Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*

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The *RHO1* gene encodes a homologue of mammalian *RhoA* small G-protein in the yeast *Saccharomyces cerevisiae*. Rho1p is required for bud formation and is localized at a bud tip or a cytokinesis site. We have recently shown that Bni1p is a potential target of Rho1p. Bni1p shares the FH1 and FH2 domains with proteins involved in cytokinesis or establishment of cell polarity. In *S.cerevisiae*, there is an open reading frame (*YIL159W*) which encodes another protein having the FH1 and FH2 domains and we have named this gene *BNR1* (*BNII* Related). Bnr1p interacts with another Rho family member, Rho4p, but not with Rho1p. Disruption of *BNII* or *BNR1* does not show any deleterious effect on cell growth, but the *bni1 bnr1* mutant shows a severe temperature-sensitive growth phenotype. Cells of the *bni1 bnr1* mutant arrested at the restrictive temperature are deficient in bud emergence, exhibit a random distribution of cortical actin patches and often become multinucleate. These phenotypes are similar to those of the mutant of *PFY1*, which encodes profilin, an actin-binding protein. Moreover, yeast two-hybrid and biochemical studies demonstrate that Bni1p and Bnr1p interact directly with profilin at the FH1 domains. These results indicate that Bni1p and Bnr1p are potential targets of the Rho family members, interact with profilin and regulate the reorganization of actin cytoskeleton.

Keywords: actin cytoskeleton/profilin/Rho

Introduction

The Rho family belongs to the small G-protein superfamily consisting of the Rho, Rac and Cdc42 subfamilies (Hall, 1994; Takai *et al.*, 1995). Evidence is accumulating that, through the reorganization of the actin cytoskeleton, Rho regulates various cell functions, such as cell morphological change, formation of stress fibres and focal adhesions, cell motility, membrane ruffling, cytokinesis, cell aggregation and smooth muscle contraction. 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 downstream 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 the GTPase-activating proteins (GAPs). 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 the reorganization of actin cytoskeleton. Very recently, one of these proteins, Rho kinase, has been shown to inhibit the myosin phosphatase activity (Kimura *et al.*, 1996), although its physiological significance remains to be clarified.

Cells of the budding yeast *Saccharomyces cerevisiae* grow by budding for cell division, and the actin cytoskeleton plays a pivotal role in the budding process (Drubin, 1991). This yeast possesses the Rho family members, including *RHO1* and *RHO2* (Madaule *et al.*, 1987), *RHO3* and *RHO4* (Matsui and Toh-e, 1992) and *CDC42* (Adams *et al.*, 1990; Johnson and Pringle, 1990). *RHO1* is a homologue of the mammalian *RhoA* gene and we have shown that the *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 with cortical actin patches, including the presumptive budding site, the bud tip and the cytokinesis site (Yamochi *et al.*, 1994). These results suggest that *RHO1* regulates the processes of bud formation. Concerning the downstream targets of Rho1p, we have shown that one of them is a homologue of mammalian protein kinase C, Pkc1p (Nonaka *et al.*, 1995), which regulates cell wall integrity through the activation of the MAP kinase cascade (Levin and Errede, 1995). We have also shown that another target of Rho1p is 1,3- β -glucan synthase (Drgonová *et al.*, 1996; Qadota *et al.*, 1996), which is involved in cell wall synthesis. Very recently, we have identified *BNII* as a third potential target of *RHO1* (Kohno *et al.*, 1996). *BNII* (Jansen *et al.*, 1996; Zahner *et al.*, 1996) and its related genes in other organisms, including *diaphanous* (Castrillon and Wasserman, 1994) and *cappuccino* (Emmons *et al.*, 1995) in *Drosophila*, *FigA* in *Aspergillus* (Marhoul and Adams, 1995) and *fus1* in *Schizosaccharomyces pombe* (Petersen *et al.*, 1995), have been shown to be involved in cytokinesis, establishment of cell polarity, or normal cell morphology. These results suggest that Bni1p or its related proteins regulate cytoskeletal reorganization, but it has not yet been demonstrated how they do so. Structural features of Bni1p- or Bnr1p-related proteins are that they contain two domains named FH1 and FH2 (Formin Homology). It should be noted that the FH1 domain

respectively (data not shown). These results suggest that the enlarged cell phenotype was due to non-polar cell growth which was caused by budding deficiency. This interpretation was supported by the delocalized staining of cortical actin patches (Figure 3A, Actin) and cell wall chitin (Figure 3B, Chitin) in the *bni1 bnr1* mutant cells, which are normally seen at the polarized growth sites and the bud scars, respectively. Staining of DNA revealed that there were more than two nuclei in ~60% of the *bni1 bnr1* mutant cells arrested at 33°C (Figure 3A, DNA), indicating that synthesis and division of nuclear DNAs are not primarily affected in the *bni1 bnr1* mutant cells. The budding pattern of the *bnr1* mutant cells was random in a haploid mutant, but not in a diploid mutant (Figure 3B, Chitin), contrasting clearly with the result that the *bni1* mutant shows a diploid-specific randomization of budding pattern (Figure 3B, Chitin) (Zahner *et al.*, 1996).

