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Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*

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The *RHO1* gene encodes a homolog of mammalian RhoA small GTP binding protein in the yeast *Saccharomyces cerevisiae*. Rho1p is localized at the growth sites, including the bud tip and the cytokinesis site, and is required for bud formation. We have recently shown that Pkc1p, a yeast homolog of mammalian protein kinase C, and glucan synthase are targets of Rho1p. Using the two-hybrid screening system, we cloned a gene encoding a protein which interacted with the GTP-bound form of Rho1p. This gene was identified as *BNI1*, known to be implicated in cytokinesis or establishment of cell polarity in *S.cerevisiae*. Bni1p shares homologous domains (FH1 and FH2 domains) with proteins involved in cytokinesis or establishment of cell polarity, including formin of mouse, *capu* and *dia* of *Drosophila* and *FigA* of *Aspergillus*. A temperature-sensitive mutation in which the *RHO1* gene was replaced by the mammalian *RhoA* gene showed a synthetically lethal interaction with the *bni1* mutation and the *RhoA bni1* mutant accumulated cells with a deficiency in cytokinesis. Furthermore, this synthetic lethality was caused by the incapability of RhoA to activate Pkc1p, but not glucan synthase. These results suggest that Rho1p regulates cytoskeletal reorganization at least through Bni1p and Pkc1p.

Keywords: actin cytoskeleton/Rho target/*S.cerevisiae*

Introduction

The Rho family belongs to the small G protein superfamily and consists of the Rho, Rac and Cdc42 subfamilies (Hall, 1994; Takai *et al.*, 1995). Rho has two interconvertible forms: GDP-bound inactive and GTP-bound active forms. The GDP-bound form is converted to the GTP-bound form by the GDP/GTP exchange reaction, which is regulated by two types of regulatory proteins: the GDP/GTP exchange proteins (GEPs), which stimulate the GDP/GTP exchange reaction, and the GDP dissociation inhibitors (GDIs), which inhibit it. The GTP-bound form interacts with its specific target and performs its cell functions. Thereafter, the GTP-bound form is converted to the GDP-bound form by the GTPase reaction, which is regulated by GTPase activating proteins (GAPs).

Evidence is accumulating that, through reorganization of the actin cytoskeleton, Rho regulates various cell functions, such as maintenance of cell morphology, formation of stress fibers and focal adhesions, cell motility, membrane ruffling, cytokinesis, cell aggregation and smooth muscle contraction (Hall, 1994; Takai *et al.*, 1995). Recently, various proteins have been identified as potential targets of Rho (Leung *et al.*, 1995; Madaule *et al.*, 1995; Amano *et al.*, 1996; Ishizaki *et al.*, 1996; Matsui *et al.*, 1996; Watanabe *et al.*, 1996). However, it remains to be clarified whether these target proteins of Rho are involved in reorganization of the actin cytoskeleton.

The budding yeast *Saccharomyces cerevisiae* possesses Rho family members, including *RHO1* (Madaule *et al.*, 1987), *RHO2* (Madaule *et al.*, 1987), *RHO3* (Matsui and Toh-e, 1992), *RHO4* (Matsui and Toh-e, 1992) and *CDC42* (Adams *et al.*, 1990; Johnson and Pringle, 1990). Cells of this yeast grow by budding and the actin cytoskeleton plays a pivotal role in the budding and cytokinesis processes (Drubin, 1991). Cortical actin patches are clustered at the growth sites, including the site of bud emergence in unbudded cells and the bud tip and the cytokinesis sites in budded cells, whereas actin fibers are generally oriented along the long axes of the mother–bud pairs (Adams and Pringle, 1984). *RHO1* is a homolog of the mammalian *RhoA* gene and we have shown that *rho1* mutants are deficient in the budding process (Yamochi *et al.*, 1994). Moreover, immunofluorescence microscopic studies indicate that Rho1p is localized at the growth site, including the presumptive budding site, the bud tip and the cytokinesis site (Yamochi *et al.*, 1994). These results suggest that *RHO1* regulates the process of bud formation through reorganization of the actin cytoskeleton. Concerning the upstream regulators of *RHO1*, we have identified and characterized Rdi1p and Rom1p/Rom2p as GDI (Masuda *et al.*, 1994) and GEP (Ozaki *et al.*, 1996) respectively. Recently, we identified a novel Rho1p interacting protein, Rom7p/Bem4p, although its role in the regulation or function of Rho1p remains to be clarified (Hirano *et al.*, 1996). Concerning the downstream targets of *RHO1*, we have recently shown that one of the targets of Rho1p is a homolog of mammalian protein kinase C, Pkc1p (Nonaka *et al.*, 1995; Kamada *et al.*, 1996), which regulates cell wall integrity through activation of the MAP kinase cascade (Levin and Errede, 1995). More recently, we have also shown that another target of Rho1p is 1,3-β-glucan synthase (glucan synthase) (Drgonová *et al.*, 1996; Qadota *et al.*, 1996), which is involved in cell wall synthesis. Although it is apparent that Pkc1p and glucan synthase are involved in bud formation, it remains to be clarified

Table I. Two-hybrid interactions between Bni1p and Rho1p

DNA binding domain fusions	Transcriptional activating domain fusions[a]	Colony color	β-Galactosidase[b] (U)
RHO1(Q68L)	BNI1	blue	81 ± 6
RHO1(Q68L)	vector	white	<1
RHO1	BNI1	white	6 ± 1
RHO1	vector	white	<1
RHO1(T24N)	BNI1	white	<1
RHO1(T24N)	vector	white	<1
RHO1(T42A Q68L)	BNI1	white	<1
RHO1(T42A Q68L)	vector	white	<1
Ras(G12V)	Raf	blue	88 ± 1

[a]Plasmid pACT-BNI1(90–489) was used as a fusion of *BNI1* with the *GAL4* transcriptional activating domain.
[b]The values are averages ± SE for three transformants.

whether these targets perform their functions through reorganization of the actin cytoskeleton.

