similar fashion, mimetics can be designed which bind to any of the other subject regulatory proteins, or mimic their binding to other proteins.

Another aspect of the invention pertains to antibodies and antibody preparations specifically reactive with at least one of the subject proteins. For example, by using peptides based on the cDNA sequence of one of the proteins represented in SEQ ID Nos. 2, 4 or 6, anti-protein/anti-peptide antisera or monoclonal antibodies can be made using standard methods. A mammal such as a mouse, a hamster or rabbit, can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic form of the protein can be administered in the presence of adjuvant. The

progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In other emobodiments, the antibodies are isolated from synthetic antibody libraries, such as antibody phage display libraries. The antibody can be a light chain, a heavy chain, a heavy chain-light chain pair, a single chain antibody, or CDR-containing fragments thereof.

In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of one of the pathogen-derived proteins of the present invention, e.g. antigenic determinants of a protein represented by one of SEQ ID Nos. 2, 4 or 6 or a closely related homolog (e.g. 90 percent homologous, more preferably at least 95 percent homologous). In yet a further preferred embodiment of the present invention, antibodies do not substantially cross react (i.e. do not react specifically) with a protein which is: e.g. less than 90 percent homologous, more preferably less than 95 percent homologous, and most preferably less than 98-99 percent homologous with one of SEQ ID Nos. 2, 4 or 6. By "not substantially cross react", it is meant that the antibody has a binding affinity for a nonhomologous protein, particularly orthologous proteins from mammalian cells, which is at least one order of magnitude, more preferably at least two orders of magnitude, and even more preferably at least three orders of magnitude less than the binding affinity of that antibody for one of the proteins of SEQ ID Nos. 2, 4 or 6.

As set out above, the present invention contemplates the use of the subject *Candida* proteins in assays for identifying anti-fungal and anti-parasitic agents, e.g. agents which act to inhibit proliferation of a pathogen by altering the activity of one or more of the subject pathogen proteins. To illustrate, inhibitors of *CaRAM2*, *CaCdc42* and/or *CaRho1*, e.g., with respect to their involvement in cell wall biosynthesis, can be used in the treatment of candidiasis - an opportunistic infection that commonly occurs in debilitated and immunosuppressed patients. Such agents could be used to treat these infections in patients with leukemias and lymphomas, in people who are receiving immunosuppressive therapy, and in patients with such predisposing factors as diabetes mellitus or AIDS, where fungal infections are a particular problem. These inhibitors can be generated for treatment of mycotic infections caused by, for example, *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida glabrata*, *Candida lusianiae*, or *Candida rugosa*. Anti-proliferative agents developed with the subject assays can also be used, for example, as preservatives in foodstuff, as a feed supplement for promoting weight gain in livestock, or in disinfectant formulations for treatment of non-living matter, e.g., for decominating hospital equipment and rooms. Furthermore, as a result of the considerable divergence between GGPTase proteins, it is likely that differential screening assays, e.g. side-by-side comparison

of inhibition of human *RAM2* relative to *Candida CaRAM2* protein, can be used to identify agents that exhibit specific inhibitory effects directed at the *Candida* GGPTase, without substantially inhibiting the corresponding enzyme in human or other animal cells. Thus, by making available purified and recombinant proteins, the present invention facilitates the development of assays which can be used to screen for drugs which are either agonists or antagonists of the normal cellular function of the subject regulatory proteins. An inhibitor, as identified in the subject assays, is an agent which is able to cause a statistically significant decrease in one or more proliferative activities of a regulatory protein of the present invention.

Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1

Activation of yeast protein kinase C by Rho1 GTPase

The abbreviations used in Example 1 are: PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MEK, MAPK-activating kinase; MEKK, MEK-activating kinase; DAG, diacylglycerol; SRF, serum response factor; JNK, Jun NH_2-terminal kinase (also known as SAPK, stress-activated protein kinase); PCR, polymerase chain reaction; HA, influenza hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione-S-transferase; PS, phosphatidylserine; PMA, phorbol myristate acetate; GS, 1,3-β-glucan synthase; MBP, myelin basic protein.

A. Overview

We have investigated the role of the essential Rho1 GTPase in cell integrity signaling in budding yeast. Conditional *rho1* mutants display a cell lysis defect that is similar to that of mutants in the cell integrity signaling pathway mediated by protein kinase C (PKC1), which is suppressed by overexpression of PKC1. *rho1* mutants are also impaired in pathway activation in response to growth at elevated temperature. PKC1 co-immuneprecipitates with Rho1 in yeast extracts, and recombinant Rho1 associates with PKC1 *in vitro* in a GTP-dependent manner. Recombinant Rho1 confers upon PKC1 the ability to be stimulated by phosphatidylserine (PS), indicating that Rho1 controls signal transmission through PKC1.

The *PKC1* gene of the budding yeast *Saccharomyces cerevisiae* encodes a homolog of mammalian protein kinase C (PKC) (ref. 1) that regulates a MAP kinase (MAPK)-

activation cascade comprised of a MEKK (Bck1), a redundant pair of MEKs (Mkk1/2), and a MAPK (Mpk1) (2, 3). Mutants in this signaling cascade, called the cell integrity pathway, undergo cell lysis resulting from a deficiency in cell wall construction that is exacerbated by growth at elevated temperatures. We have reported that thermal stress activates the cell integrity pathway, and proposed that weakness in the cell wall that develops during growth at high temperature induces the signal for pathway activation (4).

PKC1 most closely resembles the conventional isoforms of mammalian PKC, which require phospholipids, Ca^{2+}, and diacylglycerol (DAG) as cofactors to stimulate their catalytic activity (1). However, *in vitro* studies of this yeast protein kinase have failed to demonstrate stimulation by cofactors, despite the finding that mutations in *PKC1* predicted to relieve cofactor dependence have an activating effect on the enzyme (5, 6). This suggested that one or more components required for cofactor-dependent stimulation of PKC1 was missing from *in vitro* reconstitution experiments.

Members of the Rho family of small GTPases (RhoA, Cdc42, and Rac) regulate various aspects of actin cytoskeleton organization and activation of the SRF transcription factor in mammalian cells (7-10). Cdc42 and Rac, but not RhoA, stimulate the signaling pathway that contains the JNK/SAPK (Jun NH_2-terminal kinase or stress-activated protein kinase) MAPK homolog in mammalian cells (11-13). Downstream effectors of RhoA have not been identified (14, 15). The yeast *RHO1* gene encodes a homolog of mammalian RhoA that resides at sites of cell growth (16) and whose function is essential for viability (17). A *rho1*Δ mutant is partially suppressed by expression of human RhoA, but a residual cell lysis defect is apparent at high temperature (18), suggesting that *RHO1* may function within the cell integrity pathway. Additionally, an activated allele of *PKC1* was isolated recently as a dominant mutational suppressor of this defect (19), further supporting the notion that these signaling molecules act through a common pathway. In this communication, we demonstrate that Rho1 associates with PKC1 in a GTP-dependent manner, and confers upon this protein kinase the ability to respond to phosphatidylserine as an activating cofactor.

B. Experimental Procedures

Yeast strains and mutant construction--All strains used in this study were derived from YPH500 (See reference of Example 3). Error-prone PCR (21) was used to introduce random mutations into the *RHO1* sequence. The PCR-amplified *RHO1* fragment was inserted into the EcoRI/BglII gap of pY0701, and introduced into yeast strain YOC706, which harbors a *rho1*Δ and a plasmid expressing *RHO1* under the control of the *GAL1* promoter (18). We examined 4000 transformants for growth on YPD (yeast extract/peptone/dextrose) plates at 23°C and 37°C, and identified 41 $rho1^{ts}$ mutations.

Among these, 11 *rho1* alleles (designated *rho1-1 - rho1-11*) contained single or double base changes. All of these alleles were reconstructed by site-directed mutagenesis, and integrated at the *ADE3* locus (See reference of Example 3) of diploid strain YOC701 (*RHO1/rho1Δ ::HIS3*). Haploid strains used in this study (YOC764 [*RHO1*], YOC729 [*rho1-3*], and YOC755 [*rho1-5*]) were derived from YOC701 integrants by standard genetic techniques. A single copy plasmid (pYO904) that carries HA-tagged *RHO1* was constructed in vector pRS314, as described previously (16), and introduced into yeast strain YOC701. A segregant bearing *rho1Δ::HIS3* and pYO904, and a wild-type (*RHO1*) segregant lacking the plasmid were used for coimmunoprecipitation experiments.

Antibodies, extracts, immunoprecipitation, protein kinase assays and immunodetection--Anti-HA antibodies (12CA5; BAbCo, Inc.) were used for immunoprecipitation and immunodetection of ^{HA}Rho1, Mpk1^{HA}, and PKC1^{HA}. Polyvalent PKC1 antibodies (used for immunodetection of PKC1) were raised by Cocalico Biologicals (Reamstown, PA) in New Zealand white rabbits against a TrpE::PKC1 fusion protein that contains amino acids 470-664 of PKC1. This antiserum was used (at 1:3000 dilution) for immunodetection of PKC1. Secondary antibodies used were horseradish peroxidase-conjugated donkey anti-rabbit (Amersham; at 1:10,000 dilution).

Yeast extract preparation, immunoprecipitation, immunodetection and protein kinase assays of Mpk1^{HA} were conducted as described previously (4). Preparation of cell extracts and immunoprecipitations for experiments with ^{HA}Rho1 were carried out as in (4) with some modifications. Lysis buffer without *p*-nitrophenyl phosphate and with 1% NP-40 was used. The extract (700 μg protein) was precleared by incubation with 20 μl of a 50% suspension of protein A-sepharose for 1 h prior to immunoprecipitation to eliminate non-specific binding of proteins to immunecomplexes. Beads were boiled in SDS-PAGE sample buffer, and samples were applied to 7.5% (for PKC1 blots) or 15% (for ^{HA}Rho1 blots) SDS-PAGE gels. For PKC1 kinase assays, all as described previously (5), except for the addition of recombinant GTPases (see below). A synthetic peptide corresponding to the sequence surrounding Ser939 of Bck1, a phosphorylation site for PKC1, was used as substrate in PKC1 kinase assays (5)..

Recombinant Rho1 and Cdc42. Recombinant GST-Rho1 and GST-Cdc42 were expressed and purified from baculovirus-infected insect (Sf9) cells, as described (23). For *in vitro* association with PKC1^{HA}, GST-Rho1 was not eluted from the glutathione agarose beads used for purification. GST-Rho1-bound beads were incubated with cell extract in immunoprecipitation buffer (4) for 5 h at 4°C, followed by 3 washes with this buffer. For use in PKC1^{HA} protein kinase assays, GST-Rho1 and GST-Cdc42 were eluted from the beads with reduced glutathione. Purified GST-Rho1 displayed no protein kinase activity against the Bck1 peptide in the absence of PKC1 (not shown).

C. Results And Discussion

To examine the role of *RHO1* in the cell integrity signaling pathway, we isolated a set of 11 temperature-sensitive *rho1* alleles by *in vitro* random mutagenesis. Some of these mutants displayed cell lysis defects at the restrictive temperature (eg. *rho1-5*), but others did not (eg. *rho1-3*; Figure 1A). Additionally, overexpression of *PKC1* suppressed exclusively *rho1-5* (Figure 1B). Because of this allele-specific behavior, we chose *rho1-3* and *rho1-5* for further study.

The Mpk1 MAPK is activated via PKC1 in response to brief heat shock treatment (4). To determine if *RHO1* is required for cell integrity pathway signaling, we tested the ability of $rho1^{ts}$ mutants to activate Mpk1 upon heat shock. Mpk1, tagged at its COOH-terminus with the influenza hemagglutinin (HA) epitope ($Mpk1^{HA}$), was immunoprecipitated from extracts of heat shock-treated cells, and assayed for protein kinase activity *in vitro* using myelin basic protein (MBP) as substrate. Heat shock-induced activation of Mpk1 was completely blocked in the *rho1-3* mutant (Figure 2), indicating that *RHO1* function is essential for Mpk1 activation. The *rho1-5* mutant allowed some Mpk1 activation, suggesting that this allele retains some function at restrictive temperature. Residual function of the *rho1-5* allele at high temperature might also explain the allele-specific suppression of this mutant by *PKC1* overexpression if Rho1 function is required for PKC1 activation.

The yeast Cdc42 GTPase interacts with and stimulates the Ste20 protein kinase, which regulates the MAPK-activation cascade of the yeast pheromone response pathway (24, 25). Additionally, both recombinant human Cdc42 and Rac stimulate a mammalian protein kinase that is closely related to Ste20 (PAK65) (26, 27). Because Ste20 and PKC1 function at analogous positions in their respective MAPK signaling pathways (2, 3), we examined the possibility that Rho1 interacts directly with PKC1 *in vivo*. Rho1, tagged at its NH_2-terminus with the HA epitope (^{HA}Rho1), was immunoprecipitated from yeast extracts, and the resultant immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-PKC1 antibody. PKC1 was co-immunoprecipitated with ^{HA}Rho1 (Figure 3A, lanes 4 and 6), suggesting that PKC1 associates with Rho1 *in vivo*. This interaction was observed both in cells growing at 23°C and after heat shock.

To determine if the association between Rho1 and PKC1 depends on the activation state of Rho1, we examined the effect of different guanine nucleotides on this interaction *in vitro*. Recombinant glutathione-S-transferase-(GST)-Rho1, immobilized on glutathione agarose beads, was preloaded with either GTPγS or GDP prior to incubation with a yeast extract containing soluble PKC1 tagged at its COOH-terminus with the HA epitope ($PKC1^{HA}$). After washing the beads, bound $PKC1^{HA}$ was detected by SDS-PAGE and

immunoblotting with anti-HA antibody. Figure 3B shows that GTPγS-bound GST-Rho1 associated with PKC1 (lane 5), but GDP-bound protein did not (lane 3).

We also tested the possibility that PKC1 activity is stimulated by Rho1. $PKC1^{HA}$ was immunoprecipitated from yeast extracts, and its protein kinase activity was measured in the presence or absence of GST-Rho1 using a synthetic Bck1 peptide as substrate. Figure 4A shows that GST-Rho1 did not stimulate PKC1 activity alone but, when bound to GTPγS, conferred upon the protein kinase the ability to respond to activating cofactors (PS, DAG, and Ca^{2+}). This stimulatory effect is specific to Rho1, because GST-Cdc42 did not confer cofactor-dependent stimulation on PKC1. In the presence of GTP-bound GST-Rho1, PKC1 was strongly activated by phosphatidylserine (PS) as a lone cofactor (Figure 4B). The conventional isoforms of mammalian PKC are not stimulated by PS alone (28, 29). In contrast, this behavior is characteristic of the atypical ζ isoform of PKC (28, 30). No additional stimulation was observed by addition of Ca^{2+}, DAG, or phorbol ester (PMA) as a DAG substitute. This behavior is also exclusively characteristic of PKCζ (28, 30). Interestingly, the cys-rich region of PKC1, which is predicted to be a DAG-binding domain, has been reported to interact with Rho1 in two-hybrid experiments (19). Therefore, Rho1 may replace DAG in the activation of PKC1.

This study provides the first example of a PKC isoform whose stimulation by cofactors is dependent on a GTPase. We have identified recently a second role for Rho1 in the maintenance of cell integrity. Specifically, Rho1 is an essential component of the 1,3-β-glucan synthase (GS) complex (see Example 2, *infra*), the enzyme responsible for constructing polymers of 1,3-β-glucan in the cell wall. We have found that thermal induction of the *FKS2* gene, which encodes another component of the GS (32, 33), is under the control of *PKC1* and *MPK1*. Based on these findings, we propose the following model. A signal induced by weakness created in the cell wall during growth (and exacerbated at high temperature) stimulates guanine nucleotide exchange of Rho1 at the growth site. The GTP-bound Rho1 stimulates cell wall construction directly by activating GS and indirectly by stimulating PKC1-dependent gene expression in support of this process (Figure 5).