These phenotypes of the *bni1 bnr1* mutant were very similar to those of the mutant of the *PFY1* gene, which

encodes a yeast homologue of profilin (Haarer *et al.*, 1990). Another phenotype of the *pfy1* mutant is growth-sensitive to 1 M sorbitol, as are some other temperature-sensitive mutants of the actin gene (*ACT1*) (Novick and Botstein, 1985; Haarer *et al.*, 1990). This sorbitol-sensitive phenotype was also seen in the *bni1 bnr1* mutant (Figure 4). These results suggest that there are physiological interactions between profilin and Bni1p or Bnr1p. This point was further examined genetically. The *bni1* mutant was transformed with a multicopy plasmid carrying *PFY1*. Overexpression of *PFY1* inhibited growth of the *bni1* mutant, but not that of the wild-type strain (Figure 5). Although this growth inhibition was not seen in the *bnr1* mutant (data not shown), this result indicates that at least *BNI1* interacts genetically with *PFY1*.

Interactions of Bni1p and Bnr1p with profilin at their FH1 domains in the yeast two-hybrid method

The phenotypic similarities between the *bni1 bnr1* and *pfy1* mutants and the presence of proline-rich FH1 domains in Bni1p and Bnr1p suggested that Bni1p and Bnr1p interact physically with profilin. This point was examined by the yeast two-hybrid method (Fields and Song, 1989). Both Bni1p and Bnr1p indeed interacted with yeast profilin (Figure 6). The region of Bni1p or Bnr1p that interacted with profilin was delimited. Both Bni1p and Bnr1p interacted with profilin at their FH1 domains (Figure 7A and B).

Direct interaction of the FH1 domain of Bni1p with profilin

In the next set of experiments, it was examined whether Bni1p directly interacts with profilin. A maltose-binding

Fig. 2. The temperature-sensitive growth phenotype of the *bni1 bnr1* mutant. Cells of strains, OHNY1 (WT), BTY1 (*bni1*), HIY2 (*bnr1*) and HIY11 (*bni1 bnr1*), were streaked onto YPDAU plates which were subsequently incubated at 24°C or 33°C for 4 days.

Fig. 3. Morphological phenotypes of the *bni1 bnr1* mutant. (A) Staining of actin and DNA. Cells of haploid strains, OHNY1 (WT) and HIY11 (*bni1 bnr1*), were incubated for 8 h at 33°C in YPDAU medium, fixed and double-stained with rhodamine-phalloidin and DAPI for actin and DNA, respectively, followed by microscopic observation. All fields were photographed at the same magnification. (B) Staining of chitin. Cells of haploid strains, OHNY1 (WT), BTY1 (*bni1*), HIY2 (*bnr1*) and HIY11 (*bni1 bnr1*), and diploid strains OHNY3 (WT), KY4 (*bni1*), HIY3A (*bnr1*) and HIY13A (*bni1 bnr1*), were incubated for 8 h at 33°C in YPDAU medium, fixed and stained with calcofluor for chitin, followed by microscopic observation. All fields were photographed at the same magnification.

Fig. 4. The sorbitol-sensitive growth phenotype of the *bni1 bnr1* mutant. Cells of strains OHNY1 (WT), HIY11 (*bni1 bnr1*) and DCLS5-6 (*pfy1*) were streaked onto a YPD AU plate and a YPD AU plate containing 1 M sorbitol and incubated for 4 days at 24°C.

Fig. 5. Genetic interaction between *BNI1* and *PFY1*. Cells of strains OHNY1 (WT) and BTY1 (*bni1*) were transformed with YEpL-*PFY1* (*PFY1*) or YEp351 (Vector). Four independent transformants obtained from each transformation were streaked onto SD plates lacking leucine and incubated for 4 days at 33°C.

Fig. 6. Two-hybrid interactions between yeast profilin and Bni1p or Bnr1p. Cells of yeast strains were streaked on an SD plate lacking tryptophan, leucine and histidine, and incubated for 4 days at 30°C. The yeast strains streaked were TAT7 containing pBTM116-HA-PFY1 and pACTII-HK-BNI1(1-1953) [PFY1 BNI1], pBTM116-HA-PFY1 and pACTII-HK-BNR1(1-1374) [PFY1 BNR1], pBTM116-HA-PFY1 and pACTII-HK [PFY1 Vector], and pBTM116-Ras(G12V) and VP16-Raf [Ras(G12V) Raf].

protein (MBP) fused to Bni1p(1239–1650) containing the FH1 domain was expressed in *Escherichia coli* and purified. This MBP–Bni1p(1239–1650) and MBP were subjected to SDS–PAGE, blotted onto a nitrocellulose membrane filter and detected by an overlay assay using the glutathione *S*-transferase (GST)-fused yeast profilin (yeast GST–profilin) or GST. Yeast GST–profilin and GST were detected with an anti-GST antibody. GST–profilin interacted with MBP–Bni1p(1239–1650), but not with MBP (Figure 8). GST did not interact with MBP–Bni1p(1239–1650) or MBP. GST–profilin also interacted with MBP–Bni1p(1239–1328), which contains only the FH1 domain of Bni1p (data not shown). These results indicate that profilin interacts directly with Bni1p at the FH1 domain.

This direct interaction of profilin with Bni1p was further confirmed using the Bni1p-immobilized amylose resin column. The GST-fused human profilin (human GST–profilin) (Kwiatkowski and Bruns, 1988) or GST was loaded onto an amylose resin column which was prebound

to MBP–Bni1p(1239–1650) or MBP. The protein complexes were eluted with maltose and subsequently subjected to SDS–PAGE followed by protein staining. Human GST–profilin bound to MBP–Bni1p(1239–1650), but not to MBP (Figure 9A). GST did not bind to MBP–Bni1p(1239–1650) or MBP. To confirm that GST–profilin bound specifically MBP–Bni1p(1239–1650), GST–profilin or GST was recovered from each eluate with a glutathione column and subjected to SDS–PAGE followed by protein staining. GST–profilin bound specifically to MBP–Bni1p(1239–1650) (Figure 9B). When the amounts of GST–profilin and MBP–Bni1p(1239–1650) were quantified by densitometric tracing, it was found that ~2 mol of GST–profilin bound to 1 mol of MBP–Bni1p(1239–1650) (data not shown). We concluded that profilin directly binds to the FH1 domain of Bni1p.