In the present work, we have cloned the *BNI1* gene as a gene encoding a protein which specifically interacts with the GTP-bound form of Rho1p. The results of our genetic studies of *BNI1*, along with the previous identification of *BNI1* and the *BNI1*-related genes as genes involved in cytokinesis or establishment of cell polarity in various organisms, suggest that Bni1p may be specifically involved in regulation of the actin cytoskeleton as a target of Rho1p in *S.cerevisiae*.

Results

Molecular cloning of BNI1 as a potential target of RHO1

To search for a gene which encodes a potential target of *RHO1*, the two-hybrid method was used. Since it was presumed that a target of Rho1p should specifically bind to the GTP-bound form of Rho1p, Rho1p(Q68L), carrying an amino acid substitution which is likely to keep Rho1p in the GTP-bound form, was used as a bait to screen a yeast cDNA library. We have actually shown that Rho1p(Q68L) is specifically bound to Pkc1p, which is a target of Rho1p, in the two-hybrid method (Nonaka *et al.*, 1995). Among 5×10^6 total transformants, 182 positive clones (His^+ and $lacZ^+$) were identified and the library plasmids were recovered from these clones. Among these 182 plasmids, 32 clones were found to confer the His^+ and $lacZ^+$ phenotypes on L40 containing pBTM116-RHO1(Q68L). DNA sequencing of the insert DNAs of these clones revealed that one clone encoded amino acid positions 90–489 of Bni1p (GenBank accession No. L31766), which is involved in control of the budding pattern and cytokinesis in *S.cerevisiae* (Zahner *et al.*, 1996). Since we had shown that Rho1p is localized at the cytokinesis site (Yamochi *et al.*, 1994), we decided to investigate the physiological significance of the Rho1p(Q68L)–Bni1p interaction.

The specificity and extent of the interaction of Bni1p(90–489) with Rho1p were investigated further. As described in Table I, Rho1p(Q68L) interacted with Bni1p(90–489) to an extent similar to that of the interaction of Ras(G12V) with Raf, which is the target of Ras. Moreover, neither the wild-type Rho1p (the GDP-bound form) nor Rho1p(T24N) (the GDP-bound form or the nucleotide-free form) interacted with Bni1p(90–489).

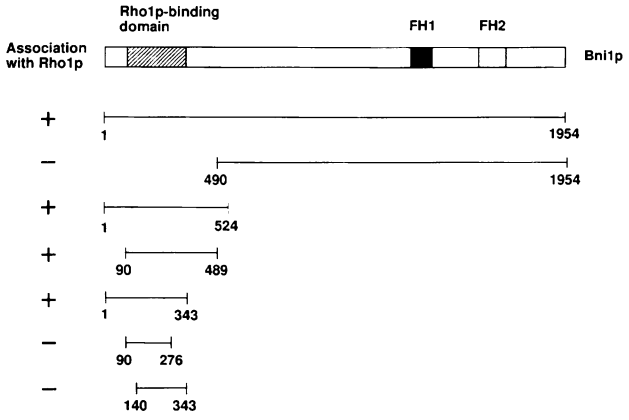

Fig. 1. Rho1p interacts with the N-terminal region of Bni1p. Various DNA fragments encoding truncated Bni1ps were cloned into the two-hybrid vector pACT-HK and the resultant plasmids were transformed into L40 containing pBTM116-RHO1(Q68L). Association of the Bni1 proteins with Rho1p(Q68L) was examined by the qualitative assay method for β-galactosidase activity. FH1 and FH2 are formin homology domains 1 and 2 respectively.

Rho1p(T42A Q68L), carrying an amino acid substitution in the effector domain, which is implicated in interaction with its target, did not interact with Bni1p(90–489) either. These results are similar to those obtained with Pkc1p (Nonaka *et al.*, 1995) and suggest that Bni1p is a target of Rho1p in *S.cerevisiae*.

Bni1p is a protein of 1954 amino acids in length and it contains FH1 and FH2 domains, which are found in several proteins involved in the establishment of cell polarity or cytokinesis, including formin IV of mouse and the products of *capu* and *dia* of *Drosophila* and *FigA* of *Aspergillus* (Castrillon and Wasserman, 1994; Emmons *et al.*, 1995; Figure 1). The region of *BNI1* which was cloned in the two-hybrid screening system corresponded to the N-terminal region. As shown in Figure 1, Rho1p(Q68L) was bound to full-length Bni1p, suggesting that the GTP-bound form of Rho1p interacts with Bni1p *in vivo*. Deletion mapping demonstrated that the region of Bni1p interacting with Rho1p is in a 253 amino acids region (amino acid positions 90–343).

Bni1p interacts directly with Rho1p

To investigate whether Bni1p interacts directly with Rho1p *in vitro*, a recombinant Bni1p fused to maltose binding protein (MBP), which contains amino acid positions 1–

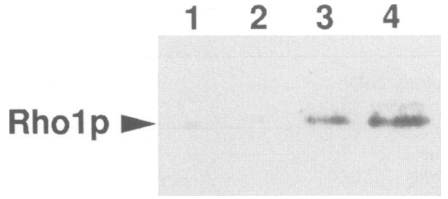

Fig. 2. Bni1p interacts directly with Rho1p. The GDP- or GTPγS-bound form of Rho1p was incubated, at a Rho1p concentration of 80 nM, with MBP or MBP–Bni1p(1–524), which was prebound to protein A–Sepahrose CL-4B beads through the anti-MBP antibody. The recovered samples were subjected to SDS–PAGE followed by Western blotting with the anti-Rho1p antibody. Lanes 1 and 2, MBP; lanes 3 and 4, MBP–Bni1p(1–524). Lanes 1 and 3, the GDP-bound form of Rho1p; lanes 2 and 4, the GTPγS-bound form of Rho1p.

Table II. Synthetic lethality between *RhoA* and *bni1* mutations

Genotype	Viable	Inviable[a]
RhoA bni1	0	32
RHO1 bni1	49	3
RhoA BNI1	39	13
RHO1 BNI1	32	0

[a]Genotypes of the inviable segregants were inferred from those of the viable segregants.

