Interaction of Bnr1p with a Novel Src Homology 3 Domain-containing Hof1p

IMPLICATION IN CYTOKINESIS IN SACCHAROMYCES CERESVIAE

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Proteins containing the formin homology (FH) domains FH1 and FH2 are involved in cytokinesis or establishment of cell polarity in a variety of organisms. We have shown that the FH proteins Bni1p and Bnr1p are potential targets of the Rho family small GTP-binding proteins and bind to an actin-binding protein, profilin, at their proline-rich FH1 domains to regulate reorganization of the actin cytoskeleton in the yeast Saccharomyces cerevisiae. We found here that a novel Src homology 3 (SH3) domain-containing protein, encoded by YMR032w, interacted with Bnr1p in a GTP-Rho4p-dependent manner through the FH1 domain of Bnr1p and the SH3 domain of Ymr032wp. Ymr032wp weakly bound to Bni1p. Ymr032wp was homologous to cdc15p, which is involved in cytokinesis in Schizosaccharomyces pombe, and we named this gene HOFL (homolog of cdc15). Both Bnr1p and Hof1p were localized at the bud neck, and both the bnr1 and hof1 mutations showed synthetic lethal interactions with the bni1 mutation. The hof1 mutant cells showed phenotypes similar to those of the septin mutants, indicating that HOFL is involved in cytokinesis. These results indicate that Bnr1p directly interacts with Hof1p as well as with profilin to regulate cytoskeletal functions in S. cerevisiae.

The Rho family belongs to the small G protein superfamily and regulates various cell functions through reorganization of the actin cytoskeleton (for reviews, see Refs. 1 and 2). Many potential targets of Rho have been identified (for a review, see Ref. 3), but it has not yet been thoroughly clarified how Rho regulates reorganization of the actin cytoskeleton through these targets.

The actin cytoskeleton plays a pivotal role in the budding processes in the yeast Saccharomyces cerevisiae (for a review, see Ref. 4). This yeast has the Rho family members, including RHO1, RHO2, RHO3, RHO4, and CDC42, which are involved in the budding processes (for reviews, see Refs. 4 and 5). We have isolated BN11 as a potential target of RHO1, which links RHO1 with the actin cytoskeleton (6). BNI1 has subsequently been shown to be a potential target of CDC42, RHO3, and RHO4 (7). BNR1 is a BNI1-related gene and is a potential target of RHO4 (8). Bni1p and Bnr1p are members of the FH family of proteins, which are defined by the presence of two formin homology domains, the proline-rich FH1 domain and the FH2 domain. The FH proteins play an important role in the actin cytoskeleton-dependent processes, including cytokinesis and establishment of cell polarity (for reviews, see Refs. 9 and 10). We have recently shown that Bni1p interacts with elongation factor 1α, which binds to and bundles actin filaments (11), and that Spa2p is required for the localization of Bni1p at the bud tip (12). Bni1p and Bnr1p, at their FH1 domains, bind to an actin monomer-binding protein, profilin, which is implicated in actin polymerization (7, 8). A proline-rich sequence also interacts with an SH3 domain, which is found in a wide variety of proteins, ranging from cytoskeletal components to signal transducing enzymes (for a review, see Ref. 13). Actually, the FH1 domain of mouse formin binds to SH3 domain-containing proteins (14), although its physiological significance remains obscure.

We show here that a novel SH3 domain-containing protein, Hof1p, directly binds to the FH1 domain of Bnr1p through the SH3 domain in a GTP-Rho4p-dependent manner. The hof1 mutant shows a deficiency in cytokinesis. Both Hof1p and Bnr1p are localized at the bud neck, and both the hof1 and bnr1 mutations show synthetic lethal interactions with the bni1 mutation. Our results suggest that Bnr1p interacts with both Hof1p and profilin at its FH1 domain to regulate reorganization of the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

Strains, Media, and Standard Methods—Wild type yeast strains OHNY1 (MATa ura3 leu2 trp1 his3 ade2) and OHNY3 (MATa ura3 ura3 leu2 leu2 trp1 trp1 his3 his3 ade2 ade2) (15) were used for cytological and genetic studies. TAT7 (MATa trp1 leu2 his3 lys2::lexA-HIS3 ura3::lexA-LEU2) was used for the two-hybrid studies. Yeast strains were usually grown in rich medium (yeast extract peptone dextrose adenine uracil, YPDAU), and yeast transformants were selected in dextrose-containing (synthetic dextrose) or galactose-containing (synthetic galactose) selection media (12). Yeast transformations were performed by the lithium acetate methods (16). Standard yeast genetic manipulations were performed as described (17). An Escherichia coli strain DH5α was used for construction and propagation of plasmids and purification of recombinant proteins.