Bnr1p as a potential target of Rho4p

The weak homology of Bnr1p to the Rho1p-binding region of Bni1p suggested that Bnr1p also interacts with Rho1p. This point was examined by the yeast two-hybrid method. Bnr1p did not interact with a dominant active Rho1p, Rho1p(Q68L), keeping Rho1p in the GTP-bound form (Bourne *et al.*, 1991) (Table I). Therefore, it was examined whether Bnr1p interacts with other Rho family members in *S.cerevisiae*, including Rho2p, Rho3p, Rho4p and Cdc42p. Dominant active mutations were introduced into *RHO2*, *RHO3*, *RHO4* and *CDC42*, and these mutant genes were cloned into a yeast two-hybrid vector along with the wild-type genes. Bnr1p did not interact with either Rho2p(Q65L), Rho2p, Rho3p(Q74L) or Rho3p (Table I). However, Bnr1p significantly interacted with Rho4p(Q70L) and Rho4p, but not with a dominant negative Rho4p, Rho4p(T25N), keeping Rho4p in the GDP-bound or nucleotide-free form (Farnsworth and Feig, 1991). In the case of *CDC42*, although background levels were high, significant interaction of Bnr1p with Cdc42p(Q12V) or Cdc42p was not observed. We have shown that the Rho1p-binding region of Bni1p resides in the N-terminal region which is amino acid positions from 90 to 343 (Kohno *et al.*, 1996). The Rho4p-binding region of Bnr1p also resided in the N-terminal region which is at least amino acid positions from 63 to 421 (data not shown). This region of Bnr1p is homologous to the Rho1p-binding region of Bni1p (23% identity). These results suggest that Bnr1p is a potential target of Rho4p.

Direct interaction of Bnr1p with Rho4p

To investigate whether Bnr1p interacts directly with Rho4p, an MBP fused to Bnr1p(63–421) and a GST fused to Rho4p were expressed in *E.coli* and purified. MBP–Bnr1p(63–421) bound to the GTPγS-bound form, but not the GDP-bound form, of GST–Rho4p (Figure 10). MBP did not bind to either form of GST–Rho4p. This result indicates that Bnr1p directly interacts with the GTP-bound form of Rho4p.

Discussion

We have shown that Bni1p and its homologue, Bnr1p, are potential targets of the Rho family members and bind to profilin at their proline-rich FH1 domains. These results, together with the genetic results that the *bni1 bnr1*

Fig. 7. Deletion mappings of the yeast profilin-interacting domains of Bni1p and Bnr1p. Various DNA fragments encoding truncated Bni1ps or Bnr1ps were cloned into the yeast two-hybrid vector, pACTII-HK, and resultant plasmids were transformed into TAT7 containing pBTM116-HA-PFY1. The interaction of Bni1p or Bnr1p with yeast profilin was examined in each transformant by the qualitative and quantitative assay methods for β -galactosidase activity. Closed bars, blue colony colour; open bars, white colony colour. The values are the average of β -galactosidase activities for three transformants. Each measured value was within 50% of the average. (A) Deletion mapping of Bni1p. (B) Deletion mapping of Bnr1p.

disruption mutant showed the phenotypes similar to those of the *pfy1* mutant, suggest that Bni1p and Bnr1p regulate the reorganization of the actin cytoskeleton through profilin. Consistently, at least Rho1p (Yamochi *et al.*, 1994) and Bni1p (Jansen *et al.*, 1996) have been shown to be localized at a bud tip, where actin cytoskeleton seems to be actively reorganized (Drubin, 1991). Recently, Wiskott-Aldrich syndrome protein (WASP) has been shown to be involved in the reorganization of actin cytoskeleton as a target of a Rho family member, Cdc42 (Aspenström *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996). Since WASP also has proline-rich sequences, it is interesting to clarify whether it also regulates the reorganization of the actin cytoskeleton through profilin.

Genetic studies have established that profilin plays a pivotal role for the reorganization of actin cytoskeleton (Sohn and Goldschmidt-Clermont, 1994), but the mode of action of profilin has not yet been clarified. Profilin has been shown to stimulate the dissociation of ADP from the ADP-bound form of an actin monomer to form the ATP-bound form, which polymerizes several times faster than the ADP-bound form. An additional mechanism by which profilin promotes actin polymerization has been reported (Pantaloni and Carlier, 1993). In the presence of

Fig. 8. Direct interaction of Bni1p(1239-1650) with yeast profilin. 40 pmol of MBP-Bni1p(1239-1650) or MBP was subjected to SDS-PAGE and transferred to a nitrocellulose membrane filter. The filter was then probed with yeast GST-profilin or GST, which was detected with an antibody raised against GST. Lanes 1 and 3, MBP-Bni1p(1239-1650); lanes 2 and 4, MBP.

thymosin β 4, which is an actin monomer-sequestering protein, and excess ATP, profilin desequesters an actin monomer from thymosin β 4 and, by lowering the critical concentration for actin, increases filament assembly. The

Fig. 9. Complex formation of MBP-Bni1p(1239–1650) with human profilin. (A) Binding of human GST-profilin to MBP-Bni1p(1239–1650). GST-profilin or GST was loaded onto an amylose resin column which was prebound to MBP-Bni1p(1239–1650) or MBP. The bound proteins were subsequently eluted with maltose and the eluted proteins were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. (B) Isolation of the MBP-Bni1p(1239–1650)–human GST-profilin complex. Each eluate obtained in (A) was loaded onto a glutathione–Sepharose 4B column and the bound proteins were subsequently eluted with reduced glutathione. The eluted proteins were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. Lanes 1 and 2, MBP; lanes 3 and 4, MBP-Bni1p(1239–1650); lanes 1 and 3, GST; lanes 2 and 4, GST-profilin.