524 of Bni1p, was expressed in *Escherichia coli* and purified. As shown in Figure 2, MBP–Bni1p(1–524), but not MBP, bound to the GTPγS-bound form of Rho1p with higher affinity than to the GDP-bound form. This result indicates that Bni1p directly interacts with Rho1p.

The bni1 mutation is synthetically lethal with the RhoA mutation

To investigate whether *BNI1* genetically interacts with *RHO1*, *BNI1* was disrupted with *HIS3* and it was found that the *bni1* mutant grew normally at 24 and 30°C, but poorly at 35°C (data not shown). We have recently shown that a mutant in which the *RHO1* gene is replaced by the mammalian *RhoA* gene shows a recessive temperature-sensitive growth phenotype (Qadota *et al.*, 1994; Yamochi *et al.*, 1994). The *bni1* mutant HKY2-1A was crossed with the *RhoA* mutant HNY78 and the resultant diploid was tetrad dissected. As described in Table II, the *bni1* mutation was synthetically lethal with the *RhoA* mutation. Since expression of *RHO1* under the control of the *GAL1* promoter suppressed the growth deficiency of the *RhoA bni1* mutant (Figure 3), this synthetic lethality was not due to a dominant effect of the *RhoA* mutation. It was also confirmed that expression of *BNI1* under the control of its promoter on a single copy plasmid suppressed the growth deficiency of the *RhoA bni1* mutant (data not shown). These results indicate that a function of Bni1p overlaps with that of a downstream component of Rho1p for vegetative cell growth and that the *RhoA* mutation is deficient in activation of this downstream component.

The physiological significance of the interaction of Rho1p with Bni1p was investigated further. If the Rho1p–Bni1p interaction is essential for the function of Bni1p, RhoA should bind to Bni1p, since the *RhoA* mutant is viable. This point was examined by the two-hybrid method and it was found that RhoA interacted with Bni1p in the same way as Rho1p interacted with Bni1p (data not

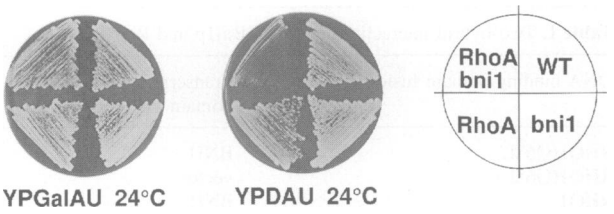

Fig. 3. The *bni1* mutation is synthetically lethal with the *RhoA* mutation. Cells of strains KY2-1-4D (*RhoA bni1*), HNY78 (*RhoA*), OHNY1 (wild-type) and KY2-1A (*bni1*) were streaked onto YPGalAU and YPDAU plates which were subsequently incubated at 24°C for 4 days.

Table III. Synthetic lethality between *pkc1* and *bni1* mutations

Genotype	Viable	Inviable[a]
pkc1 bni1	0	71
PKC1 bni1	83	2
pkc1 BNI1	77	8
PKC1 BNI1	71	0

[a]Genotypes of the inviable segregants were inferred from those of the viable segregants.

shown). Moreover, it was found that expression of Bni1p(490–1954), which lacks the Rho1p binding domain, did not complement the growth deficiency of the *RhoA bni1* mutant (data not shown). These results are consistent with the interpretation that Bni1p is a target of Rho1p.

Pkc1p, but not glucan synthase, is involved in the synthetic lethality between the RhoA and bni1 mutations

We have recently shown that Pkc1p (Nonaka *et al.*, 1995; Kamada *et al.*, 1996) and glucan synthase (Drgonová *et al.*, 1996; Qadota *et al.*, 1996) are targets of Rho1p. These results raise the possibility that the lethality of the *RhoA bni1* mutant is due to a deficiency of *RhoA* in the activation of Pkc1p or glucan synthase. In other words, *BNI1* may genetically interact with the *PKC1* or/and glucan synthase pathways.

Whether *BNI1* genetically interacts with *PKC1* was therefore examined. The *bni1* mutant BTY1 was crossed with the *pkc1* mutant TFY7 and the resultant diploid was subjected to tetrad analysis. Since the *pkc1* mutant requires osmotic support for growth (Levin and Bartlett-Heubusch, 1992), spore clones were grown on YPDAU medium containing 1 M sorbitol. As described in Table III, the *bni1* mutation clearly showed a synthetically lethal interaction with the *pkc1* mutation. Both the *bni1* and *pkc1* mutants grew normally, but the *bni1 pkc1* mutant did not form a visible colony even in the presence of 1 M sorbitol. This result suggests that the lethality of the *RhoA bni1* mutant was due to a deficiency of RhoA in activation of Pkc1p. Consistently, as shown in Figure 4, the lethality of the *RhoA bni1* mutant was suppressed by expression of the *PKC1(R398P)* mutant gene, which we isolated as a dominant suppressor of the *RhoA* mutation (Nonaka *et al.*, 1995), but was not suppressed by osmotic support.

In the next experiment, we examined whether *BNI1* genetically interacts with the glucan synthase pathway. A pair of closely related proteins, Fks1p and Fks2p, are known to be subunits of glucan synthase and at least

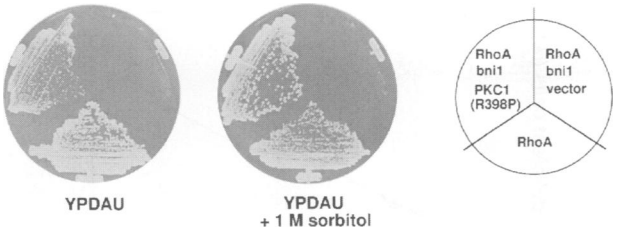

Fig. 4. The synthetic lethality of the *RhoA bni1* mutation is suppressed by the dominant active *PKC1(R398P)* mutation, but not by an osmotic support. Cells of strains KY2-1-4C transformed with pRS316-PKC1(R398P) [*RhoA bni1 PKC1(R398P)*], KY2-1-4C transformed with pRS316 (*RhoA bni1* vector) and HNY81 (*RhoA*) were streaked onto a YPDAU plate and a YPDAU plate containing 1 M sorbitol, which were subsequently incubated at 24°C for 4 days.

