Differential Activities and Regulation of *Saccharomyces cerevisiae* Formin Proteins Bni1 and Bnr1 by Bud6*  

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Formins are conserved proteins that nucleate actin assembly and tightly associate with the fast growing barbed ends of actin filaments to allow insertional growth. Most organisms express multiple formins, but it has been unclear whether they have similar or distinct activities and how they may be regulated differentially. We isolated and compared the activities of carboxyl-terminal fragments of the only two formins expressed in *Saccharomyces cerevisiae*, Bni1 and Bnr1. Bnr1 was an order of magnitude more potent than Bni1 in actin nucleation and processive capping, and unlike Bni1, Bnr1 bundled actin filaments. Profilin bound directly to Bni1 and Bnr1 and regulated their activities similarly. However, the cell polarity factor Bud6/Aip3 specifically bound to and stimulated Bni1, but not Bnr1. This was unexpected, since previous two-hybrid studies suggested Bud6 interacts with both formins. We mapped Bud6 binding activity to specific residues in the carboxyl terminus of Bni1 that are adjacent to its diaphanos autoregulatory domain (DAD). Fusion of the carboxyl terminus of Bni1 to Bnr1 conferred Bud6 stimulation to a Bnr1-Bni1 chimera. Thus, Bud6 differentially stimulates Bni1 and not Bnr1. We found that Bud6 is up-regulated during bud growth, when it is delivered to the bud tip on Bni1-nucleated actin cables. We propose that Bud6 stimulation of Bni1 promotes robust cable formation, which in turn delivers more Bud6 to the bud tip, reinforcing polarized cell growth through a positive feedback loop.

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1 The abbreviations used are: FH1 and FH2, formin homology 1 and 2, respectively; HA, hemagglutinin; DTT, dithiothreitol; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; BBS, Bud6 binding site; DAD, diaphanos autoregulatory domain.

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duces an amino-terminal 6-histidine tag. The His<sub>6</sub>-Bni1-(1227–1953) encoding DNA fragment of this plasmid was PCR-amplified and subcloned into the BglII-NotI sites of pBG007 (a URA3, 2 μ m with an insertion of the GAL promoter) to create pBG564. To construct a plasmid for expressing His<sub>6</sub>-Bnr1(FH1-COOH), DNA encoding Bnr1 (residues 757–1375) was PCR-amplified and subcloned into the Nhel-NotI sites of pBG564 to construct pBG565. To construct a plasmid for expressing Bni1(FH1FH2), DNA encoding Bni1 (residues 1227–1824) was PCR-amplified and subcloned into the BamHI-NotI sites of pBG564 to construct pBG560. The plasmid for expressing the Bnr1-Bni1 chimera (Bnr1 residues 735–1320 fused to Bni1 residues 1808–1953) was constructed by homologous recombination ("gap repair") as follows. DNA encoding Bni1 (residues 1808–1953) was PCR-amplified using primers ACAGATTGGAAAAAGTAAACAGGGATGCTGTTGATCTAC-TCTATCTAATAATGAAAAATTACGCTTGC and AATCACAATTT-TCCGGCGCCCTATATTTTGATCTGGACTGACAATATTA- TTTGAAACTACGGCTTAC and then transformed with SphI-digested pBG565 into a borA strain, a gift from Dr. David Pellman (Dana Farber Cancer Institute). The resulting gap-repaired plasmid, pBG561, was rescued from yeast and amplified in Escherichia coli. Bni1 fragments used to test Bud6 binding (see Fig. 6) were PCR-amplified and subcloned into BamHI-HindIII sites of pQE-9 for expression in E. coli as His<sub>6</sub> fusion proteins. All plasmids were verified by sequencing.

Rabbit skeletal muscle was homogenized at 4 °C using a Dounce homogenizer in 0.5% Nonidet P-40, 1 mM DTT, 350 mM NaCl, followed by three washes with PBS, 0.5% Nonidet P-40, 1 mM DTT. Cells were resuspended in 1 ml of HEKGS10 buffer or proteins in HEKGS10 buffer were mixed. After a 10-min incubation at room temperature, reactions were diluted 1:10 in 10 mM Tris, pH 7.5, 0.7 mM ATP, 0.2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.2 mM DTT, and a 10-μl aliquot was immediately spotted onto carbon-coated copper grids, negatively stained with 2% uranyl acetate, and examined in a Morgagni 268 transmission electron microscope (FEI, Hillsboro, OR). Specimens were imaged using an AMT CCD camera (Advanced Microscopy Techniques, Corp., Danvers, MA) at 200,000× magnification.