Table 1. Two-hybrid interactions between the Rho family members and Bnr1p

DNA-binding domain fusion	Transcriptional activating domain fusions ^a	
	BNR1	Vector
RHO1(Q68L)	<1	<1
RHO1	<1	<1
RHO1(T24N)	<1	<1
RHO2(Q65L)	8	8
RHO2	<1	<1
RHO3(Q74L)	9	13
RHO3	<1	<1
RHO4(Q70L)	180	<1
RHO4	83	<1
RHO4(T25N)	16	6
CDC42(Q12V)	133	158
CDC42	72	82

The values are the average of β -galactosidase activities for three transformants. Each measured value was within 50% of the average.

^aA plasmid pACTII–HK–BNR1(1–1374) was used as a transcriptional activating domain fusion with *BNR1*.

mechanism for this effect remains to be clarified, but it is suggested that profilin couples the ATPase activity of F-actin to the addition of profilin–actin complexes onto barbed ends with subsequent release of profilin. However, it is currently unknown whether profilin promotes filament assembly in this way in *S.cerevisiae*, since a homologue of thymosin β 4 has not yet been discovered in *S.cerevisiae*. We do not know at present how Bni1p and Bnr1p are involved in this profilin–actin interaction, but the genetic results that the phenotypes of the *bni1 bnr1* mutant are similar to those of the *pfy1* mutant indicate that the interactions of Bni1p and Bnr1p with profilin are important for the proper functions of profilin. Another genetic result which links *BNI1* with profilin is that overexpression of profilin inhibits the growth of the *bni1* mutant, but not that of the *bnr1* mutant or the wild-type strain. These results may suggest that a quantitative balance between

Fig. 10. Direct interaction of Bnr1p with Rho4p. MBP-Bnr1p(63–421) or MBP was loaded onto a glutathione–Sepharose 4B column which was prebound to GST or the GDP- or GTP γ S-bound form of GST–Rho4p. The bound proteins were subsequently eluted with reduced glutathione and the eluted proteins were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. Lanes 1 and 4, GST; lanes 2 and 5, GDP-GST–Rho4p; lanes 3 and 6, GTP γ S–GST–Rho4p; lanes 1, 2 and 3, MBP; lanes 4, 5 and 6, MBP–Bnr1p(63–421).

profilin and Bni1p/Bnr1p is important for the proper functions of these proteins, and that Bni1p is more abundant than Bnr1p and plays a more important role than Bnr1p. Consistent with this, the *bni1* mutant, but not the *bnr1* mutant, shows a slow growth phenotype at a higher temperature. Our genetic results indicate that biochemical studies of profilin in the presence of Bni1p or Bnr1p are worth further examination.

Our results indicate that Bni1p and Bnr1p regulate the functions of profilin through their proline-rich FH1 domains. Vasodilator-stimulated phosphoprotein (VASP) is a microfilament- and focal adhesion-associated protein which is concentrated in highly dynamic regions of the cell cortex (Reinhard *et al.*, 1992). VASP has also been shown to bind to profilin through a proline-rich domain (Reinhard *et al.*, 1995). Therefore, the proline-rich domains seem to play important roles in the reorganization of actin cytoskeleton. It is interesting to examine whether VASP also functions as a target of a Rho family member. Our results suggest that the other FH1 domain-containing proteins found in various organisms also interact with profilin. Very recently, one of these proteins, *cappuccino* in *Drosophila*, has been shown to bind to profilin in the yeast two-hybrid method (Manseau *et al.*, 1996), although it has not been clarified whether *cappuccino* binds to profilin at the FH1 domain and whether it binds directly to profilin. On the other hand, the FH1 domain of formin has been shown to bind to the SH3 or WW domain-containing proteins (Chan *et al.*, 1996). Although it remains to be shown that these interactions are physiologically significant, if formin, like Bni1p or Bnr1p, interacts with profilin at the FH1 domain, the SH3 or WW domain-containing proteins may regulate the interactions of the FH1 domain with profilin. Similarly, it should be examined whether the FH1 domain of Bni1p or Bnr1p interacts with an SH3 or WW domain-containing protein in *S.cerevisiae*.

Our present conclusion that Bni1p and Bnr1p regulate the reorganization of the actin cytoskeleton through profilin is consistent with the findings that FH1- and FH2-containing proteins are involved in cytokinesis or establishment of cell polarity (Castrillon and Wasserman, 1994; Emmons *et al.*, 1995). Recently, Bni1p has been shown