Fks1p has been shown to be immunoprecipitated with Rho1p (Qadota *et al.*, 1996). Simultaneous disruption of both genes results in lethality, but disruption of either one does not (Inoue *et al.*, 1995). Since disruption of *FKS1* results in a slow growth phenotype, the *fks1* mutant was crossed with the *bni1* mutant and the resultant diploid was subjected to tetrad analysis. The result indicated that the *fks1 bni1* mutant is viable at 23, 30 and 37°C (data not shown), suggesting that *BNI1* is not involved in the glucan synthase pathway. This point was examined further by another approach. It was found that, in contrast to the *RhoA* mutation, another temperature-sensitive mutation of *RHO1*, *rho1-104*, did not show synthetic lethality with the *bni1* mutation (Figure 5A). However, as shown in Figure 5B, glucan synthase activity was reduced in the *rho1-104* mutant, as well as in the *RhoA* mutant. These results indicate that the lethality of the *RhoA bni1* mutant is not due to a reduced level of glucan synthase activity. This conclusion is consistent with the result that the *bni1* mutant possessed a normal level of glucan synthase activity (Figure 5B). Taken together, we concluded that a function of Bni1p overlaps with that of Pkc1p, but not with that of glucan synthase, for vegetative cell growth.

Deficiency of cytokinesis in the RhoA bni1 mutant

A diploid strain homozygous for the *bni1* mutation grows normally but is partially deficient in cytokinesis and shows a random budding pattern (Zahner *et al.*, 1996). We constructed a diploid strain homozygous for both the *bni1* and *RhoA* mutations whose lethality was suppressed by the expression of *RHO1* under control of the *GAL1* promoter. This strain was incubated under *RHO1* repressed conditions for 12 h and the cells were subjected to microscopic analysis along with those of the wild-type and *bni1* mutant strains. It was found that 30% of the *bni1* mutant cells and 60% of the *RhoA bni1* mutant cells contained unseparated large buds, probably due to a deficiency in cytokinesis, and the mother–bud neck was wider in >80% of the *RhoA bni1* mutant cells than in the *bni1* mutant cells (Figure 6A, Phase). Enlargement of the mother–bud neck in the *RhoA bni1* mutant was more clearly observed by staining the chitin ring, which is present at the cytokinesis site, with Calcofluor (Figure 6A, Chitin). Moreover, staining of actin revealed that cortical actin patches, which are normally seen at the cytokinesis site, were randomly distributed throughout the cells in >90% of the cytokinesis-deficient cells of both

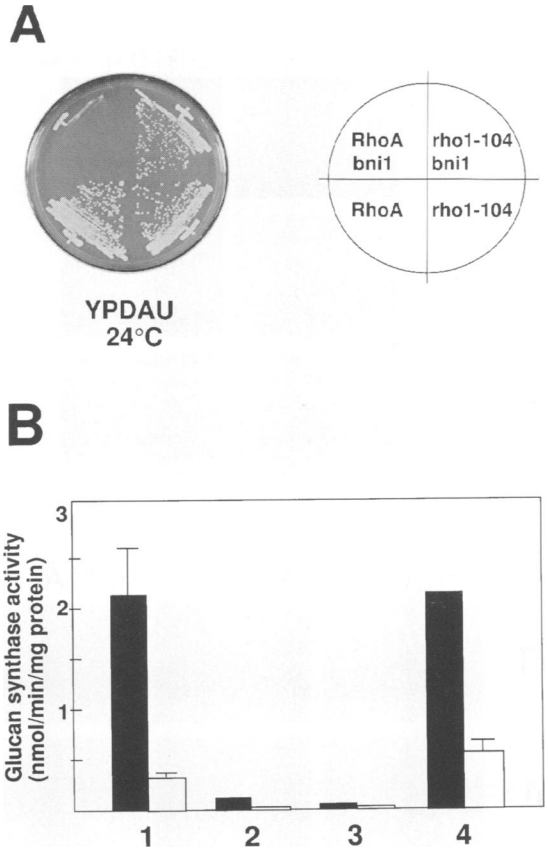

Fig. 5. *BNI1* is not involved in the glucan synthase pathway. (**A**) The *rho1-104* mutation is not synthetically lethal with the *bni1* mutation. Cells of strains KY2-1-4D (*RhoA bni1*), HNY78 (*RhoA*), HKY15-5A (*rho1-104 bni1*) and HNY21 (*rho1-104*) were streaked onto a YPDAU plate, which was subsequently incubated at 24°C for 4 days. (**B**) Glucan synthase activity is reduced in the *RhoA* and *rho1-104* mutants, but not in the *bni1* mutant. Membrane fractions of the wild-type strain (OHNY1), the *RhoA* mutant (HNY78), the *rho1-104* mutant (HNY21) and the *bni1* mutant (HKY2-1A) were cultured at 24°C and glucan synthase activity was measured in the presence or absence of 0.8 μM GTPγS. 1, OHNY1; 2, HNY78; 3, HNY21; 4, HKY2-1A. Closed bars, in the presence of GTPγS; open bars, in the absence of GTPγS. The values are averages ± SE for four experiments.

the *bni1* and *RhoA bni1* mutants (Figure 6B, Actin). Interestingly, DNA staining revealed that ~10% of the *RhoA bni1* mutant cells contained more than two nuclei, indicating that the *RhoA bni1* mutant is deficient in the migration of divided nuclei into daughter cells (Figure 6B, DNA). These results suggest that the effect of the *bni1* mutation on cytokinesis is enhanced by the *RhoA* mutation. Consistent with this, the percentage of cytokinesis-deficient cells in the *RhoA bni1* mutant increased from 30 to 75% after a shift from *RHO1*-expressed to *RHO1*-repressed conditions (Figure 7). We conclude that the *RhoA bni1* mutant does not grow mainly due to a deficiency in cytokinesis.