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1 The abbreviations used are: FH, formin homology; SH3, Src homology 3; DBDlexa, DNA-binding domain of LexA; ADgala, transcriptional activation domain of GAL4; MBP, maltose-binding protein; GST, glutathione S-transferase; kbp, kilobase pair(s).
**Terminator**

pKO11-BNR1 was made by inserting the 4.1-kbp SmaI fragment from pACTII-HK-HOF1 into the BamHI site of pUC19-HOF1, corresponding to amino acid positions from 551 to 669. Rho B.D., Rho4p-binding domain; Rho B.D., Rho1p, Rho3p, Rho4p, and Cdc42p-binding domain.

**Yeast Two-hybrid Method**—Standard molecular biological techniques were used for construction of plasmids, DNA sequencing, and polymerase chain reaction (18). Two-hybrid plasmids were constructed by inserting various truncated fragments of BNR1 or BN11 and HOF1, generated by polymerase chain reaction or digestion with restriction enzymes, into pBTM116-HA (8), encoding DBD LexA, and pACTII-HK (15), encoding AD Gal4, respectively. pKT10-RHO4ΔC, pKT10-RHO4ΔC(Q70L), pKT10-RHO3ΔC, and pKT10-RHO3ΔC(Q74L) were constructed by placing the BamHI (filled in) SmaI fragments of RHO4ΔC, RHO4ΔC(Q70L), RHO4ΔC(Q72N), RHO3ΔC, and RHO3ΔC(Q74L) (8), respectively, downstream of the TDH3 promoter of pKT10 expression vector (19). pMAL-c2-HOF1(551–669) was made by inserting the 0.4-kbp EcoR1-SalI fragment of pACTII-HK, corresponding to amino acid positions from 551 to 669 of Hof1p, into the EcoR1-SalI site of pMAL-c2 (New England Biolabs, Inc., Beverly, MA). pGEK-4T-2-BNR1(1–69) was made by inserting the 1.7-kbp BamHI-BamHI fragment of BNR1 into the BamHI site of pACTII-HK (8), encoding DBD LexA, with restriction enzymes, into pBTM116-HA (8), encoding DBD LexA, and pKT10-RHO4ΔC, respectively. pKT10-RHO4ΔC, pKT10-RHO4ΔC(Q70L), RHO1p-, Rho3p-, Rho4p-, and Cdc42p-binding domain.

**Molecular Biological Techniques and Plasmids**—Standard molecular biological techniques were used for construction of plasmids, DNA sequencing, and polymerase chain reaction (18). Two-hybrid plasmids were constructed by inserting various truncated fragments of BNR1 or BN11 and HOF1, generated by polymerase chain reaction or digestion with restriction enzymes, into pBTM116-HA (8), encoding DBD LexA, and pACTII-HK (15), encoding AD Gal4, respectively. pKT10-RHO4ΔC, pKT10-RHO4ΔC(Q70L), pKT10-RHO3ΔC, and pKT10-RHO3ΔC(Q74L) were constructed by placing the BamHI (filled in) SmaI fragments of RHO4ΔC, RHO4ΔC(Q70L), RHO4ΔC(Q72N), RHO3ΔC, and RHO3ΔC(Q74L) (8), respectively, downstream of the TDH3 promoter of pKT10 expression vector (19). pMAL-c2-HOF1(551–669) was made by inserting the 0.4-kbp EcoR1-SalI fragment of pACTII-HK, corresponding to amino acid positions from 551 to 669 of Hof1p, into the EcoR1-SalI site of pMAL-c2 (New England Biolabs, Inc., Beverly, MA). pGEK-4T-2-BNR1(1–69) was made by inserting the 1.7-kbp BamHI-BamHI fragment of BNR1 into the BamHI site of pACTII-HK (8), encoding DBD LexA, with restriction enzymes, into pBTM116-HA (8), encoding DBD LexA, and pKT10-RHO4ΔC, respectively. pKT10-RHO4ΔC, pKT10-RHO4ΔC(Q70L), RHO1p-, Rho3p-, Rho4p-, and Cdc42p-binding domain.