Table II. Yeast strains used in this study

Strain	Genotype
OHNY1	<i>MATa ura3 leu2 trp1 his3 ade2</i> (Nonaka <i>et al.</i> , 1995)
OHNY3	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2</i> (Ozaki <i>et al.</i> , 1996)
BTY1	<i>MATa ura3 leu2 trp1 his3 ade2 bni1::HIS3</i> (Kohno <i>et al.</i> , 1996)
KY4	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 bni1::HIS3/bni1::HIS3</i> (Kohno <i>et al.</i> , 1996)
HIY2	<i>MATα ura3 leu2 trp1 his3 ade2 bnr1::TRP1</i>
HIY3A	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 bnr1::TRP1/bnr1::TRP1</i>
HIY3B	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 BNR1/bnr1::TRP1</i>
HIY11	<i>MATa ura3 leu2 trp1 his3 ade2 bni1::HIS3 bnr1::TRP1</i>
HIY13A	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 bni1::HIS3/bni1::URA3 bnr1::TRP1/bnr1::TRP1</i>
DCLS5-6	<i>MATa ura3 leu2 trp1 his3 ade2 pfy1::ADE2</i>
TAT7	<i>MATa trp1 leu2 his3 LYS2::lexA-HIS3 ura3::lexA-lacZ</i>

Strains used in this study are isogenic except TAT7 and DCLS5-6.

to be involved in various aspects of cell polarity in *S.cerevisiae*. In one genetic screening, 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). *SHE5* is identical to *BNI1*, whereas *SHE1* is identical to *MYO4*, which encodes a myosin motor. *BNI1* may be involved in the formation of actin filaments on which Myo4p moves to bring Ash1p or a protein needed for the daughter cell-specific localization of Ash1p from the mother cell to its daughter cell. In another genetic screening, *BNI1* has been identified as a mutation which causes a bipolar budding-specific randomization of budding pattern (Zahner *et al.*, 1996). This result suggests that Bni1p is necessary for the initial localization of bipolar positional signals to the presumptive budding site. We have found that the *bnr1* mutant shows an axial budding-specific randomization of budding pattern. Therefore, in contrast to Bni1p, Bnr1p seems to be necessary for the localization of axial positional signals to the presumptive budding site. In this respect, it is clear that there is a functional difference between Bni1p and Bnr1p. It would be interesting to examine whether Bni1p or Bnr1p regulates the budding pattern through the profilin–actin system. The involvement of *BNI1* and *BNR1* in the control of budding patterns suggests that Rho1p and Rho4p regulate the budding patterns through Bni1p and Bnr1p, respectively. However, since Rho1p is involved in the activation of glucan synthase and Pkc1p, which are essential for cell growth, it is difficult to examine genetically whether *RHO1* is involved in the control of budding pattern. In the case of *RHO4*, we have not observed the axial budding-specific random budding phenotype in the *rho4* mutant (data not shown), suggesting that a factor other than Rho4p is involved in the Bnr1p-regulated budding pattern. This factor may be a new member of the Rho family (*YNL180C*, GenBank accession number Z71456), which has been revealed by the yeast genome sequencing project (data not shown). Further studies are necessary to clarify the interactions between the Rho family members and Bni1p or Bnr1p in the control of budding patterns.

Bni1p and Bnr1p bind preferentially to the GTP γ S-bound forms of Rho1p and Rho4p, respectively. Although Bni1p interacts significantly with the GDP-bound form of Rho1p (Kohno *et al.*, 1996), the interaction of Bnr1p with the GDP-bound form of Rho4p was negligible. The

functional significance of these interactions are currently unknown. One plausible function of the Rho family members is that they bind to Bni1p or Bnr1p to bring them to the cellular sites of action such as a bud tip. This mode of action has been proposed for the function of Ras on the regulation of its target, c-Raf-1 (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). In contrast, we and other groups have shown that the GTP-bound form of Rho1p stimulates the protein kinase activity of Pkc1p (H.Nonaka *et al.*, unpublished results; Kamada *et al.*, 1996) and the glucan synthase activity (Drgonová *et al.*, 1996; Qadota *et al.*, 1996). We have also shown that the GTP-bound form of Ras stimulates the protein kinase activity of B-Raf (Yamamori *et al.*, 1995). Therefore, the Rho family members may regulate biochemical activities of Bni1p or Bnr1p, including their affinities for profilin. However, in the current study, we have used truncated forms of Bni1p which lack the Rho1p-binding domain. To examine how Rho1p regulates the interaction of Bni1p with profilin, we have attempted to purify full-length (recombinant or native) Bni1p. However, such purification has not been successful due to Bni1p's insolubility and high susceptibility to proteolysis. Further studies are required to clarify the mode of action of the Rho family members on the regulation of Bni1p and Bnr1p.

Materials and methods

Strains, media and yeast transformations

Yeast strains used in this study are listed in Table II. Yeast strains were grown on rich media 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). Yeast transformations were performed by the lithium acetate methods (Gietz *et al.*, 1992). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco) and amino acids were supplemented to SD medium when required. Standard yeast genetic manipulations were performed as described (Sherman *et al.*, 1986). An *E.coli* strain DH5 α 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). Plasmids used in this study are listed in Table III.

DNA sequences were determined using ALFred DNA sequencer (Pharmacia Biotech, Inc., Uppsala, Sweden) and PCRs were performed using GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Point mutations were introduced by the PCR mutagenesis methods (Higuchi, 1989).