Discussion

In the present study, we have cloned *BNI1* as a gene which encodes a putative target of Rho1p. The two-hybrid study indicates that Bni1p specifically interacts, at its N-terminal 254 amino acid region, with the GTP-bound form of Rho1p to an extent similar to that of the interaction

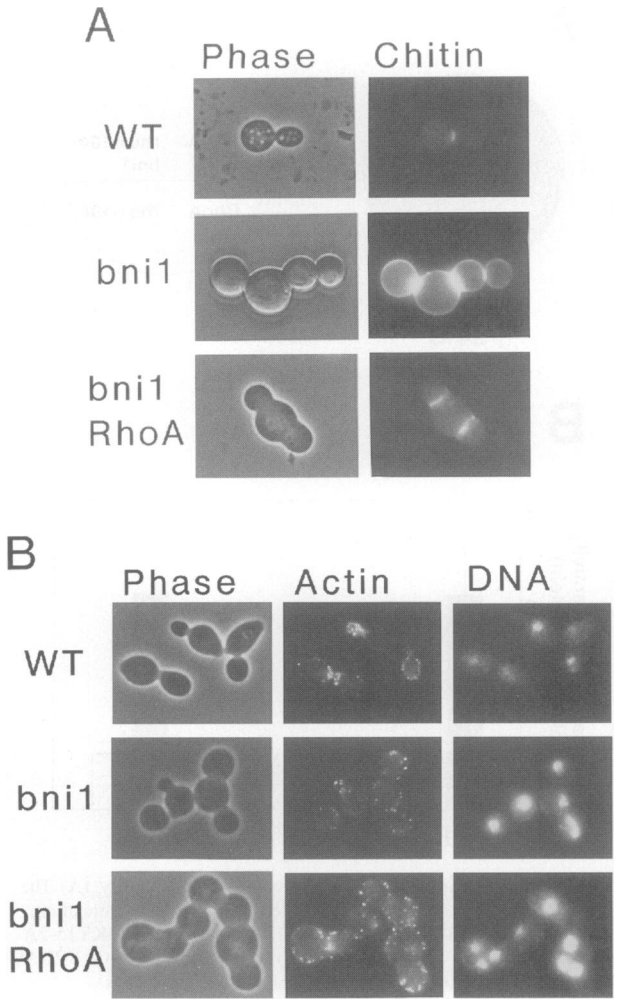

Fig. 6. Deficiency in cytokinesis of the *RhoA bni1* mutant. (**A**) Staining of chitin. Cells of diploid strains OHNY3 (wild-type), KY4 (*bni1*) and KY1 (*bni1 RhoA*), incubated at 24°C in YPDAU medium for 12 h, were fixed and stained with Calcofluor for chitin and were subsequently subjected to microscopic observation. (**B**) Staining of actin and DNA. Cells of OHNY3, KY4 and KY1, incubated as described above, were fixed and double stained with rhodamine–phalloidin and DAPI for actin and DNA respectively and were subsequently subjected to microscopic observation. All fields were photographed at the same magnification.

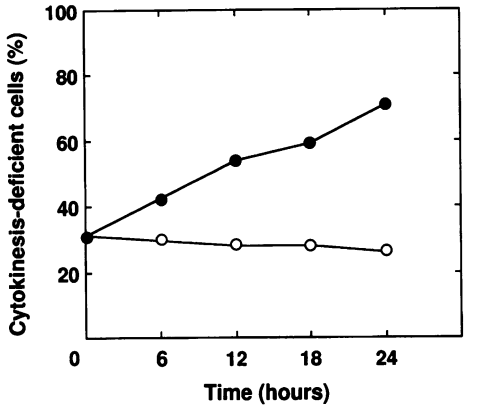

Fig. 7. Accumulation of cytokinesis-deficient cells in the *RhoA bni1* mutant. Cells of diploid strains KY1 (*RhoA bni1*) and KY4 (*bni1*), grown at 24°C in YPGalAU medium were collected, washed with H_2O and inoculated into YPDAU medium. After incubation at 24°C for various times, a portion of the culture was sampled and the percentage of cytokinesis-deficient cells was determined by observing >400 cells under a light microscope. ●, KY1; ○, KY4.

of Ras with Raf. The result with recombinant Bni1p also indicates that Bni1p binds directly to the GTPγS-bound form of Rho1p with higher affinity than to the GDP-bound form, but, in contrast to the results obtained in the two-hybrid study, Bni1p bound to the GDP-bound form of Rho1p to a significant extent. It may be speculated that *in vivo* there is an unknown factor which enables Bni1p to more specifically interact with the GTP-bound form of Rho1p. One candidate for such a factor is Rdi1p, which specifically interacts with the GDP-bound form of Rho1p (Masuda *et al.*, 1994). Rdi1p might inhibit the GDP-bound form of Rho1p from interacting with Bni1p. We have previously shown that Pkc1p (Nonaka *et al.*, 1995; Kamada *et al.*, 1996) and glucan synthase (Drgonová *et al.*, 1996; Qadota *et al.*, 1996) are targets of Rho1p. Bni1p is a candidate for the third target among the targets of Rho1p which have so far been identified in *S.cerevisiae*. Although we have shown that Pkc1p (Kamada *et al.*, 1996; H.Nonaka *et al.*, unpublished results) and glucan synthase (Drgonová *et al.*, 1996; Qadota *et al.*, 1996) are activated by the GTP-bound form of Rho1p, the mode of action of Rho1p on Bni1p remains to be clarified.

The *bni1* mutant shows a partial deficiency in cytokinesis and the *RhoA* mutation enhances this phenotype. The *BNI1* gene was originally isolated as a gene whose mutation was synthetically lethal with the *cdc12* mutation (Zahner *et al.*, 1996). Cdc12p is a protein named septin which is conserved among eukaryotes and, at least in yeast, septin probably forms a filament-like structure required for cytokinesis at the mother–bud neck (Longtine *et al.*, 1996). We have found that the *RhoA* mutation is also synthetically lethal with the *cdc12* mutation (data not shown), suggesting that Rho1p is involved in the regulation of septin functions. It has been shown that Rho regulates cytokinesis in *Xenopus* eggs (Kishi *et al.*, 1993) and in sand dollar eggs (Mabuchi *et al.*, 1993). It may be interesting to investigate whether Rho regulates cytokinesis through Bni1p-like targets and whether septin-like proteins are involved in this Rho–Bni1p system in higher eukaryotes. The involvement of Bni1p-like proteins in cytokinesis is also supported by the fact that the product of *dia*, a homolog of Bni1p, is required for cytokinesis in *Drosophila* (Castrillon and Wasserman, 1994).