**Yeast Two-hybrid Method**—A plasmid containing a gene fused to DDB LexA was transformed into TAT7, and the resultant transformant was retransformed by a plasmid containing a gene fused to AD Gal4. Cells of each transformant were cultured in synthetic dextrose medium lacking tryptophan and leucine, and grown to a mid-log phase. Cells of each transformant were placed on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl β-D-galactosidase (22). Disruption of HOF1—pUC19-hof1::LEU2 was cut with BamHI and Smal, and the digested DNA was introduced into OHNY3. The genomic DNA was isolated from each transformant, and the proper disruption of HOF1 was verified by polymerase chain reaction (data not shown). A transformant in which one HOF1 allele was disrupted was subjected to tetrad analysis. All dissected asci (11 asci) showed a 2 Leu : 2 Leu segregation pattern, and all of the Leu+ clones showed the temperature-sensitive growth phenotype. One Leu+ strain, TKUK1, was used as a hof1 mutant in this study.

**Cytological Techniques**—Cdc11p and HA-tagged proteins were stained with the anti-Cdc11p antibody (kindly provided by J. Pringle) and the anti-HA monoclonal antibody, 12CA5, respectively (23). Actin and DNA were stained with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) and 4,6-diamidino-2-phenylindole dihydrochloride (Sigma), respectively (23). Chitin was stained with Calcofluor White M2R New (Sigma) (24). Stained cells were observed with a Zeiss Axioscope microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a pellicle cooling 3CCD color camera (C5810–01; Hamamatsu Photonics KK, Hamamatsu, Japan).

**Materials and Chemicals for Biochemical Assays**—Recombinant MBP-Hof1p(551–669) was purified from DH5α carrying pMAL-c2-
HOF1(551–669) using an amylose resin column (New England Biolabs, Inc.) (25). Recombinant GST-Bnr1p(757–1326) was purified from DH5α carrying pGEX-4T-2-BNR1(757–1326) using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) (26).

Assay for the Binding of Recombinant Hof1p with Bnr1p—Purified MBP-Hof1p(551–669) or MBP (4 nmol each) in 600 μl of Buffer A (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol) was loaded onto a glutathione-Sepharose 4B column that was prebound to GST-Bnr1p(757–1326) (400 pmol) or GST (4 nmol). Each column was washed with 20 column volumes of Buffer A. GST-Bnr1p(757–1326) or GST was eluted with 300 μl of Buffer A containing 10 mM reduced glutathione. An aliquot (20 μl) of each eluate was subjected to SDS-polyacrylamide gel electrophoresis, followed by protein staining with Coomassie Brilliant Blue.

Other Procedures—SDS-polyacrylamide gel electrophoresis and determination of protein concentrations were performed as described (27, 28).

RESULTS

Direct Interaction of Bnr1p with Hof1p—We examined by the two-hybrid method whether Bnr1p(1–1374) or -Bni1p(1–1953), containing the FH1 and FH2 domains, interacted with an SH3 domain-containing protein. Among four proteins containing an SH3 domain tested, including Abp1p, Sla1p, Rvs167p, and Ymr032wp, only Ymr032wp bound to both Bnr1p and Bni1p (data not shown). Deletion analysis of Bnr1p indicated that Bnr1p bound to Ymr032wp at its FH1 domain, although full-length Bnr1p did not bind to Ymr032wp (Fig. 1A). In contrast, Bni1p(1239–1953), containing the FH1, FH2, and its C-terminal domains, weakly bound to Hof1p, but neither the FH1 domain itself nor full-length Bni1p did (Fig. 1B). Western blot analysis indicated that the levels of expression of Bni1ps were similar to those of Bnr1ps (data not shown). Deletion analysis of Ymr032wp indicated that Ymr032wp bound to Bnr1p at its SH3 domain (Fig. 1C). Ymr032wp is homologous to cdc15p, which is involved in cytokinesis in Schizosaccharomyces pombe (29). Both of the proteins have a region at the N terminus with potential to form coiled-coils, a C-terminal SH3 domain, and a PEST region, which is implicated in turnover of proteins (30). We named Ymr032wp Hof1p (homolog of cdc15).