Bud6 and Profilin Binding Assays—For the assays shown in Figs. 5F and 6A, Ni<sup>2+</sup>-NTA-agarose beads coated with either Bni1(FH1-COOH) or Bnr1(FH1-COOH) were prepared from yeast lysates as described above (prior to elution with imidazole). Control beads were prepared in parallel by incubation with a wild type yeast cell lysate. The concentration of formin bound to the beads was determined by SDS-PAGE and Coomassie Blue staining reaction (25) of F-buffer or proteins in F-buffer. The F-buffer contained 160 mM NaCl, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM DTT, 200 μ M CaCl<sub>2</sub>, and 0.5 mM EDTA. Filament elongation reactions were performed as described (6) using gel-filtered actin monomers (10% pyrene-labeled). Actin filaments were manipulated using precut pipette tips to prevent filament shearing. For the assays shown in Figs. 5F and 6A, Ni<sup>2+</sup>-NTA-agarose beads coated with either Bni1(FH1-COOH) or Bnr1(FH1-COOH) were prepared from yeast lysates as described above (prior to elution with imidazole). Control beads were prepared in parallel by incubation with a wild type yeast cell lysate. The concentration of formin bound to the beads was determined by SDS-PAGE and Coomassie Blue staining reaction (25) of F-buffer or proteins in F-buffer. The F-buffer contained 160 mM NaCl, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM DTT, 200 μ M CaCl<sub>2</sub>, and 0.5 mM EDTA. Filament elongation reactions were performed as described (6) using gel-filtered actin monomers (10% pyrene-labeled). Actin Filament Assembly and Elongation Assays—

Actin Filament Assembly and Elongation Assays—Monomeric rabbit skeletal muscle actin was prepared by gel filtration using a Sephacryl

S-200 column (AP Biotechnology) equilibrated in G-buffer (10 mM Tris (pH 8.0), 0.2 mM ATP, 0.2 mM CaCl<sub>2</sub>, and 0.2 mM DTT). Actin assembly was measured in 80-μl reactions. Gel-filtered monomeric actin (final concentration 1 or 2 μ M, 5% pyrene-labeled) was converted to Mg-ATP actin for 2 min immediately before use in the reaction as described (12). Mg-ATP-actin was then mixed with 10 μl of HEKGS<sub>10</sub> buffer or proteins in HEKGS<sub>10</sub> buffer and added immediately to 3 μl of 20× initiation mix (40 mM MgCl<sub>2</sub>, 10 mM ATP, 1 mM MgCl<sub>2</sub>) to initiate assembly. Pyrene fluorescence was monitored over time at an excitation of 365 nm and emission of 407 nm in a fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ) at 25 °C. Rates of actin assembly were calculated from the slopes of the assembly curves at 50% polymerization. Filament elongation reactions were performed as described (6) using gel-filtered actin monomers (10% pyrene-labeled).
signal West Pico Chemiluminescent Substrate (Pierce).

Phosphoprotein Staining—SDS-polyacrylamide gels were stained with Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes, Inc., Eugene, OR) and visualized according to the manufacturer’s specifications.