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Example 2

Identification of yeast Rho1 GTPase as a regulatory subunit of 1,3-β-glucan synthase

A. Overview

1,3-β-glucan synthase is a multi-enzyme complex that catalyzes the synthesis of 1,3-β-linked glucan, a major structural component of the yeast cell wall. Temperature-sensitive mutants in the essential Rho-type GTPase, Rho1, displayed thermolabile glucan synthase activity, which was restored by the addition of recombinant Rho1. Glucan synthase from mutants expressing constitutively active Rho1 did not require exogenous GTP for activity. Rho1 copurified with 1,3-β-glucan synthase and was found to associate with the Gsc1/Fks1 subunit of this complex *in vivo*. Both proteins were found to reside predominantly at sites of cell wall remodeling. Therefore, it appears that Rho1 is a regulatory subunit of 1,3-β-glucan synthase.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (1). Vegetative proliferation requires that the cell remodel its wall to accomodate growth, which during bud formation, is polarized to the bud tip. The main structural component responsible for the rigidity of the yeast cell wall is 1,3-β-linked glucan polymers with some branches through 1,6-β-linkages. The biochemistry of the yeast

enzyme catalyzing the synthesis of 1,3-β-glucan chains has been studied extensively (2,3), but little is known at the molecular level about the genes encoding subunits of this enzyme. Only a pair of closely related proteins (Gsc1/Fks1 and Gsc2/Fks2) are known to be subunits of the 1,3-β-glucan synthase (GS) (3-5). GS activity in many fungal species, including *S. cerevisiae*, requires GTP or a non-hydrolyzable analog (eg. GTPγS) as a cofactor, suggesting that a GTP-binding protein stimulates this enzyme (2,6). In this report, we demonstrate that the Rho1 GTPase is an essential regulatory component of the GS complex.

The *Saccharomyces RHO1* (*R*as *ho*mologous) gene encodes a small GTPase that resides at sites of growth (7), and whose function is essential for viability (M. S. Boguski *et al.* (1992) New Biol. 4:408). Based on phenotypic analyses of conditional *rho1* mutants, we and others have suggested that the normal function of Rho1 is to maintain cell integrity (7,9). Conditional *rho1* mutants are hypersensitive to Calcofluor white and echinocandin B, drugs that interfere with cell wall assembly, suggesting that this gene is involved in wall construction (10). To determine if Rho1 is required for glucan synthesis, we measured GS activity in extracts of temperature-sensitive *rho1* mutants grown at permissive temperature. GS activity from wild-type cells increased as a function of assay temperature from 23°C to 30°C to 37°C (Figure 6A). All of the *rho1* mutants tested displayed reduced levels of activity at each temperature relative to wild-type. Moreover, the enzyme from all but one mutant (*rho1-5*) exhibited some level of thermolability, suggesting that *RHO1* function is required for GS activity. Therefore, we tested the ability of purified, recombinant glutathione-S-transferase (GST)-Rho1 to restore GS activity to membrane fractions from the most impaired *rho1* mutant (*rho1-3*). Membranes from this mutant were virtually devoid of activity at 37°C. Figure 6B shows that GS activity was restored fully by the addition of GTPγS-bound GST-Rho1, but not GST-Cdc42, another member of the Rho-family of GTPases. GTPγS could be replaced with GTP, but not GDP (Figure 6C). These results indicate that the GS-deficient mutant membranes lack only Rho1 function.

We also examined GS activity from yeast cells expressing an constitutively active *RHO1* allele (*RHO1-Q68H*). The analogous mutation in Ras results in a protein that is impaired for the ability to hydrolyze GTP and has transforming potential in mammalian cells (11). The GTP requirement of GS activity was examined in membranes obtained from *rho1-3* cells overexpressing *RHO1* or *RHO1-Q68H* under the inducible control of the *GAL1* promoter (Figure 7). Under inducing conditions (galactose), expression of *RHO1-Q68H* resulted in GS activity that was independent of exogenous GTP. By contrast, GS activity in membranes from cells overexpressing *RHO1* was largely dependent on GTP. Similar results were obtained with another activated allele (*RHO1-G19V*; 12). Taken together, these results indicate that GS activity requires functional Rho1 in the GTP-bound state.

To determine if Rho1 is a component of the GS complex, we monitored the levels of Rho1 during purification of GS activity. The enzyme was purified by successive product entrapments following extraction from membranes (3). Figure 8 shows that both Rho1 and Gsc1/Fks1 were enriched in the partially purified GS. The specific activity of GS was increased approximately 700-fold through purification, whereas Rho1 was enriched approximately 400-fold. GS purified from the *rho1-5* mutant was deficient in GS activity despite normal levels of Rho1 and Gsc1/Fks1 proteins (data not shown). To determine if Rho1 copurifies with GS because it physically associates with the GS complex, the partially purified enzyme was immunoprecipitated with either of two monoclonal antibodies against Gsc1/Fks1. The resultant immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-Rho1 antibody. Figure 9A shows that Rho1 coimmunoprecipitates with Gsc1/Fks1.

Finally, we examined the localization of Rho1, tagged at its NH_2-terminus with the influenza hemagglutinin (HA) epitope (^{HA}Rho1), and Gsc1/Fks1 in growing yeast cells. Rho1 is known to be located at the bud tip (the site of polarized growth) during bud formation, and at the mother/bud neck (the site of septum formation) during cytokinesis (7). Indirect immunofluorescence of cells double labeled with anti-HA and anti-Gsc1/Fks1 antibodies revealed that Gsc1/Fks1 colocalizes with ^{HA}Rho1 (Figure 9B). These results strongly suggest that Rho1, like Gsc1/Fks1, is a component of the GS complex. This complex is redistributed through the cell cycle so as to reside at sites of cell wall remodeling.

We have shown recently that Rho1 interacts with and activates the PKC1 protein kinase (see Example 1, *supra*). Like *rho1* mutants, *pkc1* mutants display cell integrity defects that result from a deficiency in cell wall construction. However, several observations indicate that PKC1 is not involved in the activation of GS. First, mutants in *PKC1* display no defect in GS activity (14). Second, overexpression of PKC1 did not restore GS activity to *rho1* mutants (15). Third, PKC1 was not detected in the purified GS complex (16). Therefore, we propose that Rho1 plays at least two distinct regulatory roles in the maintenance of cell integrity. One is the activation of GS and the other is the stimulation of PKC1 for signal transduction. Rho1 may serve to coordinate, both spacially and temporally, several events required for effective cell wall remodeling. Both the GTP requirement for GS activity, and the structure of fungal PKCs are evolutionarily conserved (6,17), suggesting that the dual function of Rho1 may be conserved as well.

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10. Yeast strains YOC752 (*rho1-2*), YOC729 (*rho1-3*), YOC754 (*rho1-4*), YOC755 (*rho1-5*) and YOC764 (wild-type) were used in this study. YOC752, YOC729, and YOC755 displayed hypersensitivity to Calcofluor white and echinocandin B at 23°C.

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12. YPH499 cells carrying plasmids with wild-type *RHO1* (pYO762), *RHO1-G19V* (pYO906) under the control of the *GAL1* promoter, or vector alone (pYO761) were used. Cells were incubated in galactose medium for 10h, and GS activity associated with the membrane fraction was measured (3). Most of the GS activity from cells with pYO906 was GTPγS-independent, whereas only 15-20% of the activity was GTPγS-independent in the control strains.

14. A temperature-sensitive *pkc1* strain (SYT11-12A) and its isogenic wild-type strain (YS3-6D) [S. Yoshida *et al.*, (1992) *Mol. Gen. Genet.* 231:337] were grown in YPD (yeast extract/peptone/dextrose) at 23°C. A *pkc1*Δ strain (DL376) and its isogenic wild-type (DL100) [D. E. Levin and E. Bartlett-Heubusch, (1992) *J. Cell Biol.* 116:1221] were grown at 23°C in YPD containing 10% sorbitol. GS activities were assayed at 23°C and at 37°C.

15. Mutants used were *rho1-3* and *rho1-5* carrying *PKC1* on a multicopy plasmid (pYO910), or vector alone (pYO324).

16. Partially purified enzyme fraction (second product entrapment) was analyzed by immunoblotting with anti-PKC1 antibody (S. Yoshida, unpublished).

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18. Crude yeast extracts were prepared as described [Y. Kamada *et al.*, (1995) *Genes Dev.* 9:1559], and stored at -80°C in lysis buffer supplemented with 33% glycerol. Membrane fractions, where indicated, were obtained from crude extracts and 1,3-β-glucan synthase (GS) activity was measured as described in (2) with the following modifications: UDP-[^{3}H]glucose was used as the substrate and α-amylase (1U/40 μl) was added to reaction mixtures to eliminate the contribution of [^{3}H]glucose incorporation into glycogen. For all GS assays, the mean and standard error for four experiments is shown.

19. Recombinant GST-Rho1 and GST-Cdc42 were expressed in Sf9 insect cells, and purified as described previously [Y. Zheng *et al.*, (1994) *J. Biol. Chem.* 269:2369].

20. A series of protein sample dilutions was analyzed by immunoblotting with guinea pig anti-Rho1 antiserum or mouse anti-Gsc1/Fks1 monoclonal antibodies (T2B8; 3). The amount of antigens was estimated by densitometry.

21. Goat anti-mouse IgG-agarose (20μl; Sigma) was incubated with 500 μl media from monoclonal antibody cultures for 5h at 37°C. The agarose beads were washed 5 times with phosphate-buffered saline and twice with Buffer A (0.4 CHAPS, 0.08% cholesteryl hemisuccinate, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 8 μM GTPγS and 33% glycerol). Partially purified GS (1.8 μg) was added and the reaction mixtures were further incubated for 2h at 37°C. After washing the beads four times with Buffer A, the bound complexes

were analyzed by immunoblotting with anti-Rho1 antiserum or anti-Gsc1/Fks1 monoclonal antibodies (T2B8).

22. Cells of haploid strain YOC785, which bears a *rho1*Δ and the HA-tagged *RHO1* gene (13) on a centromere plasmid (pYO904) were double stained with mouse monoclonal antibody against Gsc1/Fks1 (T2B8) and rabbit anti HA-antibody (Boehringer), as described previously [J. R. Pringle *et al.*, (1989) *Methods Cell Biol.*, 31:357]. Secondary antibodies were FITC-conjugated anti-mouse IgG (Cappel) and TRITC-conjugated anti-rabbit IgG (Cappel). Control strains (YPH499 for ^{HA}Rho1 and *gsc1*Δ for Gsc1/Fks1) produced no signals in single staining experiments. The secondary antibodies did not cross-react with the heterologous primary antibodies. Some internal punctate staining of Gsc1/Fks1 that did not colocalize with ^{HA}Rho1 may represent secretory intermediates.

Example 3

Yeast geranylgeranyl protein transferase I is essential for membrane localization of Rho1 GTPase and 1,3-β-glucan synthase activity

The abbreviations used in Example 3 are: GGPTase I, geranylgeranyl protein transferase I; GST, glutathone-S-transferase; HA, influenza hemagglutinin; ORF open reading frame; GS, 1,3-β-glucan synthase.

A. Overview

Protein prenylation, farnesylation and geranylgeranylation, is a posttranslational reaction which requires the covalent attachment of a hydrophobic tail, isoprenoid (C15 or C20), to the C-terminal cysteine residue of the substrate proteins (1). Prenylation is necessary for many proteins to interact with membranes and to locate at proper intracellular places. Many lines of evidence have been accumulated to show that small GTPases require prenylation to gain full functionality (1, 2).

Genes encoding subunits of each prenyltransferase have been cloned in the yeast *Saccharomyces cerevisiae*. The genes *CAL1* (3) (also known as *CDC43* (4)) and *DPR1* (5) (also known as *RAM1*) encode β subunits of the yeast GGPTase I and FTase, respectively, and *RAM2* encodes the common α subunit (6). The α subunit, β subunit and component A of the yeast GGPTase II are encoded by *BET4*, *BET2* and *MSI4*, respectively (7). An alignment of the homologous regions of the three β subunit sequences (positions 159-350 of the Cal1/Cdc43 sequence) reveals 32-40% identity each other (3). This region contains novel repeat motifs (M. S. Boguski *et al.* (1992) New Biol. 4:408). The repeats have a length of 44-45 residues and there are three repeats in the Cal1p/Cdc43p sequence. The repeats are conserved in the central Gly-Gly-Phe-Gly-Gly sequence region. The α subunit of isoprenyl transferases also possesses distinct internal repetitive sequence containing tryptophan. Hydrophobic bonds between the side chains of the conserved tryptophan and

phenylalanine may be important for forming heterodimer (M. S. Boguski *et al.* (1992) New Biol. 4:408).

Among prenyltransferase mutants, a mutation in the GGPTase I β subunit gene was the first to be isolated and characterized. *cal1-1* was identified originally as a mutation resulting in a Ca^{2+}-dependent phenotype (9). The *cal1-1* mutant simultaneously exhibits a homogeneous terminal phenotype with a G2/M nucleus and a small bud at 37°C (9). Independent screening of yeast cell cycle mutants which accumulated enlarged unbudded cells identified six other alleles, *cdc43-2* ~ *cdc43-7* (10). Yeast GGPTase I is essential for yeast cell growth, since deletions of the *CAL1* gene result in a lethal phenotype (3). However, GGPTase I is no longer essential, when the dosage of the two GTPases, Rho1p (11, 12) and Cdc42p (13), are artificially elevated (14). Since the yeast GGPTase I prenylates these two GTPases, Cdc42p and Rho1p are implicated genetically as the only two essential substrates of GGPTase I (14). *CAL1/CDC43* is necessary not only for the function of the small GTPases but also for membrane localization of the small GTPases. An increase in soluble Cdc42p is observed in the *cdc43-2* strain grown at the restrictive temperature (15).

This study was undertaken to understand the molecular lesions caused by loss of the GGPTase I function, using the seven temperature-sensitive mutations in the *CAL1/CDC43* gene. All of the mutation sites were determined at the nucleotide level. An increase in soluble Rho1p was observed in the *cal1-1* strain grown at the restrictive temperature. Futhermore, GS activity was dramatically reduced in the *cal1-1* mutant strains. Several phenotypic differences were observed among the *cal1/cdc43* mutations, possibly due to the alteration of substrate specificity caused by the mutations.

B. Experimental Procedures

Materials.—YPD medium contained 1% Bacto-yeast extract (Difco Laboratories, Detroit, MI), 2% polypeptone (Nihon Chemicals, Osaka), and 2% glucose (Wako Chemicals, Tokyo). YPD supplemented with 100 mM or 300 mM $CaCl_2$ was used as Ca^{2+}-rich medium. Other standard media are described elsewhere (16).

DNA manipulation--DNA fragments containing the *cdc43* mutations were cloned by gap repair (17). The pCAL-F9 plasmid containing the 2.8 kb *SphI-PstI* fragment of the *CAL1/CDC43* gene was digested with *Nsp*(7524)V and *Eco*T22I and introduced into the *cdc43* strains (*cdc43-2* ~ *cdc43-7*). Transformation of the plasmid containing the *Nsp*(7524)V-*Eco*T22I gap resulted in repair of the gap to yield plasmids in which the gap was repaired by gene conversion with the chromosomal sequences. The gap-repaired plasmids were recovered from yeast, and its *Nsp*(7524)V-*Eco*T22I fragment was subcloned

into the *Nsp*(7524)V-*Eco*T22I gap of pCAL-F9. Then, the resulting plasmids YCpT-cdc43-2 ~ YCpT-cdc43-7 were introduced into the *cdc43* strains. Because the transformants showed a temperature-sensitive phenotype, we concluded that all of the *cdc43* mutations resided within the region between the *Nsp*(7524)V and *Eco*T22I. Nucleotide sequencing of the 1.0-kb *Nsp*(7524)V-*Eco*T22I fragment from the YCpT-cdc43-2 ~ YCpT-cdc43-7 revealed that each of the *cdc43* mutants possessed a single base pair change within the ORF.

Production of the anti-Rho1p antibody–The purified GST-Rho1p (64-209) which is a fusion protein of GST with Rho1p from amino acid positions 64 to 209 was minced and emulsified with R-700 (RIBI ImmunoChem Research, Hamilton, MT) and the resulting emulsion was used to immunize four guinea pigs. After boost was repeated five times with three-weeks intervals, blood was collected from the animals and one of the immune serum was used in this study. The anti-Rho1p antibody specifically recognized Rho1p. Western blotting analysis showed that there was no other protein band detected in the lysates of cells expressing human *rhoA* in place of *RHO1*.