To examine whether Hof1p directly interacts with Bnr1p, Hof1p(551–669), containing the SH3 domain, and Bnr1p(757–1326), containing the FH1 domain, were fused to MBP and GST, respectively, and these fusion proteins were expressed in E. coli and purified. MBP-Hof1p(551–669) bound to GST-Bnr1p(757–1326) at a molar ratio of about 1:1, but it did not bind to GST (Fig. 2). MBP did not bind to GST-Bnr1p(757–1326). This result indicates that Hof1p directly interacts with Bnr1p.

Rho4p-dependent Interaction of Bnr1p with Hof1p—We examined by the two-hybrid method whether full-length Bnr1p interacted with Hof1p in a Rho4p-dependent manner. Wild type or mutant Rho4p carrying an amino acid substitution that keeps Rho4p in the GTP-bound form (Rho4p(Q70L)) (31) or the GDP-bound or nucleotide-free form (Rho4p(T25N)) (32) was overexpressed in the two-hybrid reporter strain, TAT7, expressing full-length Bnr1p and full-length Hof1p. These Rho4ps did not possess the C-terminal lipid modification site to prevent association of Rho4ps with the membranes (8). Hof1p interacted with full-length Bnr1p when Rho4p(Q70L), but not wild type Rho4p or Rho4p(T25N), was overexpressed (Fig. 3A). We next examined whether full-length Bni1p interacted with Hof1p in a Rho family-dependent manner. However, Hof1p did not interact with full-length Bni1p even when Rho3p(Q74L) or Rho4p(Q70L) was overexpressed (Fig. 3B). Western blot analysis indicated that wild type and mutant Rho4ps and wild type and mutant Rho3ps were expressed at a similar level (data not shown). We could not examine the effect of Rho1p(Q68L) or Cdc42p(G12V) on the Bni1p-Hof1p interaction because overexpression of these proteins severely inhibited cell growth even with the C-terminal mutations.2 These results indicate that full-length Bnr1p interacts with Hof1p in a GTP-Rho4p-dependent manner but that full-length Bni1p does not interact with Hof1p, irrespective of the presence or the absence of the GTP-Rho family.

Involvement of Hof1p in Cytokinesis—The Hof1p gene was disrupted with LEU2. The hof1 mutant showed the temperature-sensitive growth phenotype (data not shown). The growth-arrested cells of the hof1 mutant were observed under a microscope and 70% of the hof1 mutant cells had a large and elongated bud (Fig. 4). Staining of DNA and chitin revealed that these hof1 mutant cells showed multinucleate and the deposition of chitin throughout the cells (Fig. 4). Staining of actin revealed that the hof1 mutant cells possessed cortical actin patches at the bud tip but not at the mother-bud neck (Fig. 4B). These phenotypes are similar to those of the septin mutants (33). The septin family members, including CDC3, CDC10, CDC11, and CDC12, are involved in cytokinesis (for reviews, see Refs. 34 and 35). Our results indicate that the hof1 mutant is deficient in cytokinesis.

The physiological significance of the interaction of Bnr1p with Hof1p was examined genetically. We have shown that the bnr1 mutation is synthetically lethal with the bni1 mutation (8). The hof1 mutant was crossed with the bni1 mutant, and the

2 T. Kamei, K. Tanaka, T. Hihara, M. Ukikawa, H. Imamura, M. Kikyo, K. Ozaki, and Y. Takai, unpublished results.
hof1 mutation was also synthetically lethal with the bni1 mutation (data not shown). In contrast, the hof1 mutation was not synthetically lethal with the bnr1 mutation (data not shown). These results suggest that HOF1 and BNR1 function in a similar signaling pathway.

**Localization of Hof1p at the Bud Neck**—The localization of Hof1p was examined by the immunofluorescence microscopic analysis. The HA-HOF1 gene was expressed under the control of the HOF1 promoter. However, a positive staining was not observed (data not shown). HA-HOF1 was therefore expressed under the control of the GAL1 promoter on a single copy plasmid. The expression of HA-Hof1p suppressed the temperature-sensitive growth phenotype of the hof1 mutant (data not shown), indicating that the addition of the HA tag does not impair the functions of HOF1. HA-Hof1p was localized at the bud neck in both small- and large-budded cells, as was Cdc11p (Fig. 5). Localization of HA-Bnr1p was similarly examined, and HA-Bnr1p was also localized at the bud neck (Fig. 5).