RESULTS

Bnr1 Potently Nucleates Actin Assembly and Processively Caps Filament Barbed Ends—To compare Bni1 and Bnr1 activities, we purified equivalent fragments of these two proteins, extending from a conserved residue at the amino-terminal boundary of the FH1 domain to their natural carboxyl termini (schematic in Fig. 1A, gel of purified proteins in Fig. 1B). We based our design on similar fragments of Bni1 and other formins (containing FH1, FH2, and carboxyl extensions) that have the strongest actin nucleation activities measured to date (3, 6, 9, 13, 14). Because we used these Bni1(FH1-COOH) and Bnr1(FH1-COOH) fragments for most of our analyses, we refer to them throughout the paper as Bni1 and Bnr1. Bni1 and Bnr1 were expressed and purified as His6 fusion proteins from their native source (S. cerevisiae), representing the first purification and characterization of formins in any species from a nonbacterial source. Bni1 expressed in bacteria and yeast had equivalent actin nucleation activities, but Bnr1 isolated from bacteria had greatly reduced activity compared with Bnr1 isolated from yeast.2 Consistent with this observation, a recent study

2 J. B. Moseley and B. L. Goode, unpublished data.
detected weak actin assembly activity for a bacterially expressed GST-Bnr1(FH1FH2) construct (22). For this reason, it was necessary to purify both formins from yeast to compare their activities. The increased activity we observed for Bnr1 purified from yeast may reflect a requirement for eukaryotic protein chaperones. However, purified Bnr1 isolated from yeast does not appear to be phosphorylated (see below).

We first compared Bni1 and Bnr1, purified from yeast, in actin assembly assays. Bni1 nucleated the polymerization of 2 μM gel-filtered Mg²⁺-actin monomers (5% pyrene-labeled) as previously reported for similar Bni1 constructs isolated from bacteria (Fig. 1C) (3, 6, 14). By comparison, Bnr1 showed a far more robust nucleation activity than Bni1 (Fig. 1D). Direct comparisons over a range of formin concentrations demonstrated that Bnr1 is >10-fold more potent than Bni1 in nucleation activity (Fig. 1E). These results raise three important points. First, the potency of Bnr1 nucleation activity is at least equivalent to the strongest reported for any formin protein to date; previous studies showed that the mouse formin mDia1 is about 7-fold more active than Bni1 (13). Second, comparisons of some bacterially expressed formins may be compromised, and accurate comparisons of formin activities may require their expression in yeast or other eukaryotes. Third, the >10-fold difference in nucleation activity for Bnr1 compared with Bni1 may be important in vivo, tailored to specific functions of these formins. For example, more robust actin nucleation activity may be required at the bud neck, where Bnr1 is positioned throughout the cell cycle.

To determine whether post-translational modifications underlie the robust nucleation activity for Bnr1 purified from yeast, we examined the phosphorylation states of our purified Bni1 and Bnr1 fragments. By two different assays, we found that Bni1, but not Bnr1, is phosphorylated (Fig. 2A). First, treatment with λ-phosphatase changes the migration of Bni1 but not Bnr1 on SDS-polyacrylamide gels. Second, a fluorescent phosphoprotein dye could detect purified Bni1 but not Bnr1 in protein gels, and the signal was abolished by treatment of Bni1 with λ-phosphatase. For these reasons, phosphorylation does not appear to contribute to the robust nucleation activity of Bnr1. We also examined the functional significance of Bni1 phosphorylation for its nucleation activity. Reversal of Bni1 phosphorylation by λ-phosphatase treatment had no apparent effect on its nucleation activity alone (Fig. 2B) or in the presence of C-Bud6 (see below).

We next tested whether Bnr1 processively caps the barbed ends of actin filaments. Bni1 and the mammalian formin mDia1 exhibit unregulated processive capping. However, the Schizosaccharomyces pombe formin Cdc12p tightly caps filament ends and blocks elongation until “gated” open by profilin (12), a protein with separate binding surfaces for actin monomers and the polyproline motifs in FH1 domains (Fig. 1A). Both Bni1 and mDia1 have roles in establishing cell polarity, whereas Cdc12p acts primarily in cytokinesis, raising the possibility that profilin gating may be a property shared by formins that direct cytokinesis in different organisms. Since Bnr1 has been linked to cytokinesis in S. cerevisiae based on its genetic interactions and its localization to the bud neck (19, 20), we tested whether Bnr1 might be profilin-gated like Cdc12p. Using a filament elongation assay that specifically measures rates of polymerization at barbed ends (6), we found that Bnr1 did not block elongation in the absence of profilin (Fig. 3A). Thus, Bnr1 acts more similarly to Bni1 than Cdc12p.