Cell fractionation experiments. Cell fractionation experiments were performed using techniques described by Ziman *et al.* (15). Briefly, cells were grown at 23°C to mid log phase, and approximately 5 x 10^8 cells were collected, washed with water, resuspended in 0.1 ml of lysis buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid pH 7.0) with 0.5 mM PMSF, and lysed on ice by vortexing with 400-500 mm acid-washed glass beads (Sigma). Greater than 80% lysis was verified by light microscopy. After addition of 0.4 ml of lysis buffer, cell lysates were spun at 390 X g for 1 min at 4°C. The supernatant was then spun at 436,000 X g for 20 min at 4°C, and the pellets were resuspended in the same volume of lysis buffer. To assess the relative amount of Rho1p and Cdc42p in each fraction, equal volumes of each fraction were loaded onto a sodium dodecyl sulfate-12.5% polyacrylamide gel for immunoblot analysis. Guinea pig polyclonal antibody against Rho1p and mouse monoclonal antibody against HA (12CA5, Boeringer Mannheim, Germany) were used at 1:500 and 1:100, respectively. Alkaline phosphatase-conjugated goat anti-guinea pig IgG and anti-mouse IgG were used at 1:5000. Antibody-antigen complexes were detected with 5-bromo-4-chloro-3-indoryl-phosphate and nitro blue tetrazolium.

C. Results and Discussion

Mutation points of *cdc43-2 ~cdc43-7* were determined after DNA fragments containing the *cdc43-2 ~ cdc43-7* mutations were cloned by the gap repair method (17) to yield YCpT-cdc43-2 ~ YCpT-cdc43-7. Based on the subcloning analysis (see Materials and Methods), we concluded that all of the *cdc43* mutations resided within the 1.0-kb region

between the *Nsp*(7524)V and *Eco*T22I, nearly corresponding to the entire coding region of *CAL1/CDC43*. Nucleotide sequencing of the 1.0-kb *Nsp*(7524)V-*Eco*T22I fragment from the YCpT-cdc43-2 ~ YCpT-cdc43-7 revealed that each of the *cdc43* mutants possessed a single base pair change within the ORF. Figure 10 shows the amino acid changes in the *cdc43* sequences. *cdc43-4* and *cdc43-6* resulted from an identical nucleotide change, and hereafter are referred to as *cdc43-6*. *cdc43-5* had a amino acid change at the same position as *cdc43-4* and *cdc43-6*, but resulted in a different amino acid change. Figure 10 shows that the four *cdc43/cal1* mutations (*cdc43-5 cdc43-6*, *cdc43-7*, *cal1-1*) were mapped within the domain homologous to the b-subunits of other protein isoprenyltransferases (a.a. position 159-350). Interestingly enough, these mutations affect the conserved amino acid residues among the subunits of GGPTase I from four different species (3, 18, 19). The other two *cdc43* mutations (*cdc43-2* and *cdc43-3*) were mapped outside of the homologous domain.

We have previously shown the functional interaction between *RHO1* and *CAL1* based on the observation that overproduction of Rho1p suppressed the temperature sensitivity of *cal1-1* (See reference of Example 3). In order to know whether the suppression by overproduction of Rho1p was seen only with the *cal1-1* allele, we examined the ability of overproduction of Rho1p to suppress the *cdc43* mutations. Since the restrictive temperatures of the *cdc43* mutants were different, effects of the Rho1p overexpression were examined at five different temperatures (23°C, 28°C, 30°C, 33°C and 37°C). We found that the *cdc43* mutations were not suppressed effectively by overproduction of Rho1p (Table 1). None of the mutations was suppressed at 37°C, while *cal1-1* was suppressed at this temperature. *cdc43-2* and *cdc43-7* with multicopy *RHO1* grew slightly faster than those with vector alone at 30°C, while *cal1-1* was suppressed completely at this temperature. Slight growth improvement of *cdc43-5* by overproduction of Rho1p was observed only at 23°C. These results indicate that among the *cal1/cdc43* mutations so far isolated, *cal1-1* is a unique mutation that is effectively suppressed by overproduction of Rho1p.

Table 1

Effect of overproduction of Rho1p and Cdc42p in the cal1/cdc43 mutants

		growth on YPD					YPD +Ca	
strain	plasmid	23°C	28°C	30°C	33°C	37°C	33°C	37°C
cal1-1	pYO324	+	+	±	-	-	++	+
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	++	++	++	+	++	+
	YEpT-CDC42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cdc43-2	pYO324	++	+	±	-	-	-	-
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	+	+	-	-	-	-

	YEpT-CDC42	++	+	±	-	-	-	-
cdc43-3	pYO324	++	++	++	±	-	-	-
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	++	++	±	-	-	-
	YEpT-CDC42	++	++	++	±	-	+	-
cdc43-5	pYO324	+	±	-	-	-	-	-
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	±	-	-	-	-	-
	YEpT-CDC42	++	++	++	++	+	++	+
cdc43-6	pYO324	++	+	±	-	-	-	-
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	+	±	±	-	-	-
	YEpT-CDC42	++	+	±	-	-	-	-
cdc43-7	pYO324	++	+	±	-	-	±	-
	YCpT-CAL1	++	++	++	++	++	++	++
	YEpT-RHO1	++	+	+	-	-	±	-
	YEpT-CDC42	++	+	±	-	-	+	-

Since overproduction of Rho1p suppressed a mutation of the *CAL1/CDC43* gene, we next attempted to examine multicopy suppression of the *cdc43* mutations by overproduction of another essential substrate of GGPTase I, Cdc42p. We found that overproduction of Cdc42p suppressed the temperature-sensitive phenotype of *cdc43-5* (Table I); the *cdc43-5* mutant with multiple copies of *CDC42* grew well at 37 °C. Among the *cdc43* mutations, *cdc43-5* was most effectively suppressed by overproduction of Cdc42p; *cdc43-3*, *cdc43-6* and *cdc43-7* were suppressed slightly by overproduction of Cdc42p at the intermediate temperature, and *cdc43-2* was not suppressed at all at any temperature examined.

Several trials to introduce multiple copies of *CDC42* into the *cal1-1* strain were unsuccessful. Reasoning that overexpression of Cdc42p might be a lethal event in the *cal1-1* strain, we attempted to increase the levels of Cdc42p by placing its expression under the control of the *GAL1* promoter that was induced by galactose in the medium. The *cal1-1* strain with *pGAL-CDC42* could grow on solid media containing glucose but did not grow on media containing galactose (Figure 11). This growth inhibition was observed at any temperature examined (23°C, 30°C and 37°C). Since *pGAL-CDC42* was not toxic in the wild-type strain and many of the other *cdc43* mutants (Figure 11), we concluded that lethality caused by the overexpression of Cdc42p is specific to the *cal1-1* mutant. Although *CDC42* on a multicopy plasmid is not toxic in *cdc43-7*, *pGAL-CDC42* is dereterious in

cdc43-7 (Figure 11). This may be due to the fact that the expression level of Cdc42p by *pGAL-CDC42* is more than that expressed by multiple copies of *CDC42*.

cal1-1 was suppressed most effectively by overexpression of Rho1p, while *cdc43-5* was suppressed by overexpression of Cdc42p. To test the possibility that the allele-specific suppression is due to substrate specificity of the mutant GGPTase I, we examined the partitioning of Rho1p and Cdc42p in the *cal1-1* and *cdc43-5* mutant strains. It was already shown that soluble Cdc42p increases in the *cdc43-2* strain grown at the restrictive temperature (15), suggesting that membrane localization of small GTPases is dependent on geranylgeranyl modification. We found that the proportion of Rho1p found in the soluble fraction of *cal1-1* dramatically increases after the temperature shift (Figure 12). Rho1p from *cdc43-5* strain grown at 37°C for 2 hr was almost exclusively in the particulate fraction, indicating that increase of soluble Rho1p is specific to *cal1-1*. The proportion of HA-tagged Cdc42p found in the soluble fraction of *cdc43-5* increased after 2 hr incubation at 37°C, while *cal1-1* did not affect partitioning of HA-tagged Cdc42p (Figure 12). Temperature-shift itself did not affect the partitioning of these GTPases in the wild-type control strain. These results suggested that *cal1-1* and *cdc43-5* specifically impair geranylgeranylation of Rho1p and Cdc42p, respectively.

We have previously shown that Rho1p is a regulatory subunit of 1,3-β-glucan synthase (see Example 2 above). To directly examine involvement of GGPTase I in the Rho1 function, we measured GS activity in membrane fractions of the *cal1-1* and *cdc43-5* mutant cells grown at permissive temperature (Figure 13). We found that *cal1-1* displayed dramatically reduced activity relative to wild-type. *cdc43-5* mutant instead displayed only slightly reduced activity, probably due to the fact that *cdc43-5* impairs geranylgeranylation of Cdc42p more than geranylgeranylation of Rho1. We tested whether purified, recombinant GST-Rho1 restored GS activity to the membrane fraction of the *cal1-1* mutant. GS activity was restored by the addition of constitutively activated Rho1. These results indicate that the GS-deficient *cal1-1* mutant membrane lack the Rho1 function.

Multiple copies of either Rho1p or Cdc42p suppressed specific alleles of *cal1/cdc43* (Table 2): *cal1-1* was suppressed effectively by multicopy *RHO1*, while *cdc43-5* was suppressed effectively by multicopy *CDC42*. Given both Rho1p from the *cal1-1* strain and Cdc42p from the *cdc43-5* strain accumulate in the soluble fraction, substrate specificipy of the mutant GGPTase I likely accounts for the allele-specific suppression. In our current model, *cal1-1* and *cdc43-5* selectively impair the *in vivo* geranylgeranylation of Rho1p and Cdc42p, respectively. This is consistent with observation of the mutant phenotypes; terminal phenotypes of *cdc43-5* and *cdc42* are undistinguished, and those of *cal1-1* and temperature-sensitive *rho1* strains are somewhat similar. This is also consistent with our observation that overexpression of Cdc42p is lethal specifically in the *cal1-1* strain, because

overexpression of Cdc42p likely sequesters the *cal1-1* GGPTase I to further impair geranylgeranylatikn of Rho1p. GS activity was dramatically reduced in *cal1-1* but not in *cdc43-5*. Taken together, our genetic and biochemical results suggest that the *CAL1/CDC43* GGPTase I has an ability to prenylate the substrate GTPases by some domain-specific, substrate-specific recognition mechanisms.

Table 2 Summary of the effect of the GTPases in the cal1/cdc43 mutants

	overproduction	
Phenotype	Cdc42p	Rho1p
suppression	*cdc 43-5* (*cdc43-3, -4, -7*)	*cal1-1* (*cdc43-2, -5, -7*)
deleterious	*cal1-1* (*cdc43-7*)	

C. References in Example 3

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Example 4

Cloning of a Candida Rho1-like GTPases

Isolation of *CaRho1* cDNA:

A *Candida* Rho1 GTPase cDNA was isolated from a *C. albicans* cDNA library in λZAP. Briefly, a probe was prepared by ^{32}P-labelling a random primed *S. cerevisae* Rho1 cDNA by standard methods (NEBlot Kit, New England Biolabs). 50,000 plaque forming units of a *C. albicans* cDNA library in λZAP was screened. Hybridization was performed overnight in Church's buffer (7% SDS, 250mM NaP pH7, 1mM EDTA pH7) at 43°C. The filters were wased twice at the same temperature in a buffer containing 2x SSC and 0.1% SDS. Three positive clones were obtained, their pSK phagemids were derived by *in vivo* excision using Strategene's instructions. Sequences were obtained using an ABI (Applied Biosystems) sequencing kit and DNA sequencer according to the manufacturer's instructions. An apparent Rho1 homolog was identified.

Isolation of *CaCdc42* cDNA:

A *Candida* cdc42 GTPase cDNA was isolated from a *C. albicans* cDNA library by PCR using degenerate primers based on conserved regions of other cdc42 proteins. Briefly, two degenerate primers, CDC42-F2 (GTNGTNGGNGAYGGNGCNGTNGG) and CDC42-R2 (ATNGCYTCRTCRAANACRTTYTT) based on the conserved regions VVGDGAVG and KNVFDEAI, respectively, were used to PCR amplify *C. albicans* genomic DNA from the strain 3153A. The amplification program consisted of 30 cycles: 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. The PCR reactions were performed with Taq DNA polymerase using 1x amplification buffer, 3mM $MgCl_2$, 250mM dNTPs, 100ng of template DNA, and 2μM primers. The PCR products were separated on 1.5% agarose gel, and a PCR product of the about 500bp was excised from the gel and reamplified using the same conditions as above. Gel purified reaction products were cloned into the pCRTMII vector (TA cloning system, Invitrogen). Sequence analysis of the cloned inserts showed that they encoded a partial ORF of an apparent cdc42 homolog.

50,000 plaque forming units of a *C. albicans* cDNA library in λZAP were screened. Hybridization was performed overnight in buffer containing 5x Denhardt, 4x SSC, 30% formamide, 1% SDS, and 2mg/ml salmon sperm DNA at 42°C and filters were washed

twice at the same termperature in a buffer containing 2x SSC and 0.1% SDS. Two positive clones were obtained, their pSK phagemids were derived by *in vivo* excision using Strategene's instructions. Sequences were obtained using an ABI (Applied Biosystems) sequencing kit and DNA sequencer according to the manufacturer's instructions. An apparent cdc42 homolog was identified.

Example 5

Cloning of a Candida GGPTase I subunit (α subunit)

A cDNA encoding the α subunit of *Candida* GGPTase I was cloned by PCR employing degenerate primers designed on the basis of sequence similarity between known RAM2 genes. Forward and reverse primers were designed corresponding to six distinct regions of RMA2 of 7 to 12 amino acids showing various degrees of conservation. A variety of primer sets were use. Only one PCR reaction out of approximately 250 yielded a product which had detectable homology with other RAM2 proteins

Briefly, genomic DNA from *C. albicans* (strain caf3-1) was isolated using standard protocols (see "Methods in Yeast Genetics: A laboratory manual", CSH Press, 1990), except that zymolase treatment was carried out for 2.5 hours. DNA fragments were purified from agarose gels using the GeneClean II kit (Bio101). DNA fragments were cloned into pCRscript using the pCR-Script Amp SK+ Cloning kit (Stratagene) according to the manufacturers instructions.