**DISCUSSION**

We have tested here the possible interactions of Bnr1p with four SH3 domain-containing proteins, Hof1p, Abp1p, Sla1p, and Rvs167p, and shown that Bnr1p interacts with only Hof1p. Abp1p, Sla1p, and Rvs167p are co-localized with cortical actin patches or actin cables (for a review, see Ref. 4), whereas Bnr1p and Hof1p are co-localized at the bud neck through the cell cycle, suggesting that Bnr1p specifically interacts with Hof1p among many SH3 domain-containing proteins.

We have shown that this interaction of Bnr1p with Hof1p is direct and GTP-Rho4p-dependent. GTP-Cdc42 stimulates an actin-depolymerizing activity of its target, neural Wiskott-Aldrich syndrome protein (36). This finding, together with the present results that although the FH1 domain of Bnr1p interacts with Hof1p even in the absence of GTP-Rho4p, full-length Bnr1p interacts with it only in a GTP-Rho4p-dependent manner, indicates that the binding of Rho family to each target molecule makes its conformation active as described for the Ras family-Raf interaction system (for a review, see Ref. 37).

We have shown that Hof1p also interacts with Bni1p, but this interaction is very weak. We have shown that Hof1p also interacts with Bni1p, but this interaction is very weak. The FH1 domain of Bni1p was not sufficient for the interaction with Hof1p, and full-length Bni1p did not interact with Hof1p in a GTP-Rho family-dependent manner. Moreover, Bni1p is mainly localized at the bud tip (12). Therefore, the physiological significance of the Bni1p-Hof1p interaction remains obscure.

The FH1 domain of Bnr1p(765–806) contains four proline-rich sequences, PQLPPPPPPPPPPPPPPPLP, PAPPPLP, PPPPPPPP-
PLP, and PPAPPLP. The SH3 domain of Hof1p interacted with a region of Bnr1p containing the former two (Bnr1p765–806) but not that containing the latter two (Bnr1p818–850) in the two-hybrid method, although the interaction was relatively weak (data not shown). The FH1 domain of Bnr1p(765–806) also interacted with profilin in the two-hybrid method (data not shown). These results indicate that the FH1 domain of Bnr1p binds to both Hof1p and profilin and are consistent with an earlier finding that both the SH3 domain and profilin possess a similar ligand-binding surface, which consists of an elongated patch of aromatic residues (for a review, see Ref. 38). In contrast, the FH1 domain of Bni1p contains four proline-rich sequences, PPPPPPPPVP, PAPPPPPPPPPP, PPPPPLP, and PPAPPLP, and PPAPP, but the former two proline-rich sequences do not possess a leucine residue at the −1 position from the C terminus. The failure of the FH1 domain of Bni1p to interact with Hof1p may be due to this structural difference. In S. pombe, cdc15p (29), cdc10p (profilin) (39), and cdc12p (an FH protein) (40) are involved in cytokinesis. It has not yet been shown that cdc15p interacts with cdc12p, but cdc3p interacts with cdc12p (40). Interactions of an FH protein with both profilin and an SH3 domain-containing protein may be a general feature in the modes of action of FH proteins.

We have lastly shown that the disruption mutant of HOF1 is deficient in cytokinesis. This result is consistent with the facts that cdc15 is also involved in cytokinesis in S. pombe (29) and that Hof1p is localized at the bud neck, as is a septin, Cdc11p. Hof1p shares homologous regions, other than the SH3 domain, with cdc15p and uncharacterized proteins in tapeworm and mouse (29). These proteins may be generally involved in cytokinesis. A protein related to mammalian IQGAP, Iqg1p/Cyk1p, has recently been implicated in cytokinesis in S. cerevisiae (41, 42). Iqg1p/Cyk1p is localized at the bud neck and associates with actin filaments. It would be interesting to examine whether Hof1p physically or functionally interacts with Iqg1p/Cyk1p.

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