Recently, five genes, *SHE1–SHE5*, have been isolated as genes needed for the accumulation in daughter nuclei of Ash1p, a repressor of *HO*, which is specifically transcribed in mother cells (Jansen *et al.*, 1996). In this study, *SHE5* has been shown to be identical to *BNI1* and *SHE1* encodes a myosin motor, Myo4p, suggesting that Bni1p is also involved in control of the actin cytoskeleton. Furthermore, an indirect immunofluorescence microscopic study has indicated that Bni1p is localized in buds (Jansen *et al.*, 1996). This result, together with the result that Rho1p is also localized in buds (Yamochi *et al.*, 1994), supports our present conclusion that Bni1p is a target of Rho1p. *BNI1* has also been identified in a genetic screen for mutants displaying a bipolar budding-specific randomization of budding pattern (Zahner *et al.*, 1996). Based on precise phenotypic analysis of the *bni1* mutant, it has

been presumed that Bni1p is necessary for the initial localization of bipolar positional signals to the presumptive bud site. These results suggest that Bni1p is involved in the establishment of cell polarity in yeast, but this conclusion does not seem to be consistent with the fact that the *bni1* mutant shows a partial deficiency in cytokinesis. However, one plausible idea is that the Rho1p–Bni1p system is required to assemble a component required for cytokinesis at the presumptive budding site, since the budding site is the future cytokinesis site in cells of *S.cerevisiae*. Taken together, the Rho1p–Bni1p system seems to be involved in the localization of numerous proteins at the budding site or in the bud. That the *RhoA bni1* mutant showed an abnormal localization of divided nuclei may indicate that the Rho1p–Bni1p system is also required for localization of a machinery needed for the migration of nuclei into daughter cells.

Bni1p shares two conserved domains, FH1 and FH2 domains, with a variety of proteins, including formin IV of mouse and fus1p of *Shizosaccharomyces pombe* and the products of *capu* and *dia* of *Drosophila* and *FigA* of *Aspergillus* (Castrillon and Wasserman, 1994; Emmons *et al.*, 1995). Of these proteins, Capu is required for the establishment of cell polarity (Emmons *et al.*, 1995), Dia is involved in cytokinesis (Castrillon and Wasserman, 1994), FigA is required for normal cell morphology or polarity and fus1p is required for cell conjugation (Petersen *et al.*, 1995). These results suggest that FH1- and FH2-containing proteins are involved in regulation of cytoskeletal reorganization. In this respect, it is noteworthy that the FH1 domains consist of proline-rich sequences (Castrillon and Wasserman, 1994). The proline-rich sequences have been shown to interact with an actin binding protein, profilin (Tanaka and Shibata, 1985), and the SH3 domains, which are often found in proteins implicated in regulation of the actin cytoskeleton (Pawson and Schlessinger, 1993). In fact, it has been demonstrated that a fusion protein containing 28 amino acids from the FH1 domain of formin bound *in vitro* to the SH3 domains of Abl and Src (Ren *et al.*, 1993). Recently, a target of Cdc42Hs, another member of the Rho family of small GTP binding proteins, has been identified to be the Wiskott–Aldrich syndrome protein (WASP) (Aspenström *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996) and WASP has been shown to induce actin polymerization when expressed in cells (Symons *et al.*, 1996). It should be noted that WASP contains the same proline-rich sequence as does Bni1p and another protein, vasodilator-stimulated phosphoprotein (VASP), which has homologous regions to WASP, has actually been shown to bind to profilin through the proline-rich sequence (Reinhard *et al.*, 1995). These results, together with our present results, suggest that Rho1p and Cdc42Hs may regulate reorganization of the actin cytoskeleton by a similar mechanism.

The present results of genetic analyses indicate that the Bni1p functions in cell growth overlap with Pkc1p, but not with glucan synthase. This may be the first demonstration of a functional interaction between the two targets of a small GTP binding protein. We also examined whether Bni1p physically interacted with Pkc1p by the two-hybrid method, which it did not (data not shown). It has been shown that Pkc1p regulates cell wall integrity through the MAP kinase cascade, which is composed of Bck1p (MAP kinase kinase kinase), Mkk1p/Mkk2p (MAP kinase kinase) and Mpk1p (MAP kinase) in that order (Levin and Errede, 1995). Therefore, it should be determined whether Pkc1p carries out its Bni1p-related function through the MAP kinase cascade. Based on the lethality of the *pkc1* mutant being caused by cell lysis and suppressed in the presence of 1 M sorbitol, it was proposed that *PKC1* regulates cell wall integrity (Levin and Bartlett-Heubusch, 1992). However, the lethality of the *pkc1 bni1* mutant was not suppressed by 1 M sorbitol, indicating that there is another function of Pkc1p which is not related to regulation of cell wall integrity, but is related to regulation of cytoskeletal reorganization by Bni1p.

In conclusion, this study is the first report of the identification of a target of Rho which probably links Rho with the actin cytoskeleton. The region of Bni1p that interacts with Rho1p is present in the N-terminal 254 amino acid region. It would be intriguing to examine whether FH1- and FH2-containing proteins found in other organisms interact with Rho.

Materials and methods

Strains, media and yeast transformations

Yeast strains used in this study are listed in Table IV. Yeast strains were grown on rich medium that contained 2% Bacto-peptone (Difco Laboratories, Detroit, MI), 1% Bacto-yeast extract (Difco), 0.04% adenine sulfate, 0.02% uracil and 2% glucose (YPDAU) or 3% galactose and 0.2% sucrose (YPGalAU). Yeast transformations were performed by the lithium acetate method (Gietz *et al.*, 1992). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco), with SD medium supplemented with amino acids when required. Standard yeast genetic manipulations were performed as described (Sherman *et al.*, 1986). *Escherichia coli* strain DH5α was used for construction and propagation of plasmids.