Primers were generated as follows: Ram2F3: GAYDSNAARAAYTAYCAYGYN-TGG (corresponding to DS/AKNYHV/AW) and Ram2R2 CAANWRRTYYTTNCKDATN-GKRTC (corresponding to WL/YN/EKRIP/TD. Two sequential rounds of PCR were required to amplify the partial CaRAM2 sequence using the Ram2F2 and Ram2R2 primers. A 50μL PCR reaction contained 100-150ng *C albicans* genomic DNA, 1μM each primer, 1x Taq polymerase buffer, 3mM $MgCl_2$, 200μM dNTPs and 2.5U Taq polymerase. The PCR cycling conditions involved a "hot start" at 94°C for 2 minutes, followed by the 30 cycle program: 94°C for 30 seconds, 38°C for 1 minute, 72°C for 45 seconds. The products from the first PCR reaction were electrophoresed on 2% agarose gel. A small piece of gel was removed from the area harboring DNA fragments in the size range of 400-500bp and used as the template in the second round of PCR using the same conditions as described above. The products from the second PCR round were run on a 2% agarose gel, and a product in the 400-500bp range was excised. The DNA was purifed and cloned into pCRscript. After transformation into the XL-1 blue *E coli* strain, plasmids were checked for the presence of an insert which was sequenced. An apparent RAM2 homolog was identified.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Mitotix, Inc.
(B) STREET: One Kendall Square, Building 600
(C) CITY: Cambridge
(D) STATE: MA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02139
(G) TELEPHONE: (617) 225-0001
(H) TELEFAX: (617) 225-0005

(i) APPLICANT:
(A) NAME: The Johns Hopkins University
(B) STREET: 3400 North Charles Street
(C) CITY: Baltimore
(D) STATE: MD
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 21218
(G) TELEPHONE: (410) 516-8137
(H) TELEFAX: (410) 955-2926

(ii) TITLE OF INVENTION: Assays and Reagents for Identifying Anti-Fungal Agents, and Uses Related Thereto

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (text)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/631,319
(B) FILING DATE: 10-APR-1996

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 985 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 114..707

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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                                                           Met
                                                            1

GTT AAC GGT CCA GCT GAA CTT CGT AGA AAA TTA GTC ATT GTC GGT GAT       164
Val Asn Gly Pro Ala Glu Leu Arg Arg Lys Leu Val Ile Val Gly Asp
            5                  10                  15

GGT GCT TGT GGT AAG ACT TGT TTA TTA ATT GTT TTT TCA AAA GGT ACT       212
Gly Ala Cys Gly Lys Thr Cys Leu Leu Ile Val Phe Ser Lys Gly Thr
        20                  25                  30

TTC CCA GAA GTT TAT GTC CCA ACA GTT TTT GAA AAT TAC GTT GCT GAT       260
Phe Pro Glu Val Tyr Val Pro Thr Val Phe Glu Asn Tyr Val Ala Asp
    35                  40                  45

GTT GAA GTT GAT GGT AGA AAA GTT GAA TTG GCA TTA TGG GAT ACT GCT       308
Val Glu Val Asp Gly Arg Lys Val Glu Leu Ala Leu Trp Asp Thr Ala
50                  55                  60                  65

GGT CAA GAA GAT TAT GAT AGA TTA AGA CCA TTA TCT TAT CCA GAT TCT       356
Gly Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro Asp Ser
                70                  75                  80

AAT GTT ATT TTG ATT TGT TTT TCA GTT GAT TCA CCA GAT TCT TTA GAT       404
Asn Val Ile Leu Ile Cys Phe Ser Val Asp Ser Pro Asp Ser Leu Asp
            85                  90                  95

AAC GTT TTA GAA AAA TGG ATT TCT GAA GTT TTA CAT TTC TGT CAA GGT       452
Asn Val Leu Glu Lys Trp Ile Ser Glu Val Leu His Phe Cys Gln Gly
        100                 105                 110

GTT CCA ATC ATT TTA GTT GGT TGT AAA TCT GAT TTA AGA GAT GAT CCT       500
Val Pro Ile Ile Leu Val Gly Cys Lys Ser Asp Leu Arg Asp Asp Pro
    115                 120                 125

CAT ACT ATT GAA GCC TTG AGA CAA CAA CAA CAA CAA CCA GTC TCA ACT       548
His Thr Ile Glu Ala Leu Arg Gln Gln Gln Gln Gln Pro Val Ser Thr
130                 135                 140                 145

TCT GAA GGC CAA CAA GTT GCT CAA AGA ATT GGT GCT GCT GAT TAC TTG       596
Ser Glu Gly Gln Gln Val Ala Gln Arg Ile Gly Ala Ala Asp Tyr Leu
                150                 155                 160

GAA TGT TCT GCT AAA ACC GGT AGA GGT GTT AGA GAA GTG TTT GAA GCT       644
Glu Cys Ser Ala Lys Thr Gly Arg Gly Val Arg Glu Val Phe Glu Ala
            165                 170                 175

GCT ACT AGA GCT TCT TTA AGA GTT AAA GAA AAG AAG GAA AAG AAG AAG       692
Ala Thr Arg Ala Ser Leu Arg Val Lys Glu Lys Lys Glu Lys Lys Lys
        180                 185                 190

AAA TGT GTT GTC TTG TAAATGAAAC AACAACTAAA AGAACAAGAA GAAGAAGAAG       747
Lys Cys Val Val Leu
    195
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AAGCACTAGC AATAGCAAAA GCTAAAAGAA AAAAATAAAG TCAAGCAAAT ACAACAAAAG 807

GCAAAGTCAG AATAGAAAGA AACCTGAAGC CCTCTTATGA GTTGTGGTTT TCTTTCTTAT 867

TCTTTTTTTT TATTCATTTC ATTATGTTTT ATCCTATACT TTTTTTTTAG TTTCAGCACT 927

AGATTTTAAA GAATTTTGTT ATTTAATTAA TATTAATATT ATTAAAAAAA AAAAAAAA 985

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 198 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

Asp Gly Ala Cys Gly Lys Thr Cys Leu Leu Ile Val Phe Ser Lys Gly
20 25 30

Thr Phe Pro Glu Val Tyr Val Pro Thr Val Phe Glu Asn Tyr Val Ala
35 40 45

Asp Val Glu Val Asp Gly Arg Lys Val Glu Leu Ala Leu Trp Asp Thr
50 55 60

Ala Gly Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro Asp
65 70 75 80

Ser Asn Val Ile Leu Ile Cys Phe Ser Val Asp Ser Pro Asp Ser Leu
85 90 95

Asp Asn Val Leu Glu Lys Trp Ile Ser Glu Val Leu His Phe Cys Gln
100 105 110

Gly Val Pro Ile Ile Leu Val Gly Cys Lys Ser Asp Leu Arg Asp Asp
115 120 125

Pro His Thr Ile Glu Ala Leu Arg Gln Gln Gln Gln Gln Pro Val Ser
130 135 140

Thr Ser Glu Gly Gln Gln Val Ala Gln Arg Ile Gly Ala Ala Asp Tyr
145 150 155 160

Leu Glu Cys Ser Ala Lys Thr Gly Arg Gly Val Arg Glu Val Phe Glu
165 170 175

Ala Ala Thr Arg Ala Ser Leu Arg Val Lys Glu Lys Lys Glu Lys Lys
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Lys Lys Cys Val Val Leu
195

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 921 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...918

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 1               5                  10                  15

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            20                  25                  30

AAA CAA ATT ATG GGG TTA TTA CTT GCA CTT ATG AAA GCT GAA GAG TAT     144
Lys Gln Ile Met Gly Leu Leu Leu Ala Leu Met Lys Ala Glu Glu Tyr
        35                  40                  45

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TCA CAT TAT ACA ATT TGG ATC TAT CGA TTT AAT ATT TTG AAA AAC TTA     240
Ser His Tyr Thr Ile Trp Ile Tyr Arg Phe Asn Ile Leu Lys Asn Leu
65                  70                  75                  80

CCC AAT AGA AAC CTT TAT GAT GAA TTG GAT TGG TGT GAA GAA ATT GCT     288
Pro Asn Arg Asn Leu Tyr Asp Glu Leu Asp Trp Cys Glu Glu Ile Ala
                85                  90                  95

TTG GAC AAT GAA AAA AAC TAT CAG ATT TGG AAT TAT CGA CAA TTA ATT     336
Leu Asp Asn Glu Lys Asn Tyr Gln Ile Trp Asn Tyr Arg Gln Leu Ile
            100                 105                 110

ATT GGT CGA ATT ATG GAA TTG AAT AAT AAT GAC TTT GAC CCA TAT CGA     384
Ile Gly Arg Ile Met Glu Leu Asn Asn Asn Asp Phe Asp Pro Tyr Arg
        115                 120                 125

GAA TTC CTT ATA TTA GAA GCA ATG TTA AGT TCA GAC CCC AAG AAC CAT     432
Glu Phe Leu Ile Leu Glu Ala Met Leu Ser Ser Asp Pro Lys Asn His
    130                 135                 140

CAT GTT TGG TCG TAT CGT AAG TGG TTG GTT GAT ACG TTT GAT TTA CAT     480
His Val Trp Ser Tyr Arg Lys Trp Leu Val Asp Thr Phe Asp Leu His
145                 150                 155                 160

AAT GAC GCA AAA GAA TTA TCG TTT GTT GAT AAA GTC ATC GAT ACT GAT     528
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Asn Asp Ala Lys Glu Leu Ser Phe Val Asp Lys Val Ile Asp Thr Asp
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AAG AAA CAT TTG GCC ACC GAT AAT ACA ATT GAT GAG GAG CTA AAT TAT      624
Lys Lys His Leu Ala Thr Asp Asn Thr Ile Asp Glu Glu Leu Asn Tyr
        195                 200                 205

GTT AAA GAT AAG ATT GTT AAA TGT CCA CAG AAT CCA AGT ACT TGG AAT      672
Val Lys Asp Lys Ile Val Lys Cys Pro Gln Asn Pro Ser Thr Trp Asn
    210                 215                 220

TAT TTA TTG GGG ATT CAT GAA CGG TTT GAT CGA TCA ATT ACT CAA TTA      720
Tyr Leu Leu Gly Ile His Glu Arg Phe Asp Arg Ser Ile Thr Gln Leu
225                 230                 235                 240

GAA GAG TTT AGT TTG CAA TTT GTT GAT TTG GAA AAA GAT CAA GTG ACG      768
Glu Glu Phe Ser Leu Gln Phe Val Asp Leu Glu Lys Asp Gln Val Thr
                245                 250                 255

AGT TCA TTT GCT TTG GAG ACA TTG GCA AAA ATA TAC ACA CAA CAA AAG      816
Ser Ser Phe Ala Leu Glu Thr Leu Ala Lys Ile Tyr Thr Gln Gln Lys
            260                 265                 270

AAA TAC AAT GAG GCT AGA ACT GTT TAT GAT TTG TTG AAA TCT AAA TAT      864
Lys Tyr Asn Glu Ala Arg Thr Val Tyr Asp Leu Leu Lys Ser Lys Tyr
        275                 280                 285

GAT CCA ATT AGA TCC AAT TTC TGG GAT TAT CAG ATT TCC AAA CTC ACA      912
Asp Pro Ile Arg Ser Asn Phe Trp Asp Tyr Gln Ile Ser Lys Leu Thr
    290                 295                 300

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 306 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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            20                  25                  30
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        35                  40                  45
Ser Glu Arg Ala Leu His Ile Thr Glu Leu Gly Ile Asn Glu Leu Ala
    50                  55                  60
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Ser His Tyr Thr Ile Trp Ile Tyr Arg Phe Asn Ile Leu Lys Asn Leu
65                  70                  75                  80
Pro Asn Arg Asn Leu Tyr Asp Glu Leu Asp Trp Cys Glu Glu Ile Ala
                85                  90                  95
Leu Asp Asn Glu Lys Asn Tyr Gln Ile Trp Asn Tyr Arg Gln Leu Ile
            100                 105                 110
Ile Gly Arg Ile Met Glu Leu Asn Asn Asn Asp Phe Asp Pro Tyr Arg
        115                 120                 125
Glu Phe Leu Ile Leu Glu Ala Met Leu Ser Ser Asp Pro Lys Asn His
    130                 135                 140
His Val Trp Ser Tyr Arg Lys Trp Leu Val Asp Thr Phe Asp Leu His
145                 150                 155                 160
Asn Asp Ala Lys Glu Leu Ser Phe Val Asp Lys Val Ile Asp Thr Asp
                165                 170                 175
Leu Lys Asn Asn Ser Ala Trp Ser His Arg Phe Phe Leu Leu Phe Ser
            180                 185                 190
Lys Lys His Leu Ala Thr Asp Asn Thr Ile Asp Glu Glu Leu Asn Tyr
        195                 200                 205
Val Lys Asp Lys Ile Val Lys Cys Pro Gln Asn Pro Ser Thr Trp Asn
    210                 215                 220
Tyr Leu Leu Gly Ile His Glu Arg Phe Asp Arg Ser Ile Thr Gln Leu
225                 230                 235                 240
Glu Glu Phe Ser Leu Gln Phe Val Asp Leu Glu Lys Asp Gln Val Thr
                245                 250                 255
Ser Ser Phe Ala Leu Glu Thr Leu Ala Lys Ile Tyr Thr Gln Gln Lys
            260                 265                 270
Lys Tyr Asn Glu Ala Arg Thr Val Tyr Asp Leu Leu Lys Ser Lys Tyr
        275                 280                 285
Asp Pro Ile Arg Ser Asn Phe Trp Asp Tyr Gln Ile Ser Lys Leu Thr
    290                 295                 300
Ser Val
305
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 934 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 260..832

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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ACTTTTGCTT TCAGTTTTTT CTTTTTTTAG CACAAGAGAA AAGTATTCAG CTCATAAATA     240
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                       1               5                  10

GGT GCC GTT GGT AAA ACT TGC TTA TTA ATC TCG TAT ACC ACT AGT AAA      340
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            15                  20                  25

TTT CCA GCT GAT TAT GTT CCT ACT GTT TTT GAT AAT TAT GCT GTA ACC      388
Phe Pro Ala Asp Tyr Val Pro Thr Val Phe Asp Asn Tyr Ala Val Thr
        30                  35                  40

GTG ATG ATA GGA GAC GAA CCA TTT ACC TTG GGA TTA TTT GAT ACT GCT      436
Val Met Ile Gly Asp Glu Pro Phe Thr Leu Gly Leu Phe Asp Thr Ala
    45                  50                  55

GGT CAA GAA GAT TAC GAC AGA TTA AGG CCT TTG TCA TAT CCA TCG ACT      484
Gly Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro Ser Thr
60                  65                  70                  75

GAT GTA TTC CTT GTT TGT TTT TCC GTC ATT TCT CCT GCT TCG TTT GAA      532
Asp Val Phe Leu Val Cys Phe Ser Val Ile Ser Pro Ala Ser Phe Glu
                80                  85                  90

AAT GTT AAA GAA AAA TGG TTC CCA GAA GTT CAT CAC CAT TGT CCC GGT      580
Asn Val Lys Glu Lys Trp Phe Pro Glu Val His His His Cys Pro Gly
            95                  100                 105

GTG CCA ATA ATT ATT GTC GGT ACC CAA ACT GAT TTA CGA AAC GAT GAT      628
Val Pro Ile Ile Ile Val Gly Thr Gln Thr Asp Leu Arg Asn Asp Asp
        110                 115                 120

GTT ATT TTA CAG AGA TTG CAC AGA CAA AAA TTG TCC CCA ATC ACC CAG      676
Val Ile Leu Gln Arg Leu His Arg Gln Lys Leu Ser Pro Ile Thr Gln
    125                 130                 135

GAA CAG GGT GAA AAA TTG GCT AAG GAA TTG AGA GCT GTC AAG TAT GTT      724
Glu Gln Gly Glu Lys Leu Ala Lys Glu Leu Arg Ala Val Lys Tyr Val
140                 145                 150                 155

GAG TGT TCT GCA TTG ACT CAA AGA GGA TTG AAA ACA GTG TTT GAC GAG      772
Glu Cys Ser Ala Leu Thr Gln Arg Gly Leu Lys Thr Val Phe Asp Glu
                160                 165                 170

GCT ATA GTA GCT GCA TTA GAA CCT CCT GTA ATT AAA AAA TCG AAA AAG      820
Ala Ile Val Ala Ala Leu Glu Pro Pro Val Ile Lys Lys Ser Lys Lys
            175                 180                 185

TGT ACT ATT TTA TAGGTCGGCG ATACTAGAAG ATAGAGGATA TTGGAAATAG          872
Cys Thr Ile Leu
        190

GGCATACATG AGATATTGAA TATCTATCAT TAAATATATA ATTAGTTTTT TTCAAAAAAA    932

AA                                                                   934
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Gln Thr Ile Lys Cys Val Val Val Gly Asp Gly Ala Val Gly Lys
 1               5                  10                  15

Thr Cys Leu Leu Ile Ser Tyr Thr Thr Ser Lys Phe Pro Ala Asp Tyr
            20                  25                  30

Val Pro Thr Val Phe Asp Asn Tyr Ala Val Thr Val Met Ile Gly Asp
        35                  40                  45

Glu Pro Phe Thr Leu Gly Leu Phe Asp Thr Ala Gly Gln Glu Asp Tyr
    50                  55                  60

Asp Arg Leu Arg Pro Leu Ser Tyr Pro Ser Thr Asp Val Phe Leu Val
65                  70                  75                  80

Cys Phe Ser Val Ile Ser Pro Ala Ser Phe Glu Asn Val Lys Glu Lys
                85                  90                  95

Trp Phe Pro Glu Val His His His Cys Pro Gly Val Pro Ile Ile Ile
            100                 105                 110

Val Gly Thr Gln Thr Asp Leu Arg Asn Asp Asp Val Ile Leu Gln Arg
        115                 120                 125

Leu His Arg Gln Lys Leu Ser Pro Ile Thr Gln Glu Gln Gly Glu Lys
    130                 135                 140

Leu Ala Lys Glu Leu Arg Ala Val Lys Tyr Val Glu Cys Ser Ala Leu
145                 150                 155                 160

Thr Gln Arg Gly Leu Lys Thr Val Phe Asp Glu Ala Ile Val Ala Ala
                165                 170                 175

Leu Glu Pro Pro Val Ile Lys Lys Ser Lys Lys Cys Thr Ile Leu
            180                 185                 190
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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. An assay for identifying compounds having potential anti-fungal activity, comprising:

(a) forming a reaction mixture including:

(i) a fungal geranylgeranyl transferase (GGPTase),

(ii) a GGPTase substrate, and

(iii) a test compound; and

(b) detecting interaction of the GGPTase substrate with the GGPTase,

wherein a statistically significant decrease in the interaction of the GGPTase substrate and GGPTase in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential anti-fungal activity for the test compound.