Table III. Plasmids used in this study

Plasmid	Characteristics
pBTM116	DBD _{LexA} -TRP1, 2 µm; (Bartel <i>et al.</i> , 1993)
pBTM116-HA	DBD _{LexA} -TRP1, 2 µm; made by inserting the synthetic oligonucleotides encoding the HA epitope, YPYDVPDYA, which is derived from the influenza haemagglutinin protein, into the <i>EcoRI</i> - <i>PstI</i> site of pBTM116
pBTM116-RHO1ΔC	DBD _{LexA} -RHO1ΔC, TRP1, 2 µm; (Nonaka <i>et al.</i> , 1995)
pBTM116-RHO1ΔC(Q68L)	DBD _{LexA} -RHO1ΔC(Q68L), TRP1, 2 µm; (Nonaka <i>et al.</i> , 1995)
pBTM116-RHO1ΔC(T24N)	DBD _{LexA} -RHO1(T24N), TRP1, 2 µm; (Nonaka <i>et al.</i> , 1995)
pBTM116-HA-RHO2ΔC	DBD _{LexA} -RHO2ΔC, TRP1, 2 µm; made by inserting the 564 bp <i>BamHI</i> - <i>SmaI</i> PCR fragment containing the <i>RHO2</i> open reading frame into the <i>BamHI</i> - <i>SmaI</i> site of pBTM116-HA. The region encoding C-terminal five amino acids (CCIL) of Rho2p was deleted in this construct
pBTM116-HA-RHO2ΔC(Q65L)	DBD _{LexA} -RHO2ΔC(Q65L), TRP1, 2 µm; a derivative of pBTM116-HA-RHO2ΔC. A codon for amino acid position 65 of Rho2p, CAA (Q), was mutated to TTA (L) by PCR mutagenesis
pBTM116-HA-RHO3ΔC	DBD _{LexA} -RHO3ΔC, TRP1, 2 µm; made by inserting the 678 bp <i>BamHI</i> - <i>SmaI</i> PCR fragment containing the <i>RHO3</i> open reading frame into the <i>BamHI</i> - <i>SmaI</i> site of pBTM116-HA. The region encoding C-terminal six amino acids (SSCTIM) of Rho3p was deleted in this construct
pBTM116-HA-RHO3ΔC(Q74L)	DBD _{LexA} -RHO3ΔC(Q74L), TRP1, 2 µm; a derivative of pBTM116-HA-RHO3ΔC. A codon for amino acid position 74 of Rho3p, CAA (Q), was mutated to TTA (L) by PCR mutagenesis
pBTM116-HA-RHO4ΔC	DBD _{LexA} -RHO4ΔC, TRP1, 2 µm; made by inserting the 682 bp <i>BamHI</i> - <i>SmaI</i> PCR fragment containing the <i>RHO4</i> open reading frame into the <i>BamHI</i> - <i>SmaI</i> site of pBTM116-HA. The region encoding C-terminal four amino acids (CIIM) of Rho4p was deleted in this construct
pBTM116-HA-RHO4ΔC(Q70L)	DBD _{LexA} -RHO4ΔC(Q70L), TRP1, 2 µm; a derivative of pBTM116-HA-RHO4ΔC. A codon for amino acid position 70 of Rho4p, CAA (Q), was mutated to TTA (L) by PCR mutagenesis
pBTM116-HA-RHO4ΔC(T25N)	DBD _{LexA} -RHO4ΔC(T25N), TRP1, 2 µm; a derivative of pBTM116-HA-RHO4ΔC. A codon for amino acid position 25 of Rho4p, ACG (T), was mutated to AAT (N) by PCR mutagenesis
pBTM116-CDC42ΔC	DBD _{LexA} -CDC42ΔC, TRP1, 2 µm; made by inserting the 575 bp <i>EcoRI</i> - <i>PstI</i> PCR fragment containing the <i>CDC42</i> open reading frame into the <i>EcoRI</i> - <i>PstI</i> site of pBTM116. The region encoding C-terminal four amino acids (CTIL) of Cdc42p was deleted in this construct
pBTM116-CDC42ΔC(G12V)	DBD _{LexA} -CDC42ΔC(G12V), TRP1, 2 µm; a derivative of pBTM116-CDC42ΔC. A codon for amino acid position 12 of Cdc42p, GGT (G), was mutated to GTT (V) by PCR mutagenesis
pBTM116-HA-PFY1	DBD _{LexA} -PFY1, TRP1, 2 µm; made by inserting the 381 bp <i>BamHI</i> - <i>SmaI</i> PCR fragment containing the <i>PFY1</i> open reading frame, in which the intron sequence has been deleted, into the <i>BamHI</i> - <i>SmaI</i> site of pBTM116-HA
pACTII-HK	AD _{GAL4} -LEU2, 2 µm; (Ozaki <i>et al.</i> , 1996)
pACTII-HK-BNI1(1-1953)	AD _{GAL4} -BNI1(1-1953), LEU2, 2 µm; (Kohn <i>et al.</i> , 1996)
pACTII-HK-BNI1(1-1238)	AD _{GAL4} -BNI1(1-1238), LEU2, 2 µm; made by inserting the 3.7 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 1 to 1238 of Bni1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNI1(1239-1953)	AD _{GAL4} -BNI1(1239-1953), LEU2, 2 µm; made by inserting the 2.1 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 1239 to 1953 of Bni1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNI1(1239-1750)	AD _{GAL4} -BNI1(1239-1750), LEU2, 2 µm; made by deleting the 0.6 kbp <i>SacII</i> - <i>SmaI</i> fragment from pACTII-HK-BNI1(1239-1953)
pACTII-HK-BNI1(1751-1953)	AD _{GAL4} -BNI1(1751-1953), LEU2, 2 µm; made by deleting the 1.5 kbp <i>BamHI</i> - <i>SacII</i> fragment from pACTII-HK-BNI1(1239-1953)
pACTII-HK-BNI1(1239-1650)	AD _{GAL4} -BNI1(1239-1650), LEU2, 2 µm; made by inserting the 1.2 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 1239 to 1650 of Bni1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNI1(1239-1328)	AD _{GAL4} -BNI1(1239-1328), LEU2, 2 µm; made by deleting the 0.9 kbp <i>NaeI</i> - <i>SmaI</i> fragment from pACTII-HK-BNI1(1239-1650)
pACTII-HK-BNI1(1329-1650)	AD _{GAL4} -BNI1(1329-1650), LEU2, 2 µm; made by deleting the 0.3 kbp <i>BamHI</i> - <i>NaeI</i> fragment from pACTII-HK-BNI1(1239-1650)
pACTII-HK-BNI1(1239-1291)	AD _{GAL4} -BNI1(1239-1291), LEU2, 2 µm; made by inserting the 0.2 kbp <i>BamHI</i> - <i>NcoI</i> (filled in) fragment from pACTII-HK-BNI1(1239-1328) into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNI1(1292-1328)	AD _{GAL4} -BNI1(1292-1328), LEU2, 2 µm; made by deleting the 0.2 kbp <i>BamHI</i> - <i>NcoI</i> fragment from pACTII-HK-BNI1(1239-1328)
pACTII-HK-BNI1(1292-1953)	AD _{GAL4} -BNI1(1292-1953), LEU2, 2 µm; made by inserting the 2.0 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 1292 to 1953 of Bni1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(1-1374)	AD _{GAL4} -BNR1(1-1374), LEU2, 2 µm; made by inserting the 4.1 kbp <i>SmaI</i> - <i>SmaI</i> PCR fragment containing the <i>BNR1</i> open reading frame into the <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(63-421)	AD _{GAL4} -BNR1(63-421), LEU2, 2 µm; made by inserting the 1.1 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 63 to 421 of Bnr1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(612-1374)	AD _{GAL4} -BNR1(612-1374), LEU2, 2 µm; made by inserting the 2.3 kbp <i>NaeI</i> - <i>SmaI</i> fragment from pACTII-HK-BNR1(1-1374) into the <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(1-755)	AD _{GAL4} -BNR1(1-755), LEU2, 2 µm; made by inserting the 2.3 kbp <i>SmaI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 1 to 755 of Bnr1p into the <i>SmaI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(756-1165)	AD _{GAL4} -BNR1(756-1165), LEU2, 2 µm; made by inserting the 1.2 kbp <i>BamHI</i> - <i>SmaI</i> PCR fragment encoding amino acid positions from 756 to 1165 of Bnr1p into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(756-887)	AD _{GAL4} -BNR1(756-887), LEU2, 2 µm; made by inserting the 0.4 kbp <i>BamHI</i> - <i>EcoRV</i> fragment from pACTII-HK-BNR1(756-1165) into the <i>BamHI</i> - <i>SmaI</i> site of pACTII-HK
pACTII-HK-BNR1(756-807)	AD _{GAL4} -BNR1(756-807), LEU2, 2 µm; made by inserting the 0.2 kbp <i>BglII</i> - <i>BglII</i> fragment from pACTII-HK-BNR1(756-887) into the <i>BglII</i> - <i>BglII</i> site of pACTII-HK
pBS-BNI1	<i>BNI1</i> ; (Kohn <i>et al.</i> , 1996)
pBS-BNR1	<i>BNR1</i> ; made by inserting the 4.1 kbp <i>SmaI</i> - <i>SmaI</i> fragment from pACTII-HK-BNR1(1-1374) into the <i>SmaI</i> site of pBluescript KS(+)