Molecular biological techniques

Standard molecular biological techniques were used for construction of plasmids, DNA sequencing and PCR (Sambrook *et al.*, 1989). DNA sequences were determined using an ALFred DNA sequencer (Pharmacia Biotech Inc.) and PCR was performed using a GeneAmp PCR System 2400 (Perkin Elmer). Plasmids used in this study are listed in Table V.

Screening for a target of Rho1p by the two-hybrid method

A strain L40 carrying pBTM116-RHO1(Q68L) (Nonaka *et al.*, 1995) was transformed with a yeast cDNA library made in pACT (kindly provided Stephen J.Elledge). Transformants were screened for growth on SD plate medium lacking tryptophan, leucine and histidine, but containing 5 mM 3-amino-1,2,4-triazole, which is a specific inhibitor of the *HIS3* gene product. His^+ colonies were then placed on a nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described (Vojtek *et al.*, 1993). From the His^+ and $lacZ^+$ positive clones obtained with this screening, library plasmids were recovered through *E.coli* transformation. The recovered plasmids were transformed again into L40 containing pBTM116-RHO1(Q68L) to select clones which conferred the His^+ and $lacZ^+$ phenotypes on L40 containing pBTM116-RHO1(Q68L). The nucleotide sequences of the insert DNAs of selected clones were determined. For quantitative assay for β-galactosidase activity, cells of each transformant were cultured in SD-Trp-Leu medium and the β-galactosidase activity was measured according to the ONPG assay method (Guarente, 1983).

Disruption of BNI1

pBS-bni1::HIS3 was cut with *Bam*HI and *Sma*I and the digested DNA was introduced into a diploid strain OHNY3. The genomic DNA was isolated from each transformant and proper disruption of *BNI1* was verified by PCR (data not shown). A diploid strain in which one *BNI1* allele was disrupted was named HKY1 and was subjected to tetrad analysis. All dissected asci (15) showed a 2 His^-:2 His^+ segregation pattern and all of the His^+ clones grew normally at 23 and 30°C, but

Table IV. Yeast strains used in this study

Strain	Genotype
OHNY1	MATa *ura3 leu2 trp1 his3 ade2* (Nonaka *et al.*, 1995)
OHNY3	MATa/MATα *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2*
HNY78	MATa *ura3 leu2 trp1 his3 ade2 rho1::RhoA-URA3* (Nonaka *et al.*, 1995)
HNY81	MATa *ura3 leu2 trp1 his3 ade2 rho1::RhoA-HIS3* (Nonaka *et al.*, 1995)
HNY21	MATa *ura3 leu2 trp1 his3 ade2 rho1-104* (Yamochi *et al.*, 1994)
TFY7	MATα *ura3 leu2 trp1 his3 ade2 pkc1::LEU2*
HKY1	MATa/MATα *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 BNI1/bni1::HIS3*
HKY2-1A	MATα *ura3 leu2 trp1 his3 ade2 bni1::HIS3*
BTY1	MATa *ura3 leu2 trp1 his3 ade2 bni1::HIS3*
KY2-1-4D	MATα *ura3 leu2 trp1 his3 ade2 bni1::HIS3 rho1::RhoA-HIS3* YCp-LEU2-GAL1-RHO1
KY2-1-4C	MATα *ura3 leu2 trp1 his3 ade2 bni1::HIS3 rho1::RhoA-HIS3* YCp-LEU2-GAL1-RHO1
HKY15-5A	MATa or MATα *ura3 leu2 trp1 his3 ade2 bni1::HIS3 rho1-104*
KY1	MATa/α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 bni1::HIS3/bni1::HIS3 rho1::RhoA-URA3/rho1::RhoA-URA3* YCp-LEU2-GAL1-RHO1
KY4	MATa/α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 bni1::HIS3/bni1::HIS3*
L40	MATa *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*

Strains used in this study are isogenic except L40.