2. The assay of claim 1, wherein GGPTase substrate comprises target polypeptide comprising a fungal Rho-like GTPase, or a polypeptide portion thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase.

3. The assay of claim 1, wherein the reaction mixture is a prenylation system including an activated geranylgeranyl group, and the step of detecting the interaction of the GGPTase substrate with the GGPTase comprises detecting conjugation of the geranylgeranyl group to the GGPTase substrate.

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4. The assay of any of claims 3 or 23, wherein at least one of the geranylgeranyl group and the GGPTase substrate comprises a detectable label, and the level of geranylgeranyl group-conjugated to the GGPTase substrate is quantified by detecting the label in at least one of the GGPTase substrate, the geranylgeranyl group, and geranylgeranyl-conjugated GGPTase substrate.

5. The assay of claim 1, wherein the step of detecting the interaction of the GGPTase substrate with the GGPTase comprises detecting the formation of complexes including the GGPTase substrate with the GGPTase.

6. The assay of claim 5, wherein at least one of the GGPTase and the GGPTase substrate comprises a detectable label, and the level of GGPTase/GGPTase substrate complexes formed in the reaction mixture is quantified by detecting the label in at least one of the GGPTase substrate, the GGPTase, and GGPTase/GGPTase substrate complexes.

7. The assay of any of claims 4 or 6, wherein the label group is selected from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

8. The assay of claim 4, wherein the substrate target comprises a fluorescent label, the fluorescent characterization of which is altered by the level of prenylation of the substrate target.

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9. The assay of claim 8, wherein the substrate target comprises a dansylated peptide substrate of the fungal GGPTase.

10. The assay of any of claims 3 or 23, wherein conjugation of the geranylgeranyl group to the GGPTase substrate is detected by an immunoassay.

11. The assay of claim 5, wherein the formation of protein-protein complexes including the GGPTase substrate with the GGPTase is detected by an immunoassay.

12. The assay of any of claims 1 or 23, wherein the reaction mixure is reconstituted protein mixture.

13. The assay of any of claims 1 or 23, wherein the reaction mixure comprises a cell lysate.

14. The assay of any of claims 2 or 23, wherein the fungal Rho-like GPTase is selected from the group consisting of Rho1, Rho2, Rsr1/Bud1 and Cdc42, and homologs thereof.

15. The assay of claim 1, wherein the reaction mixture is a whole cell comprising heterologous nucleic acid recombinantly expressing one or more of the fungal GGPTase subunits and GGPTase substrate.

16. The assay of claim 1, wherein the reaction mixture is a whole cell comprising a heterologous reporter gene construct comprising a reporter gene in operable linkage with a transcriptional regulatory sequence sensitive to intracellular signals transduced by interaction of the GGPTase substrate and GGPTase.

17. The assay of any of claims 1, 23, 24, 25, or 26 wherein the assay is repeated for a variegated library of at least 100 different test compounds.

18. The assay of any of claims 1, 23, 24, 25, or 26 wherein the test compound is selected from the group consisting of small organic molecules, and natural product extracts.

19. The assay of any of claims 2 or 23, wherein one or more of the GGPTase and target polypeptide are derived from a human pathogen which is implicated in mycotic infection.

20. The assay of claim 19, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis.

21. The assay of claim 19, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*,

Aspergillus nidulans, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

22. The assay of claim 19, wherein the human pathogen is *Pneumocystis carinii*.

23. An assay for identifying compounds having potential anti-fungal activity, comprising:

(a) forming a cell-free reaction mixture including:

(i) a fungal geranylgeranyl transferase (GGPTase),

(ii) a target polypeptide comprising a fungal Rho-like GTPase, or a polypeptide portion thereof including a prenylation site

(iii) an activated geranylgeranyl group,

(iv) a divalent cation, and

(v) a test compound;

(b) detecting conjugation of the gernaylgernayl group of the target polypeptide in the reaction mixture,

wherein a statistically significant decrease in the prenylation of the target polypeptide and GGPTase in the presence of the test compound, relative to the level of prenylation in the absence of the test compound, indicates a potential anti-fungal activity for the test compound.

24. A method of identifying an agent which disrupts the ability of an geranylgeranyl protein transferase (GGPTase) to bind to a fungal GTPase, comprising:

i. providing an interaction trap system including

(a) a first fusion protein comprising at least a portion of a fungal GGPTase subunit,

(b) second fusion protein comprising at least a portion of a fungal GTPase, and

(c) a reporter gene, including a transcriptional regulatory sequence sensitive to interactions between the GGPTase portion of the first fusion protein and the GTPase portion of the second polypeptide;

ii. contacting the interaction trap system with a candidate agent;

iii. measuring the level of expression of a reporter gene in the presence of the candidate agent; and

iv. comparing the level of expression of the reporter gene in the presence of the candidate agent to a level of expression in the absence of the candidate agent,

wherein a decrease in the level of expression of the reporter gen in the presence of the candidate agent is indicative of an agent that inhibits interaction of the GGPTase and GTPase.

25. An assay for identifying compounds having potential anti-fungal activity, comprising:

(i) providing a first recombinant cell including a first prenylation substrate derived from a Rho-like GTPase which is a substrate for a geranylgeranyl transferase expressed by the cell;

(ii) providing a second recombinant cell including a second prenylation substrate identical to the first prenylation substrate except that it is mutated to be a substrate for a farnesyl transferase expressed by the recombinant cell;

(iii) contacting the first and second cells with a candidate agent; and

(iv) comparing the level of prenylation of the Rho-like GTPases in first and second cells,

wherein a statistically significant decrease in the prenylation of the first prenylation substrate, relative to the level of prenylation of the second prenylation substrate, is indicative of an agent that inhibits interaction of a GGPTase and GTPase.

26. An assay for screening test compounds to identify agents which modulate the interaction of a fungal geranylgeranyl transferase (GGPTase) with a fungal Rho-like GTPase, comprising:

(i) providing a cell expressing a recombinant form of one or more of a fungal GGPTase and a fungal Rho-like GTPase;

(ii) contacting the cell with a test compound; and

(iii) detecting the level of interaction of the GGPTase and Rho-like GTPase,

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wherein a statistically significant change in the level of interaction of the GGPTase and Rho-like GTPase is indicative of an agent that modulates the interaction of those two proteins.

27. The assay of claim 23, wherein one or both of a GGPTase subunit or the Rho-like GTPase are fusion proteins.

28. The assay of claim 23, wherein the level of interaction of the GGPTase and Rho-like GTPase is detected by detecting prenylation of the Rho-like GTPase.

29. The assay of claim 25, wherein the Rho-like GTPase is a fusion protein further comprising a transcriptional regulatory protein, and level of prenylation of the Rho-like GTPase is detected by measuring the level of expression of a reporter gene construct which is sensitive to the transcriptional regulatory protein portion of the fusion protein, wherein inhibition of prenylation of the fusion protein results in loss of membrane partitioning of the fusion protein and increases expression of the reporter gene construct.

30. The assay of any of claims 1, 20, 21, 22 or 23, which comprises a further step of preparing a pharmaceutical preparation of one or more compounds identified as having potential antifungal activity.

31. An assay for identifying compounds having potential antifungal activity, comprising:

(i) forming a reaction mixture including a fungal Rho-like GTPase, a fungal protein kinase C (PKC), and a test compound; and

(ii) detecting interaction of the Rho-like GTPase and PKC, wherein a statistically significant decrease in the interaction of the Rho-like GTPase and PKC in

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the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential antifungal activity for the test compound.

32. The assay of claim 31, wherein the reaction mixture is a kinase system including ATP and a PKC substrate, and the step of detecting interaction of the GTPase and PKC comprises detecting phosphorylation of the PKC substrate by a PKC/GTPase complex.

33. The assay of claim 32, wherein at least one of the PKC substrate and ATP comprises a detectable label, and the level of phosphorylation of the PKC substrate is quantified by detecting the label in at least one of the PKC substrate or ATP.

34. The assay of claim 31, wherein the step of detecting the interaction of the GTPase with the PKC comprises detecting the formation of protein-protein complexes including the GTPase and PKC.

35. The assay of claim 34, wherein at least one of the PKC and GTPase comprises a detectable label, and the level of PKC/GTPase complexes formed in the reaction mixture is quantified by detecting the label in at least one of the GTPase, the PKC, and PKC/GTPase complexes.

36. The assay of any of claims 33 or 35, wherein the label group is selected from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

37. The assay of claim 36, wherein the detectable label is a protein having a measurable activity, and one of the PKC or GTPase is fusion protein including the detectable label.

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38. The assay of claim 33, wherein the PKC substrate comprises a fluorescent label, the fluorescent characterization of which is altered by the level of phosphorylation of the PKC substrate.

39. The assay of claim 33, wherein phosphorylation of the PKC substrate is detected by immunoassay.

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40. The assay of claim 34, wherein the formation of protein-protein complexes including the GTPase and PKC is detected by an immunoassay.

41. The assay of claim 31, wherein the reaction mixure is reconstituted protein mixture.

42. The assay of claim 31, wherein the reaction mixure comprises a cell lysate.

43. The assay of claim 31, wherein the GPTase is selected from the group consisting of Rho1, Rho2, Rsr1/Bud1 and Cdc42, and fungal homologs thereof.

44. The assay of claim 31, wherein the reaction mixture is a whole cell comprising heterologous nucleic acid recombinantly expressing one or more of the PKC and GTPase.

45. The assay of claim 31, wherein the reaction mixture is a whole cell comprising a heterologous reporter gene construct comprising a reporter gene in operable linkage with a transcriptional regulatory sequence sensitive to intracellular signals transduced by interaction of the GTPase and PKC.

46. The assay of claim 31, wherein the assay is repeated for a variegated library of at least 100 different test compounds.

47. The assay of claim 31, wherein the test compound is selected from the group consisting of small organic molecules, and natural product extracts.

48. The assay of claim 31, wherein one or more of the PKC and GTPase are derived from a human pathogen which is implicated in mycotic infection.

49. The assay of claim 48, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis.

50. The assay of claim 48, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

51. The assay of claim 48, wherein the human pathogen is *Pneumocystis carinii*.

52. The assay of claim 31, which comprises a further step of preparing a pharmaceutical preparation of one or more compounds identified as having potential antifungal activity.

53. An assay for identifying compounds having potential antifungal activity, comprising:

(i) forming a reaction mixture including a fungal Rho-like GTPase, a fungal glucan synthase complex or subunit thereof (GS protein), and a test compound; and

(ii) detecting interaction of the Rho-like GTPase and GS protein, wherein a statistically significant decrease in the interaction of the Rho-like GTPase and GS protein in the presence of the test compound, relative to the level of interaction in the absence of the test compound, indicates a potential antifungal activity for the test compound.

54. The assay of claim 53, wherein the reaction mixture is a glucan synthesis system including a GTP and a UDP-glucose, and the step of detecting interaction of the GTPase and GS protein comprises detecting formation of glucan polymers in the reaction mixture.

55. The assay of claim 54, wherein the UDP-glucose comprises a detectable label, and the level of glucan polymer formation is quantified by detecting the labelled glucan polymers.

56. The assay of claim 53, wherein the step of detecting the interaction of the GTPase with the GS protein comprises detecting the formation of protein-protein complexes including the GTPase and GS protein.

57. The assay of claim 56, wherein at least one of the GS protein and GTPase comprises a detectable label, and the level of GS protein/GTPase complexes formed in

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the reaction mixture is quantified by detecting the label in at least one of the GTPase, the GS protein, and GS protein/GTPase complexes.

58. The assay of any of claims 53 or 57, wherein the label group is selected from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

59. The assay of claim 56, wherein the formation of protein-protein complexes including the GTPase and GS protein is detected by an immunoassay.

60. The assay of claim 53, wherein the reaction mixture is reconstituted protein mixture.

61. The assay of claim 53, wherein the reaction mixture comprises a cell lysate.

62. The assay of claim 53, wherein the GTPase is selected from the group consisting of Rho1, Rho2, Rsr1/Bud1 and Cdc42, and fungal homologs thereof.

63. The assay of claim 53, wherein the reaction mixture is a whole cell comprising heterologous nucleic acid recombinantly expressing one or more of the GS protein and GTPase.

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64. The assay of claim 53, wherein the assay is repeated for a variegated library of at least 100 different test compounds.

65. The assay of claim 53, wherein the test compound is selected from the group consisting of small organic molecules, and natural product extracts.

66. The assay of claim 53, wherein one or more of the GS protein and GTPase are derived from a human pathogen which is implicated in mycotic infection.

67. The assay of claim 66, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis.

68. The assay of claim 66, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

69. The assay of claim 66, wherein the human pathogen is *Pneumocystis carinii*.

70. The assay of claim 53, which comprises a further step of preparing a pharmaceutical preparation of one or more compounds identified as having potential antifungal activity.

71. A recombinant cell comprising (i) exogenous nucleic acid encoding one or more subunits of a fungal geranylgeranyl protein transferase (GGPTase), and (ii) exogenous nucleic acid encoding a fungal Rho-like GTPase or a fragment thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase.

72. The cell of claim 71, wherein one or more of the nucleic acids encoding the GGPTase and GTPase are derived from a human pathogen which is implicated in mycotic infection.

73. The cell of claim 72, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis.

74. The cell of claim 72, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

75. The cell of claim 72, wherein the human pathogen is *Pneumocystis carinii*.

76. The cell of claim 71, which cell is a recombinantly manipulate yeast cell selected from the group consisting of *Kluyverei spp*, *Schizosaccharomyces spp*, *Ustilaqo spp* and *Saccharomyces spp*.

77. The cell of claim 71, which cell is a recombinantly manipulate *Schizosaccharomyces cerivisae* cell.

78. The cell of claim 71, which cell is constitutively or inducibly defective for an endogenous activity corresponding to one or more of the GGPTase and GTPase encoded by the exogenous nucleic acids.

79. A reconstituted protein mixture or a cell lysate mixture comprising (i) a recombinant fungal geranylgeranyl protein transferase (GGPTase), and (ii) a recombinant fungal Rho-like GTPase or a fragment thereof including at least one of (a) a prenylation site which can be enzymatically prenylated by the GGPTase, or (b) a GGPTase binding sequence which specifically binds the GGPTase.

80. The mixture of claim 79, wherein one or more of the recombinant GGPTase and GTPase are derived from a human pathogen which is implicated in mycotic infection.

81. The mixture of claim 80, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis.

82. The mixture of claim 80, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

83. The mixture of claim 80, wherein the human pathogen is *Pneumocystis carinii*.

84. A recombinant cell comprising (i) exogenous nucleic acid encoding a fungal Rho-like GTPase, and (ii) exogenous nucleic acid encoding a fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or one or more subunits of a fungal glucan synthase.

85. A reconstituted protein mixture or a cell lysate mixture comprising (i) a recombinant fungal Rho-like GTPase, and (ii) a recombinant fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or a fungal glucan synthase.

86. A recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen.

87. A recombinant cell comprising exogenous nucleic acid encoding a Rho-like GTPase cloned from a human fungal pathogen.

88. A recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a glucan synthase cloned from a human fungal pathogen.

89. A recombinant cell comprising exogenous nucleic acid encoding a protein kinase C cloned from a human fungal pathogen.

90. A reconstituted protein mixture or a cell lysate mixture comprising one or more subunits of a recombinant geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen.