Table III. Continued

Plasmid	Characteristics
pBS-bni1::URA3	A derivative of pBS-BNI1; made by replacing the 561 bp <i>Bgl</i> II- <i>Bgl</i> II internal fragment of <i>BNI1</i> corresponding to amino acid positions from 1228 to 1414 of Bni1p with the 1.1 kbp <i>URA3</i> fragment
pBS-bnr1::TRP1	A derivative of pBS-BNR1; made by replacing the 365 bp <i>Bgl</i> II- <i>Bgl</i> II internal fragment of <i>BNR1</i> corresponding to amino acid positions from 685 to 707 of Bnr1p with the 0.8 kbp <i>TRP1</i> fragment
YEpl-PFY1	<i>PFY1</i> , <i>LEU2</i> , 2 μ m; made by inserting the 4 kbp <i>PFY1</i> fragment into the <i>Nhe</i> I- <i>Sph</i> I site of pQR325
YEpl351	<i>LEU2</i> , 2 μ m (Hill <i>et al.</i> , 1986)
pMAL-c2-BNI1(1239–1650)	MBP-BNI1(1239–1650); made by inserting the 1.2 kbp <i>Bam</i> HI- <i>Sma</i> I DNA fragment of <i>BNI1</i> from pACTII-HK-BNI1(1239–1650) into the <i>Bam</i> HI- <i>Sma</i> I site of pMAL-c2 (New England BioLabs, Inc.)
pMAL-c2-BNR1(63–421)	MBP-BNR1(63–421); made by inserting the 1.1 kbp <i>Bam</i> HI- <i>Bgl</i> II DNA fragment of <i>BNR1</i> from pACTII-HK-BNR1(63–421) into the <i>Bam</i> HI site of pMAL-c2
pGEX-4T-2-HA	GST-HA; made by inserting the oligonucleotides encoding the HA epitope into the <i>Bam</i> HI site of pGEX-4T-2
pGEX-4T-2-HA-PFY1	GST-HA-PFY1; made by inserting the 0.4 kbp <i>Bam</i> HI- <i>Sma</i> I DNA fragment of <i>PFY1</i> from pACTII-HK-PFY1 into the <i>Bam</i> HI- <i>Sma</i> I site of pGEX-4T-2-HA
pGEX-2T-profilin	GST-profilin; made by inserting the 0.4 kbp <i>Bam</i> HI- <i>Bam</i> HI PCR fragment containing the human profilin I open reading frame into the <i>Bam</i> HI site of pGEX-2T
pGEX-4T-2-HA-RHO4	GST-HA-RHO4; made by inserting the 0.7 kbp <i>Bam</i> HI- <i>Bam</i> HI PCR fragment containing the <i>RHO4</i> open reading frame into the <i>Bam</i> HI site of pGEX-4T-2-HA