Table V. Plasmids used in this study

Plasmids	Characteristics[a]
pBTM116-RHO1	DBD_{LexA}-RHO1[b], *TRP1*, 2μm (Nonaka *et al.*, 1995)
pBTM116-RHO1(Q68L)	DBD_{LexA}-RHO1(Q68L), *TRP1*, 2μm (Nonaka *et al.*, 1995)
pBTM116-RHO1(T24N)	DBD_{LexA}-RHO1(T24N), *TRP1*, 2μm (Nonaka *et al.*, 1995)
pBTM116-RHO1(T42A, Q68L)	DBD_{LexA}-RHO1(T42A, Q68L), *TRP1*, 2μm (Nonaka *et al.*, 1995)
pACT-BNI1(90–489)	AD_{GAL4}[b], *LEU2*, 2μm; isolated in this study from the yeast cDNA library provided by S.Elledge
pACTII-HK	AD_{GAL4}, *LEU2*, 2μm (Ozaki *et al.*, 1996)
pACTII-HK-BNI1(1–1954)	AD_{GAL4}-BNI1(1–1954), *LEU2*, 2μm; made by inserting the 5.9 kb *Bam*HI–*Sma*I PCR fragment containing the *BNI1* ORF into the *Bam*HI–*Sma*I site of pACTII-HK. The 5.9 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 1, 5′-GCCCGGATCCATGTTGAAGAATTCAGGCTCCAAAC, and downstream primer 1, 5′-GCCCCCCGGGTTATTTGAAACTTAGCCTGTTACC
pACTII-HK-BNI1(490–1954)	AD_{GAL4}-BNI1(490–1950), *LEU2*, 2μm; made by inserting the 4.4 kb *Bam*HI–*Sma*I PCR fragment encoding amino acid positions 490–1954 of Bni1p into the *Bam*HI–*Sma*I site of pACTII-HK. The 4.4 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 2, 5′-GCGCGGATCCATGAACTTCAAAAAAATGCC-CCAG, and downstream primer 1
pACTII-HK-BNI1(1–524)	AD_{GAL4}-BNI1(1–524), *LEU2*, 2μm; made by inserting the 1.6 kb *Bam*HI–*Sma*I PCR fragment encoding amino acid positions 1–524 of Bni1p into the *Bam*HI–*Sma*I site of pACTII-HK. The 1.6 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 1 and downstream primer 2, 5′-GCGCCCCGGGTCATCGGCCGTCTAAAGTTTGTTC
pACTII-HK-BNI1(1–343)	AD_{GAL4}-BNI1(1–343), *LEU2*, 2μm; made by inserting the 1.0 kb *Bam*HI–*Sma*I PCR fragment encoding amino acid positions 1–343 into the *Bam*HI–*Sma*I site of pACTII-HK. The 1.0 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 1 and downstream primer 3, 5′-GCGCCCCGGGTCATCGGCCGTGCACATAATGTATCGGT-GGTC
pACTII-HK-BNI1(90–276)	AD_{GAL4}-BNI1(90–276), *LEU2*, 2μm; made by inserting the 0.5 kb *Bam*HI–*Sma*I PCR fragment encoding amino acid positions 90–276 of Bni1p into the *Bam*HI–*Sma*I site of pACTII-HK. The 0.5 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 3, 5′-TCTAGGATCCACACAAAACTTGTCTCAATAT, and downstream primer 4, 5′-GCGCCCCGGGTCAAGTTGAGGTGGGAGAAGAGGCGCT
pACTII-HK-BNI1(140–343)	AD_{GAL4}-BNI1(140–303), *LEU2*, 2μm; made by inserting the 0.6 kb *Bam*HI–*Sma*I PCR fragment encoding amino acid positions 140–343 of Bni1p into the *Bam*HI–*Sma*I site of pACTII-HK. The 0.6 kb BNI1 DNA fragment was amplified by PCR using upstream primer 4, 5′-GCGCGGATCCCAGCATACCGGGCAAAGTCACTCC, and downstream primer 3
pBS-BNI1	*BNI1*; made by inserting the 6.5 kb PCR fragment of *BNI1* into the *Eco*RV site of pBluescript KS(+). The 6.5 kb *BNI1* DNA fragment was amplified by PCR using upstream primer 5, 5′-TATAGGATCCAGTTGGTATGGATAGAGCCAGAATGTAAAACAAGGTGGCA, and downstream primer 4, 5′-CTCTCCCGGGCTAGTGCTTGTTTGGATGTTTGTTTTGGTATTACTGTTGT
pBS-bni1::HIS3	a derivative of pBS-BNI1; made by replacing the 561 bp *Bgl*II–*Bgl*II internal fragment of *BNI1* corresponding to amino acid positions 1228–1414 of Bni1p with the 1.8 kb *HIS3* fragment
YCp-LEU2-GAL1-RHO1	*GAL1-RHO1*, *LEU2*, *CEN4* (Yamochi *et al.*, 1994)
pRS316	*URA3*, *CEN6* (Sikorski and Hieter, 1989)
pRS316-PKC1(R398P)	*URA3*, *CEN6*, *PKC1(R398P)*; made by inserting the 4.2 kb *Sph*I–*Sph*I (filled in) *PKC1(R398P)* fragment into the *Sma*I site of pRS316
pMAL-c2-BNI1(1–524)	MBP-BNI1(1–524); made by inserting the 1.5 kb *Bam*HI–*Bgl*II DNA fragment of *BNI1* from pACTII-HK-BNI1(1–524) into the *Bam*HI site of pMAL-c2 (New England BioLabs Inc.)

[a]Underlined sequences are portions of the *BNI1* gene.
[b]DBD_{LexA} and AD_{GAL4} are the DNA binding domain of LexA and the transcriptional activation domain of GAL4 respectively.

poorly at 35°C, compared with the His$^-$ clones. These *bni1* mutant strains were used for further genetic studies.

Cytological techniques

Actin and DNA were stained with rhodamine–phalloidin (Molecular Probes Inc., Eugene, OR) or 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Chemical Co., St Louis, MO) respectively, as described (Yamochi *et al.*, 1994). Chitin was stained with Calcofluor White M2R New (Sigma Chemical Co.) as described (Pringle, 1991). Stained cells were observed and photographed on Neopan Super Presto film (Fuji Photo film, Tokyo, Japan) using a Zeiss Axiophoto microscope (Carl Zeiss, Oberkochen, Germany).

Materials and chemicals for biochemical assays

Recombinant MBP–Bni1p(1–524) was purified from *E.coli* as a MBP fusion protein using an amylose column as described (Guan *et al.*, 1987). The lipid-modified form of Rho1p was purified from the membrane fraction of Sf9 insect cells as described previously (Mizuno *et al.*, 1991). The anti-MBP antibody was an affinity-purified rabbit polyclonal antibody raised against MBP (K.Shimizu *et al.*, unpublished results). The anti-Rho1p antibody has been described previously (Hirano *et al.*, 1996). Protein A–Sepharose CL-4B was purchased from Pharmacia Biotech Inc.

Assay for the binding of recombinant Bni1p with Rho1p

MBP (40 pmol) or MBP–Bni1p(1–524) (10 pmol) was incubated with the anti-MBP antibody (75 pmol) at 4°C for 2 h in buffer A (150 μl) containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 12 mM $MgCl_2$ and 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The antigen–antibody complexes were recovered with 5 mg protein A–Sepharose CL-4B. The GDP- or GTPγS-bound form of Rho1p was prepared as described previously (Yamamoto *et al.*, 1990) and 10 pmol of each form was incubated at 4°C for 1.5 h with immobilized MBP (25 pmol) or MBP–Bni1p(1–524) (5 pmol) in buffer A (120 μl) containing 5 μM GDP (for the GDP-bound form of Rho1p) or GTPγS (for the GTPγS-bound form of Rho1p). The Rho1p–MBP or Rho1p-MBP–Bni1p(1–524) complex-containing beads were recovered by centrifugation, washed three times with buffer A and the protein samples eluted and subjected to SDS–PAGE followed by Western blotting with the anti-Rho1p antibody.

Assay for glucan synthase activity

Yeast cells were grown in YPDAU medium at 24°C to an OD_{600} of 1.0. Cells were harvested by centrifugation and glucan synthase activity in the membrane fraction was measured in the presence or absence of 0.8 μM GTPγS as described (Inoue *et al.*, 1995).

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