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91. A reconstituted protein mixture or a cell lysate mixture comprising a recombinant Rho-like GTPase cloned from a human fungal pathogen.

92. A reconstituted protein mixture or a cell lysate mixture comprising one or more recombinant subunits of a glucan synthase cloned from a human fungal pathogen.

93. A reconstituted protein mixture or a cell lysate mixture comprising a recombinant protein kinase C cloned from a human fungal pathogen.

94. A cell of any one of claims 86-89, or a reconstituted protein mixture or a cell lysate of any one of claims 90 to 93 wherein the human pathogen is selected from a group consisting of *Candida albicans, Candida stellatoidea, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida pseudotropicalis, Candida quillermondii, Candida rugosa, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Rhizopus arrhizus, Rhizopus oryzae, Absidia corymbifera, Absidia ramosa,* and *Mucor pusillus.*

95. A purified and/or recombinant polypeptide comprising a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1 or SEQ ID No. 5, the GTPase sequence (i) directing the binding of said polypeptide to a glucan synthase subunit, (ii) directing the binding of said polypeptide to PKC, (iii)

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serving as a substrate for prenylation by a GGPTase, or (iv) having a GTP hydrolytic activity, or a combination thereof.

96. A purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 3, the RAM2 sequence (i) directing the binding of said polypeptide to a GGPTase or FPTase β subunit, or (ii) directing the binding of said polypeptide to a Rho1-like GTPase, or a combination thereof.

97. The polypeptide of any of claims 95 or 96, wherein said GTPase sequence is at least 80% identical to the polypeptide represented by SEQ ID No. 2 or SEQ ID No. 6, or said RAM2 sequence is at least 80% identical to the polypeptide represented by SEQ ID No. 4.

98. The polypeptide of claim 97, wherein said GTPase sequence is at least 90% identical to the polypeptide represented by SEQ ID No. 2 or SEQ ID No. 6, or said RAM2 sequence is at least 90% identical to the polypeptide represented by SEQ ID No. 4.

99. The polypeptide of claim 98, wherein said GTPase sequence is identical to the polypeptide represented by SEQ ID No. 2 or SEQ ID No. 4, or said RAM2 sequence is identical to the polypeptide represented by SEQ ID No. 4.

100. The polypeptide of any of claims 95 or 96, wherein said GTPase sequence or said RAM2 sequence, as appropriate, is is encoded by an endogenous gene from *Candida spp*.

101. The polypeptide of claim 100, wherein said *Candida spp* is selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii* and *Candida rugosa*.

102. The polypeptide of any of claims 95 or 96, which polypeptide is a fusion protein.

103. The polypeptide of claim 100, wherein said fusion protein further includes, in addition to said RAM2 sequence, a RAM1 or cdc43 sequence, said fusion protein possessing prenylation activity.

104. The polypeptide of claim 102, wherein said fusion protein further includes, in addition to said GTPase or RAM2 sequence, as appropriate, a second polypeptide portion selected from the group consisting of a DNA binding domain and a transciptional activitation domain, said fusion protein being functional in a two-hybrid assay.

105. The polypeptide of any of claims 95 or 96, which polypeptide is a recombinant protein.

106. The polypeptide of any of claims 95 or 96, which polypeptide is substantially purified.

107. A purified protein complex comprising the polypeptide of any of claims 95 or 96.

108. The protein complex of claim 107, wherein said complex comprises, in addition to the polypeptide having a GTPase sequence, a glucan synthase subunit, a PKC, a GGPTase, or a combination thereof.

109. The protein complex of claim 107, wherein said complex comprises, in addition to the polypeptide having a RAM2 sequence, a GGPTase β subunit, an FPTase β subunit, a Rho1-like GTPase, or a combination thereof.

110. An isolated nucleic acid comprising a coding sequence encoding the polypeptide of any of claims 95 or 96.

111. An expression construct comprising the nucleic acid of claim 110.

112. A host cell transformed with the expression construct of claim 111.

113. A method for producing a recombinant protein comprising culturing the host cell of claim 112 under conditions sufficient to produce a cell culture expressing said polypeptide, and isolating said polypeptide from said cell culture.

114. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 1 (sense or antisense) and selectively detects a *CaRho1* gene encoding a protein having GTP hydrolytic activity.

115. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 5 (sense or antisense) and selectively detects a *CaCdc42* gene encoding a protein having GTP hydrolytic activity.

116. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID No. 3 (sense or antisense) and selectively detects a *CaRAM2* gene encoding a GGPTase or FPTase subunit.

117. A diagnostic test kit for detecting *Candida* cells comprising the nucleic acid of claim 114, 115 or 116.

118. The nucleic acid of claim 114, 115 or 116, wherein said nucleic acid is an antisense oligonucleotide which hybridizes to *CaRho1*, *CaCdc42* or *CaRam2* gene, as appropriate, and inhibits expression of the gene.

119. The nucleic acid of claim 114, 115 or 116, wherein the nucleic acid is at least 25 nucleotides in length.

120. The nucleic acid of claim 114, 115 or 116, wherein the nucleic acid is at least 50 nucleotides in length.

121. The kit of claim 117, wherein the nucleic acid is labelled with a detectable label.

122. The kit of claim 121, wherein the detectable label is selected from the group consisting of: an enzyme, an enzyme substrate, a coenzyme, an enzyme inhibitor, a fluorescent marker, a chromophore, a luminescent marker, and a radioisotope.

123. The kit of claim 117, wherein said *Candida* cell is selected from the group consisting of *Candida albicans, Candida stellatoidea, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida pseudotropicalis, Candida quillermondii* and *Candida rugosa.*

124. A isolated, recombinant and/or monoclonal antibody which is specificallly cross-reactive with the GTPase sequence of claim 95 or the RAM2 sequence of claim 96.

125. The antibody of claim 124, wherein the antibody is labelled with a detectable label.

126. The antibody of claim 125, wherein the detectable label is selected from the group consisting of: an enzyme, an enzyme substrate, a coenzyme, an enzyme inhibitor, a fluorescent marker, a chromophore, a luminescent marker, and a radioisotope.

127. A method for detecting the presence of *Candida* cells comprising detecting, in a sample, the presence of one or more of a (i) a nucleic acid encoding a *Candida* protein selected from the group consisting of *CaRho1, CaCdc42* and *CaRAM2*, and (ii) a *Candida* protein selected from the group consisting of *CaRho1, CaCdc42* and *CaRAM2*.

128. The method of claim 127, wherein the sample is a sample of cells, tissue and/or bodily fluid.

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129. The method of claim 127, wherein the nucleic acid is detected by hybrization and/or amplification using one or more probes comprising a nucleic acid of claim 119.

130. The method of claim 127, wherein the protein is detected by immunoassay using an antibody of claim 124.

131. An assay for identifying compounds having potential anti-fungal activity, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

132. A method of identifying an agent which disrupts the ability of a geranylgeranyl protein transferase (GGPTase) to bind to a fungal GTPase, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

133. An assay for screening test compounds to identify agents which modulate the interaction of a fungal geranylgeranyl transferase (GGPTase) with a fungal Rho-like GTPase, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

134. A recombinant cell comprising (i) exogenous nucleic acid encoding one or more subunits of a fungal geranylgeranyl protein transferase (GGPTase), and (ii) exogenous nucleic acid encoding a fungal Rho-like GTPase or a fragment thereof, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

135. A reconstituted protein mixture of a cell lysate mixture comprising (i) a recombinant fungal geranylgeranyl protein transferase (GGPTase), and (ii) a

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recombinant fungal Rho-like GTPase or a fragment thereof, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

136. A recombinant cell comprising (i) exogenous nucleic acid encoding a fungal Rho-like GTPase and (ii) exogenous nucleic acid encoding a fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or one or more subunits of a fungal glucan synthase, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

137. A reconstituted protein mixture or a cell lysate mixture comprising (i) a recombinant fungal Rho-like GTPase and (ii) a recombinant fungal protein selected from the group consisting of a fungal protein kinase C (PKC) or a fungal glucan synthase, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

138. A recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

139. A recombinant cell comprising exogenous nucleic acid encoding a Rho-like GTPase cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

140. A recombinant cell comprising exogenous nucleic acid encoding one or more subunits of a glucan synthase cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

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141. A recombinant cell comprising exogenous nucleic acid encoding a protein kinase C cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

142. A reconstituted protein mixture or a cell lysate comprising one or more subunits of a recombinant geranylgeranyl protein transferase (GGPTase) cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

143. A reconstituted protein mixture or a cell lysate mixture comprising a recombinant Rho-like GTPase cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

144. A reconstituted protein mixture or a cell lysate mixture comprising one or more recombinant subunits of a glucan synthase cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

145. A reconstituted protein mixture or a cell lysate mixture comprising a recombinant protein kinase C cloned from a human fungal pathogen, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

146. A purified and/or recombinant polypeptide comprising a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

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147. A purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

148. A purified protein complex comprising the polypeptide of any one of (a) a purified and/or recombinant polypeptide comprising a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, or (b) a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

149. An isolated nucleic acid comprising a coding sequence encoding the polypeptide of any one of (a) a purified and/or recombinant polypeptide comprising GTPase sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, or (b) a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

150. An expression construct comprising nucleic acid comprising a coding sequence encoding the polypeptide of any one of (a) a purified and/or recombinant polypeptide comprising a GTPase sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 2, or (b) a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by nucleic acid which

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hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

151. A host cell transformed with a expression construct comprising a nucleic acid comprising a coding sequence encoding the polypeptide of any one of (a) a purified and/or recombinant polypeptide comprising a GTPase sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, or (b) a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

152. A method for producing a recombinant protein comprising culturing the host cell transformed with the expression construct comprising a nucleic acid comprising a coding sequence encoding the polypeptide of any one of (a) a purified and/or recombinant polypeptide comprising a GTPase sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, or (b) a purified and/or recombinant polypeptide comprising a RAM2 sequence encodable by nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

153. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID NO 1 (sense or antisense), substantially as herein described with reference to one or more of the examples but excluding comparative examples.

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154. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID NO 5 (sense or antisense), substantially as herein described with reference to one or more of the examples but excluding comparative examples.

155. An isolated nucleic acid which specifically hybridizes to the nucleic acid sequence of SEQ ID NO 3 (sense or antisense), substantially as herein described with reference to one or more of the examples but excluding comparative examples.

156. A diagnostic test kit for detecting *Candida* cells, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

157. An isolated, recombinant and/or monoclonal antibody which is specifically cross-reactive with a GTPase sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 1 or SEQ ID NO 5, or a RAM2 sequence encodable by a nucleic acid which hybridizes under stringent conditions to SEQ ID NO 3, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

158. A method for detecting the presence of *Candida* cells, substantially as herein described with reference to one or more of the examples but excluding comparative examples.

DATED this 27th day of September 1999

MITOTIX, INC. AND THE JOHNS HOPKINS UNIVERSITY

Attorney: DAVID A ADAMTHWAITE
Fellow Institute of Patent Attorneys of Australia
of BALDWIN SHELSTON WATERS

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Figure 1A

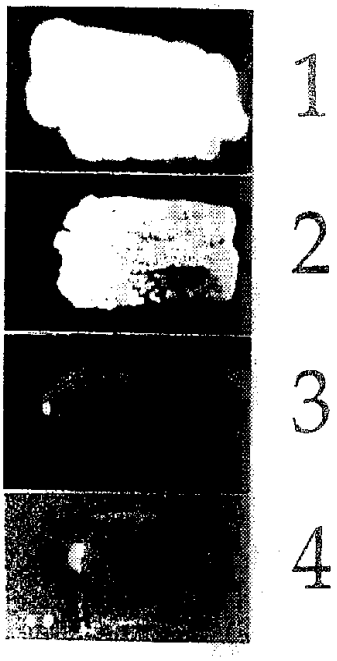


Figure 1B

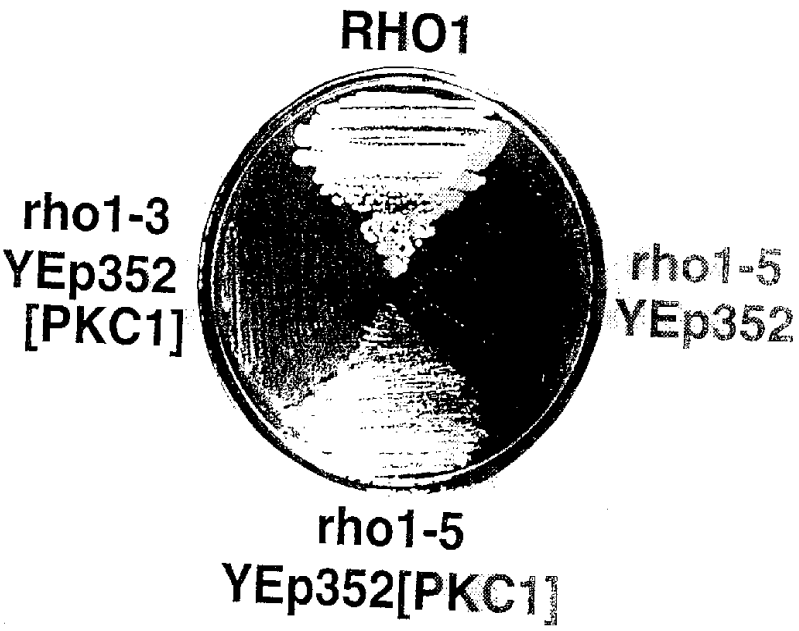


Figure 2

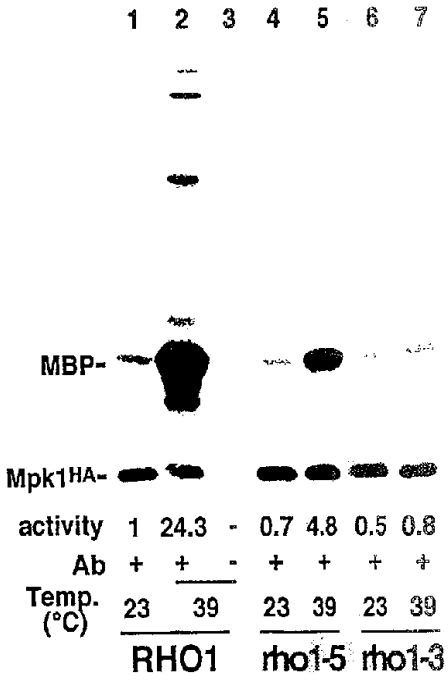

1 2 3 4 5 6 7
MBP-
Mpk1HA-
activity 1 24.3 - 0.7 4.8 0.5 0.8
Ab + + - + + + +
Temp. (°C) 23 39 23 39 23 39
RHO1
rho1-5
rho1-3

Figure 3

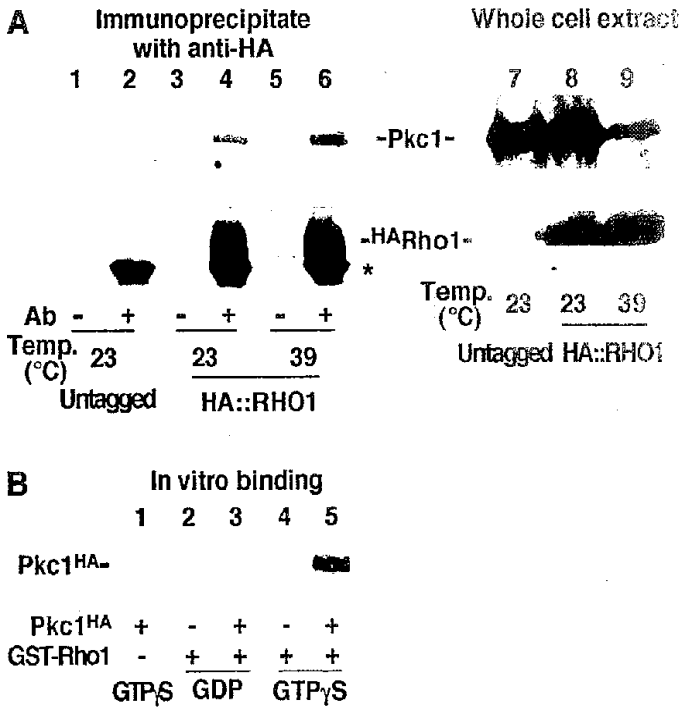
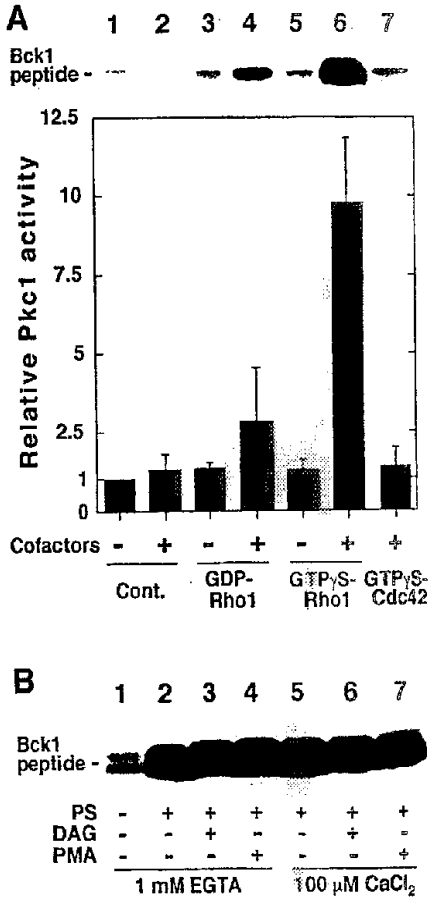