Disruptions of *BNI1* and *BNR1*

BNI1 was disrupted with *URA3* using a plasmid pBS-bni1::URA3 as described (Kohno *et al.*, 1996). *BNR1* was disrupted as follows. pBS-bnr1::TRP1 was cut with *Sma*I and the digested DNA was introduced into a wild-type diploid strain OHNY3. The genomic DNA was isolated from each transformant and the proper disruption of *BNR1* was verified by PCR (data not shown). A diploid strain in which one *BNR1* allele was disrupted was named HIY3B and was subjected to tetrad analysis. All dissected asci (16 asci) showed a 2 Trp⁻: 2 Trp⁺ segregation pattern and all of the Trp⁺ clones grew normally at 24°C, 30°C and 33°C. These *bnr1* mutant strains were used for further genetic studies.

Cytological techniques

Actin and DNA were stained with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) and 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 with a Zeiss Axiophoto microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a peltier cooling 3CCD colour camera (C5810-01; Hamamatsu Photonics KK., Hamamatsu, Japan).

Yeast two-hybrid method

A plasmid containing a gene fused to the *LexA* DNA-binding domain (DBD_{LexA}) was transformed into a yeast strain TAT7 and the resultant transformants were retransformed by a plasmid containing a gene fused to the *GAL4* transcriptional activating domain (AD_{GAL4}). Cells of each transformant were placed on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside for β -galactosidase activity as described (Vojtek *et al.*, 1993). For quantitative assay for β -galactosidase activity, cells of each transformant were cultured in SD medium lacking tryptophan and leucine and the β -galactosidase activity was measured according to the ONPG assay method described (Guarente, 1983). Two-hybrid interaction was also examined by the growth phenotype of each transformant on a SD plate medium lacking tryptophan, leucine and histidine.

Materials and chemicals for biochemical assays

Recombinant Bni1p(1239–1650) and Bnr1p(63–421) were purified from overexpressing *E. coli* DH5 α as MBP fusion proteins using an amylose resin column (New England BioLabs, Inc., Beverly, MA) as described (Guan *et al.*, 1987). Recombinant yeast or human profilin and Rho4p were purified from overexpressing *E. coli* DH5 α as GST fusion proteins using a glutathione-Sepharose 4B column (Pharmacia L-L Bio-chemicals Inc., Milwaukee, WI) as described (Kikuchi *et al.*, 1992). The anti-GST antibody (sc-138) was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The BA-85 (0.45 μ m pore size) nitrocellulose membrane filter was purchased from Schleicher & Schuell (Dassel, Germany).

Assay for the binding of recombinant Bni1p(1239–1650) with profilin

MBP-Bni1p(1239–1650) or MBP (40 pmol) was subjected to SDS-PAGE and subsequently electroblotted onto the BA-85 nitrocellulose

membrane filter. The filter was blocked overnight at 4°C in TBS (50 mM Tris-HCl, pH 7.5 and 200 mM NaCl) containing 5% skimmed milk and subsequently incubated for 1 h at 4°C in TBS containing 5% skimmed milk and 230 nM of yeast GST-profilin or GST. After three 5 min washes of the filter in TBS containing 0.05% Tween 20, the filter was incubated for 1 h at 4°C in TBS containing 5% skimmed milk and the 1000-fold diluted anti-GST antibody. After three 5 min washes of the filter in TBS containing 0.05% Tween 20, the filter was incubated for 1 h at 4°C with an appropriate secondary antibody followed by enhanced chemiluminescence detection with the ECL Western blotting detection system (Amersham Corp., Arlington Heights, IL).

Formation of the Bni1p-profilin complex

Purified human GST-profilin or GST (5 nmol) in 500 μ l of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 1 mM DTT) containing 150 mM NaCl was loaded onto an amylose resin column which was prebound to MBP-Bni1p(1239–1650) (600 pmol) or MBP (1200 pmol) and the column was then washed with 20 column volumes of buffer A containing 150 mM NaCl. MBP-Bni1p(1239–1650) or MBP was eluted with 300 μ l of buffer A containing 10 mM maltose and an aliquot (30 μ l) of each eluate was subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. Another aliquot (100 μ l) of each eluate was loaded onto a glutathione-Sepharose 4B column to recover GST-profilin or GST as described (Kikuchi *et al.*, 1992). The recovered samples were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue.

Assay for the binding of recombinant Bnr1p(63–421) with Rho4p

Purified MBP-Bnr1p(63–421) or MBP (2.3 nmol) in 500 μ l of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 5 mM MgCl₂) was loaded onto a glutathione-Sepharose 4B column which was prebound to the GDP- or GTP γ S-bound form of GST-Rho4p (2 nmol) or GST (4 nmol) and the column was then washed with 20 column volumes of buffer B. The bound proteins were subsequently eluted with 350 μ l of buffer B containing 10 mM reduced glutathione and an aliquot (8 μ l) of each eluate was subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. The GTP γ S- or GDP-bound form of GST-Rho4p was prepared as described (Yamamoto *et al.*, 1990).

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