A
Immunoprecipitate with anti-HA
Whole cell extract
1 2 3 4 5 6
7 8 9
-Pkc1-
-HARho1-
*
Ab - + - + - +
Temp. (°C) 23 23 39
Untagged HA::RHO1
Temp. (°C) 23 23 39
Untagged HA::RHO1
B
In vitro binding
1 2 3 4 5
Pkc1HA-
Pkc1HA + - + - +
GST-Rho1 - + + + +
GTPγS GDP GTPγS

Figure 4


A
1 2 3 4 5 6 7
Bck1 peptide -
12.5
10
7.5
5
2.5
1
0
Relative Pkc1 activity
Cofactors - + - + - + +
Cont.
GDP-Rho1
GTPγS-Rho1
GTPγS-Cdc42
B
1 2 3 4 5 6 7
Bck1 peptide -
PS - + + + + + +
DAG - - + - - + -
PMA - - - + - - +
1 mM EGTA
100 μM CaCl2

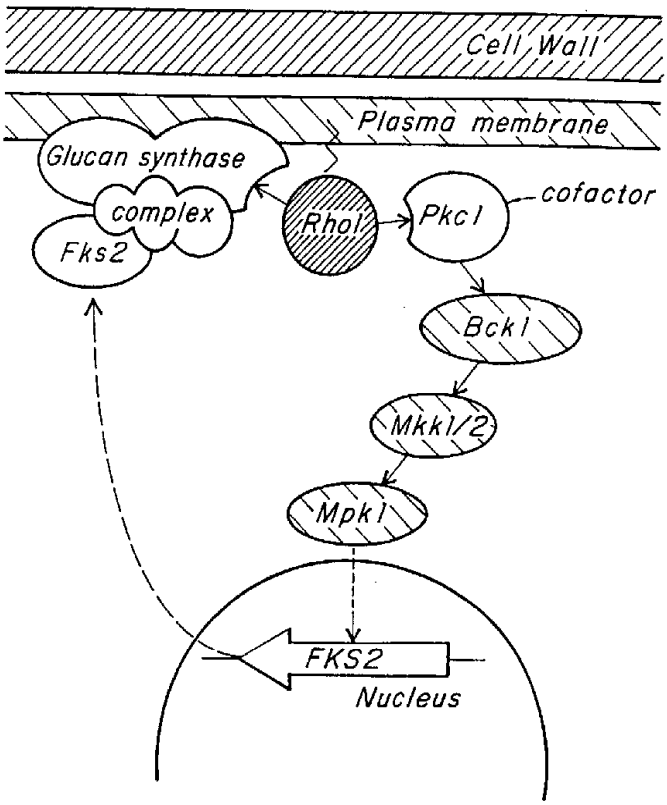

Cell Wall
Plasma membrane
Glucan synthase
complex
Fks2
Rho1
Pkc1
cofactor
Bck1
Mkk1/2
Mpk1
FKS2
Nucleus

FIG. 5

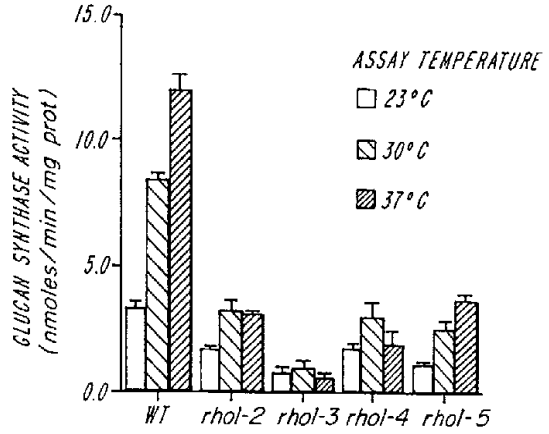

15.0
10.0
5.0
0.0
GLUCAN SYNTHASE ACTIVITY (nmoles/min/mg prot)
ASSAY TEMPERATURE
23°C
30°C
37°C
WT
rho1-2
rho1-3
rho1-4
rho1-5

FIG. 6A

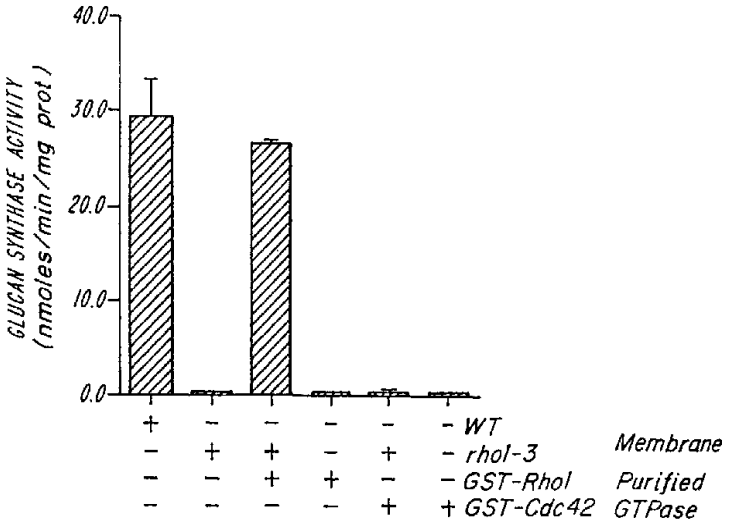

40.0
30.0
20.0
10.0
0.0
GLUCAN SYNTHASE ACTIVITY (nmoles/min/mg prot)
+ - - - - - WT
- + + - + - rho1-3
- - + + - - GST-Rho1
- - - - + + GST-Cdc42
Membrane
Purified
GTPase

FIG. 6B

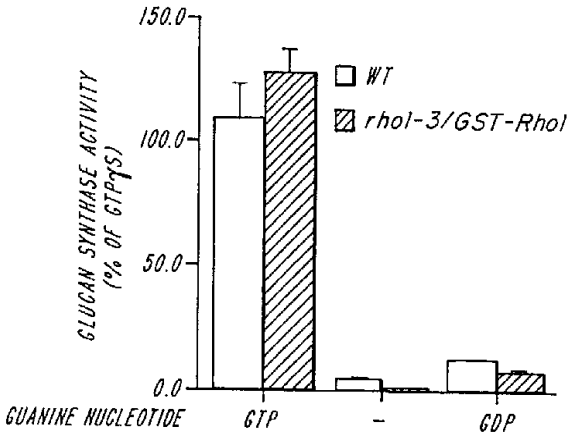

150.0
100.0
50.0
0.0
GLUCAN SYNTHASE ACTIVITY
(% OF GTPγS)
WT
rho1-3/GST-Rho1
GUANINE NUCLEOTIDE
GTP
-
GDP

FIG. 6C

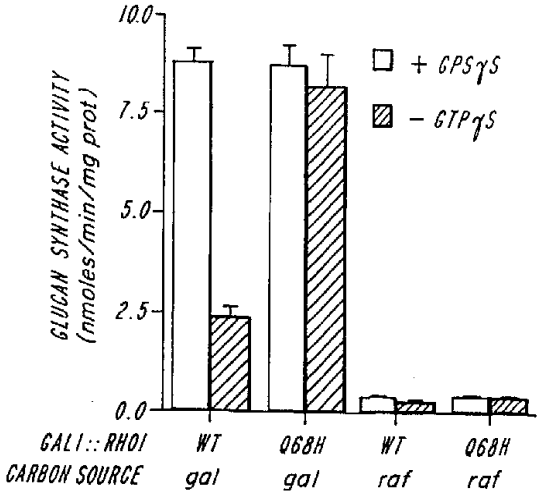
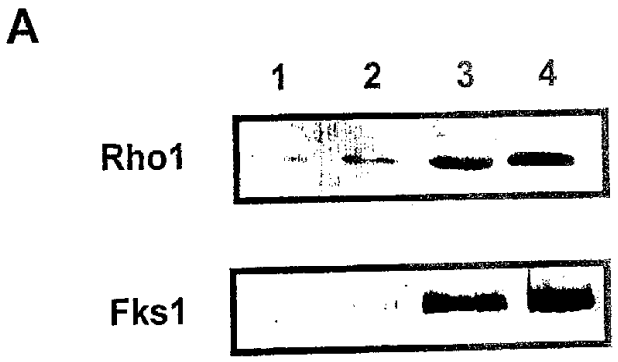
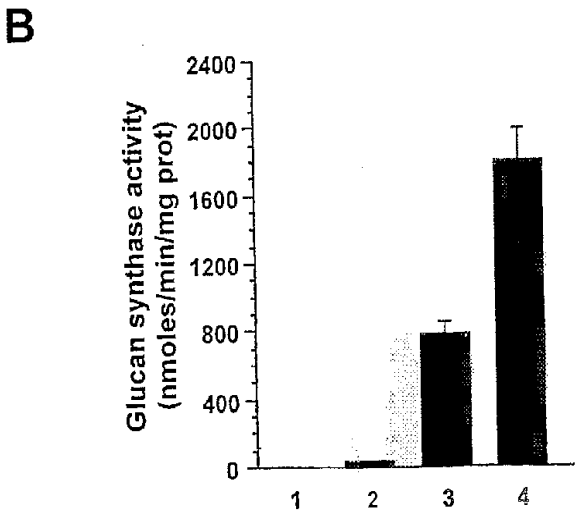

10.0
7.5
5.0
2.5
0.0
GLUCAN SYNTHASE ACTIVITY
(nmoles/min/mg prot.)
+ GPSγS
- GTPγS
GAL1::RHO1
CARBON SOURCE
WT
gal
Q68H
gal
WT
raf
Q68H
raf

FIG. 7

Figure 8


A
1
2
3
4
Rho1
Fks1


B
Glucan synthase activity
(nmoles/min/mg prot)
2400
2000
1600
1200
800
400
0
1
2
3
4

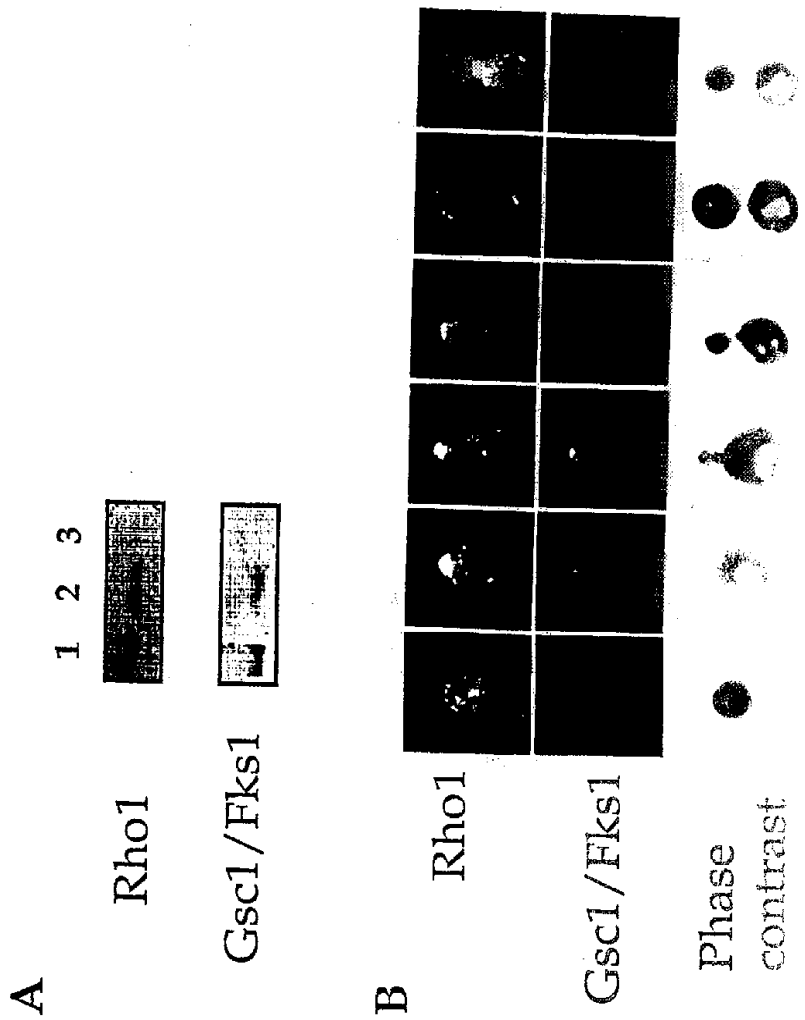
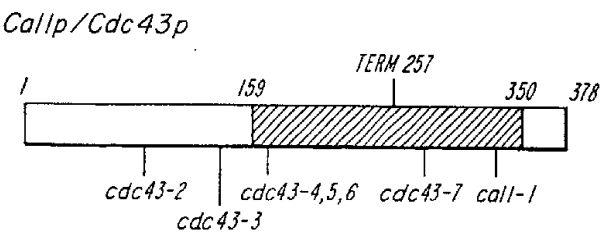


Figure 9



Cdc43-2p (S85F)	TENTVIS--GFVG
S. pombe	kEskglkysGFqa
RAT	edrsnldrcGFrG
HUMAN	edrsnlnrcGFrG
CDC43-3P (C138Y)	ARFVSKCQRPDRG
S. POMBE	knFVelCktsq-G
RAT	Aglral-QleD-G
HUMAN	Aglral-QleD-G
CDC43-4,5,6P (A171V/T/V)	LRFCYIAVAILYI
S. POMBE	mRqlYmA-----t
RAT	mRFvYcA-----s
HUMAN	mRFvYcA-----s
CDC43-7P (R280C)	DGGFQGRENKFAD
S. POMBE	sGGlnGRtNKdvD
RAT	qnGyhGRpNKpvD
HUMAN	qnGyhGRpNKpvD
CAL1-1P (G328S)	QKTLTGGFSKNDE
S. POMBE	Qh-alGGFSKTPG
RAT	dr-LvGGFaKwpd
HUMAN	dr-LvGGFaKwpd

FIG. 10

FIG. 11B

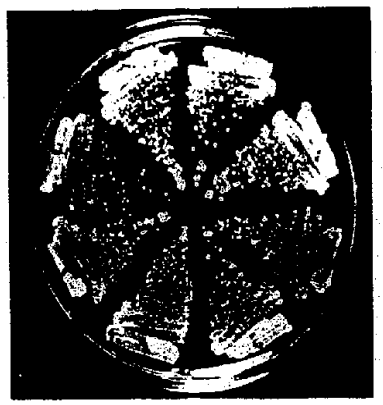


FIG. 11A

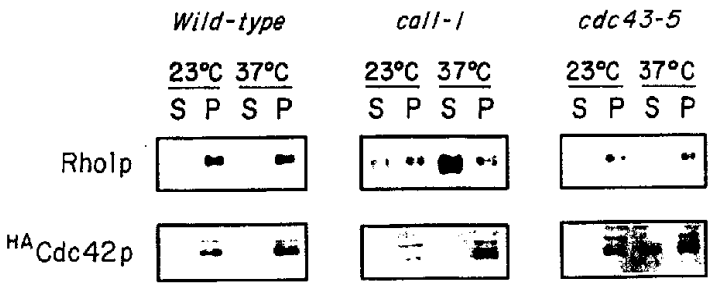


FIG. 12

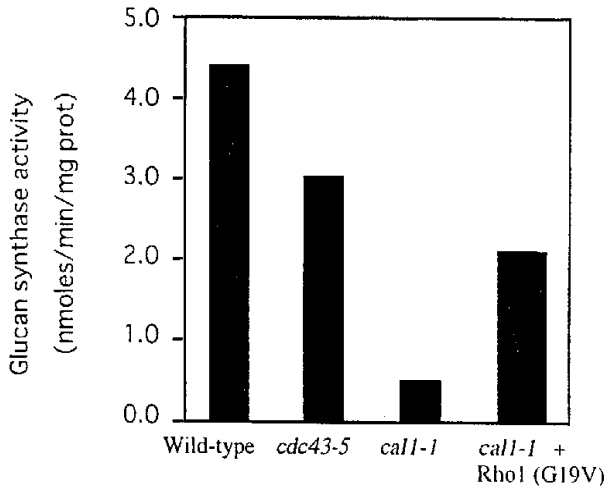

Glucan synthase activity
(nmoles/min/mg prot)
5.0
4.0
3.0
2.0
1.0
0.0
Wild-type
cdc43-5
cal1-1
cal1-1 +
Rho1 (G19V)

FIG. 13

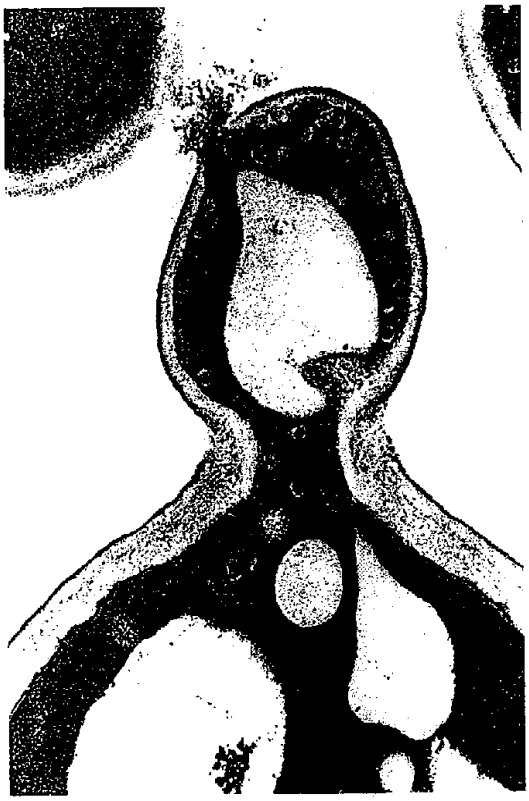


FIG. 14

Figure 15

CCGTCGATCCGATATAGCCAAATAGAATATATCTAGCTTGGAATTTATAT
ATAAATACATACATTCATGGAGTATTATTACTAATTAAAACTTATTTTC
CTCTCTACCACTGGCAAACATGTTAAGTCCATAGTAACCTTGCCACATACTT
TCTTTATCTGTCAAATACCCAACAAGTCCGGTCTTGTCACCTGCCGACAATT
TGTCGTAAATTGTGTACACTGAGTTATAATCTTTCCATCCTAAATTCTTGCG
AAGGTCTTTCAATTCATCAGCGTTTAATTCCACTTGAGCTGCAGTACCGTAT
TTCAATGGACCAACATTTATCCAATAGCCCCTTTCTATGTTACCTGATGGTA
ATGTCAAATTCTTTATAACATCCAAATAATCCAACAAATTCTCAGCCGTG
TCAATGAAAAATGCGGTTACCACCACAATGTTCTTATATGAATCTCTATTG
GGGATTTTGAAATGTCTAAAATCTTGATTATGTATATGCAACGTTTCTGGG
ATTTCACCTAGAGGTTTAAATTCAAAAGTTCTCAATTGAGATTCGGTGCTG
TAAAAATCAGAACAAGTATGAACATACGGGTACAATGTATAATTTTTGT
CTGATTTGGTATAGTTGAAATCAACAAAAGCATTCATCAACCCGGAATAT
TCAATCGAATGAACAGCACCAAAATCCCATTTAGCAAATGCGTGGGCTAA
TCTACCTAATCCTGACCCTGGGAAAACCAAACAAGTATCTTTTTTGGCATC
ATAAGGGATCAACGCCGAGAGTTGCAGGGCAACATATTCGTACATGGGTA
ATAATTCCAATCCAATATTTTCTGGATGCCAATCACGAGTATAATGACCT
AAAGCTTCAATTACTCGATAATTGGAAGCAGATGTTGTACTTGCATTTTTC
TTTTCTGAATTTAATAAATCGAAATCTTTTAACGAAATTCCATATTTAGC
GATAGCATGGTCAGCAACATCATTAACAAATTTGTAATTGGCACTGATAG
CATTATCGATTTTATCCAATTTTTTCAAATATCCAATATCTTTACATGCTT
TCTGCTGTTTGTATGACATGCGTTGGAATAGTTTTCTTCTCCGGTCGTTTTGA
GCTTTGGAACTGGTTTCATAGCTTTTCAAAGATTGAATTGCCGTTAAAACC
TCACGTTTTTGAACATCATTCAAGTTGACGAAATTTTGATAATTGGTGCCC
GCGGATTTAGGGATATTGGTGATTTGTTGTTTCAATGTCAGTTGAGCATTTT
CAACGATTGATCTGAACATGCTTGTACGAGAAATCATGTTTGTGAAAATA
AATGTTGAAACTAATATAGGGACTTGTGATTTCTGATAGACCAAAGATA
GTAGGTAGTAGACAATTATACAAAGAGGGACTGTTGTTGATATAAGTTG
AACATCCAGTAACATTATTCAAGAAGAAAGGTGAAATAGTGGGAATTA
AATATTACCCCAAATTACGGAGGAAACGGGAAAGATTGCTGTGGTATGGG
AGGAGGTGTAAAAAATGGTGGAAATAAGAAGACTGCAAATGTGCTTTAA
TCGACAATCGTTCCATCACCTTCCATTGTGAGGAAAGGGAGGAAGGAAGAG
ATGGTTCGTTTTTTTTTCTCGGAGAAGATAATCTGCTTACAAAAGAATAA
CAGTGGGGTGTTAGAGTATGTTTTCCATTATGTACAGTATGGTATAACTTC
AGGCTTTCTGAAAATCAATTTAAATGATATATTTATTGGAAAACTCCACC
ATTGAAACCATTAAACCACTTTCTCTTCATAATCTTATGTGGTATGGCGGA
AACAAGAATACAATAAGGTTTTTGTAGTAGCACACGATATTTTTTCATAG
AACCGCACTTTTCAATTGTCAATTACATAAACGGAAATTATCATGAACTT
TTCAAACAAACCAATACCATCTGATAGTATAAATAATATAGAATTTGCA
TTCATCCCATATACTTTGAAGAAATTTTTTTGATCACGAAAGCTAGACAT

Figure 15 (cont)

TCATTCCACCAACTCAACCATTatgacagactccaaatatgactattctgacattactcctgt
cgatataaacactgaagagcctcaaatatgtcaaattttgtatgacgaagattacaaacaaattatgg
ggttattacttgcacttatgaaagctgaagagtattctgaacgtgctttacatatcactgaattgggcat
taacgaactagcttcacattatacaatttggatctatcgatttaatattttgaaaaacttacccaataga
aacctttatgatgaattggattggtgtgaagaaattgctttggacaatgaaaaaaactatcagatttg
gaattatcgacaattaattattggtcgaattatggaattgaataataatgactttgacccatatcgaga
attccttatattagaagcaatgttaagttcagaccccaagaaccatcatgtttggtcgtatcgtaagtg
gttggttgatacgtttgatttacataatgacgcaaaagaattatcgtttgttgataaagtcatcgatact
gatttgaaaaataatagtgcttggtctcatcgattctttctattgtttagtaagaaacatttggccaccg
ataatacaattgatgaggagctaaattatgttaaagataagattgttaaatgtccacagaatccaagt
acttggaattatttattggggattcatgaacggtttgatcgatcaattactcaattagaagagtttagtt
tgcaatttgttgatttggaaaaagatcaagtgacgagttcatttgctttggagacattggcaaaaatat
acacacaacaaaagaaatacaatgaggctagaactgtttatgatttgttgaaatctaaatatgatcca
attagatccaatttctgggattatcagatttccaaactcacatcggtgtaaTTACCAAGGTAGA
GGGTAAGCAAAATAAATGAAGAAATTTTATACTTTCTTGTTTTCAATTGT
TTAACTAGGTAAATCATTGTATACCACCGATATTACCAATTAAAAAAAA
AATAAAAAGAGAATTTTTTTAGGAATGATTGCAAATCAATTAAGTAATT
GTGTATAAAATAGTACGTATTATATCAAGTTATTGTTATTCTACCAAGCTC
TTTGAGTGTGTATGTGTGTATCTGCTTTCTCCATATTTCTTGTATTGTTTGA
ATTATACATACGAATTCCGAATTTGACACGTTTTCGGGTTACGTTGTTTCA
TTTAGACCCCAATGTGGTGATCCATATCAATTATTTAGAAAAGTGGACAG
CTAGAGGCTTTTGAGATATGGCGGGGAATCAATTTTCTCCTAGTTGACACCA
TCCAAATATTGCCGCAAAACATCAAATGTTATTTGGCGTTATCTAATTGA
TTGAATCTACCATTTTGTTTAATCTAAGAGCAAGCTGAAATTCAGATTCGT
TATCGACTTGTATGAAATCTTAAGTTGTGTTGATAAGCAACTTTAGGGGT
AATTTTTGAATATCAGCATCAATTCTACACGTGATATACTGAGAATGAA
AAAAAAGACGAAGTAATTGTGCCAATTTCAAGAGAATTGGAGCTGATCG
ACC

Figure 16

GGAAGGGCGATCGGTGCGGCCTCTTCGCTATTAACGCCAGCTGGCGAAAGGGG
GATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG
ACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGA
ATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCT
GCAGGAATTCGATATCAAGCTTATCGATACCGTCGATCCTAAGCCATATTC
AACTGAACAGAATTTAGTTTTAAGGGGGGCAAAGTTTTTTTTTTTACTGA
CGTGTGACACCAAAAAAAAAAATAAATTAAACAACAACAAAATTGGTTG
TAAGAATTTTTTATCAAATTAGACGATATGTTAATTGATTTGTGATTTCC
AAATTACATTCTCTACACACATTTACAATGTTTGTCATATTACGAAGTAT
TTGGAAATGACAAACCTCAGTAATTTTATCACTTGAATTGAATACCTTTA
GAGGTAGATAATTTGACTCTTTAGTGAAGACTATGGAAAACTGATATCGT
GTAGGTCGTGTTAAGCTGGCTAAATCAATGTAGAGATTTCGTGTGAGTTGA
AAATAACTAACCACATCAAGTAACAACAACAACAGGGCTTCCAAAGAGA
TCAAGAGTAAGAATTATTAACATTAGATCTACTATTATAATAGATTGTT
AATTATTAAGACATATGCCTAACAATCTTTCTGTTATATCCAGTTTCACAT
TTGATTAGTCGAGAGAGAAAGGCCTAAACTAAATACAAAAGGAATGTTT
TGTTTTGATTTTGTTCCCCTTTTAAAAATAGTTTTACTTTACTTTTTTTTTG
GTTTTCGGCCCTATCGTTTGATTTTGGTTAAAATCAATTACTCATATATTCG
ATTGCAGTCGATATTAGAGAAGACCAATTAAATATTATTCTCTACATCTA
ATTAATTATCTTGAAACTAATATACATCTAGTAGTAATAGTATTATCCA
AATTAAATTGAATAACACATTACAATTTGTTTTTTATTATTTATTATTT
TTTAGTCGCCTGAATTGATTCTTTTTTTTTTTACTTCCCAGCCAAACACCAA
AAACTTTTTTCTCTCTCACACTCTCAAAATTTCTTCCAACAACAAACCTTTT
ACTGAAAGAAAAAAAAAAAATTTATTATAATTTAGTTCCCTCTTTCTCT
TTCTCTCTCACTCTCTTTTTCTTTGATTCCATATATATTTTTAATCCCTTTAT
TTAATTACTTTCAACAACAACCACCCTACCTTCCTCCCCTCCCCTCTTCCCCTT
TTAATAATACATCTATCAAATATAACATATAAACTTACATAatggttaacg
gtccagctgaacttcgtagaaaattagtcattgtcggtgatggtgcttgtggtaagacttgtttattaat
tgtttttttcaaaaggtactttcccagaagtttatgtcccaacagttttgaaaattacgttgctgatgttg
aagttgatggtagaaaagttgaattggcattatgggatactgctggtcaagaagattatgatagatta
agaccattatcttatccagattctaatgttatttttgatttgttttttcagttgattcaccagattctttagata
acgttttagaaaaatggatttctgaagttttacatttctgtcaaggtgttccaatcattttagttggttgt
aaatctgatttaagagatgatcctcatactattgaagccttgagacaacaacaacaacaaccagtctc
aacttctgaaggccaacaagttgctcaaagaattggtgctgctgattacttggaatgttctgctaaaac
cggtagaggtgttagagaagtgtttgaagctgctactagagcttctttaagagttaaagaaaagaag
gaaaagaagaagaaatgtgttgtcttgtaaATGTAACAACAACTAAAAGAACAACA
AGAAGAAGAAGAAGCATTAGCAAAAGCTAAAAGAAAAAAAAAAGTCAA
GCAAATACAACAAAAGGCAAAGTCAGAATAGAAAGAAACCTGAAGCCCT
CTTATGAGTTGTGGTTTTCTTTCTTATTCTTTTTTTTTATTCATTTCATTATG
TTTTATCCTATACTTTTTTTTTTTAGTTTCAGCACTAGATTTTAAAGAATT

Figure 16 (cont)

TTGTTATTTAATTAATATTAATATTATTACTATTTTAAAATAAAACTAC
TGCGGTGATCAGGGGTTTAACTTCTCCTGATACTTTTATATTTGATCCGTTT
TGAATATATTCATATATTTTGTTCTACAAAAAGAGTTTAACCTCTCCACA
GTTACTATATATATATATATTTCCACTGTAAATTGATAACTACTCCCTTA
TCACCGATTGCCTCATCTACCTCCTCCAAGTTAGTCTTTATACTGCCAGTACA
TATGTTAGTGTGGTAGTGGTAGTGGTGGTGTTTGTGTTTGTGTTCGTGTGTGT
GTCCGTACCAAAGGAGGATTCGACGAAATCATTCAAAGAAACTTGTAAA
AAAGGACACACACGAAAAATTAACAACAACAACAACAACAGCGACAAA
TCTTTAGGTGAAACGAAATCAAATCAAATCAAATCACACTTCCCAATATC
CACCACACACCCAACACCATGGCATCACTTAAATCATTTATTAAAAGTGTT
AGAAAAGCCAAAACCATTGCTGATGAAAGATCAGTCGTGCAAAAGGAAT
CGGCAGCAATCAGAACATCATTCAGAGACCCTGGTCTTGATCAAACCACTA
GACGTATCAACATTTCCAAACTTTTATACTTGTATATAATGGGGGAGAAA
ACACATTTTGGTCAAGTTGAATGTCTCAAATTATTAGCATCACCAAGATT
TGCTGATAAAAGATTAGGTTATTTGGCGTGTATGTTAATTTTGGATGAAA
ATCAAGAAGTTTTAACTTTATTGACCAATTCATTAGATAATGACATGCA
ACATCCTAATTCTTTTATAGTTGGATTAGCTCTTTGTTGTCTTGGTAATATT
GCTTCACCAGAATTGGCTAGAGATTTATATACCAATGTTGAAACCATTAT
TGATTCGAAAAATGTTTATTTAAAAAAGAAAGCTTGTATAGTGGCCGCT
AAATTAATTGAAAAGGAACCCGAATTGGCGAATTTTTCCATTACTAAAA
TCAATTCCAT