In elongation assays, the processive capping activity of Bni1 protects growing barbed ends in a dose-responsive manner from the inhibitory effects of capping protein (Cap1/2) (5, 6). Further, the concentration of formin required for half-maximal protection from Cap1/2 can serve as an index of its strength of association with barbed ends. Previously, we have shown that Bni1 has a half-maximal protection activity of 20–25 nM (6). Here, we measured the barbed end protection activity of Bnr1. The addition of low concentrations (1 nM) of Bnr1 to reactions containing excess (500 nM) Cap1/2 partially restored elongation (Fig. 3A). Thus, Bnr1 acts as a tight processive cap like Bni1. Higher concentrations of Bnr1 (above 1 nM) nucleated new filament assembly (i.e. stimulated actin assembly in reactions lacking seeds), even when low actin monomer concentrations (0.5 μM) were used in the assays, precluding measurement of elongation rates at higher concentrations of Bnr1.

For this reason, we next performed filament elongation assays in the presence of 10 μM profilin, which strongly suppresses actin nucleation without significantly affecting filament elongation rates.² In these reactions, the half-maximal protection activity for Bni1 was 25 nM (Fig. 3, B and C). Similarly, Bnr1 protected growing barbed ends from Cap1/2 in a
From these data, we draw two conclusions. First, a higher affinity for actin (indicated by elongation assays) probably explains the 10-fold more potent nucleation activity of Bnr1 compared with Bni1. Second, since Bni1 and Bnr1 are the only two formins expressed in budding yeast, profilin gating (as observed for S. pombe Cdc12p) is not required in budding yeast. Therefore, budding yeast do not use differences in processive versus profilin-gated capping to tailor their formins for distinct cellular functions.

**Bnr1 Bundles Actin Filaments**—All formin proteins examined to date exhibit a high affinity for the barbed ends of actin filaments. However, one formin, FRLα, additionally binds with lower affinity to the sides of actin filaments (7) and has recently been found to bundle filaments. Since F-actin bundling by specific formin isoforms may be important for the individual roles in cells, we tested the ability of Bni1 and Bnr1 to bundle actin filaments using a low speed centrifugation assay (Fig. 4A). A known F-actin bundling protein, yeast coronin (Crn1), was included as a positive control (32). Whereas Bni1 showed no bundling activity, Bnr1 concentrations greater than 500 nM induced actin filament bundling. Consistent with the low speed pelleting data above, reactions containing 100 nM Bnr1 contained bundles of actin filaments. Consistent with the low speed pelleting data above, reactions containing 100 nM Bnr1 contained no visible bundles. Thus, in two independent assays, concentrations of Bnr1 above 500 nM induced actin filament bundling. These data suggest that Bnr1 binds to the sides of and bundles actin filaments, like FRLα. Although the physiological importance of bundling by formins is not yet clear, this difference in

3 E. Harris and H. Higgs, personal communication.
activity between Bni1 and Bnr1 points further to the separation of their cellular functions, consistent with their distinct localizations, phenotypes, and genetic interactions.

Regulation of Bni1 and Bnr1 by Profilin—We next asked whether Bni1 and Bnr1 functions are differentially regulated by their in vivo ligands. First, we examined profilin regulation of Bnr1 activity. Depending on the specific arrangement of profilin-binding sequences in their FH1 domains, formins can be regulated differentially by profilin to produce highly variable rates of actin filament elongation (6, 9, 10, 12, 14). Therefore, we compared actin assembly by Bni1 and Bnr1 in the presence of 10 μM profilin. Profilin, which suppresses spontaneous nucleation by actin, inhibited the rate of actin filament assembly similarly in the presence of Bni1 and Bnr1 (over a range of formin concentrations), about 75% for Bni1 and 80% for Bnr1 (Fig. 5, A and B). Because profilin is highly abundant in cells and most free actin monomers are thought to be associated with profilin in mammals (11) and yeast, these data show that Bni1 and Bnr1 both can assemble actin filaments from the physiologically available substrate, profilin-bound actin monomers. Similar to its effects in the absence of profilin, Bnr1 nucleated actin assembly more potently (by ~4-fold) than Bni1 in the presence of profilin (Fig. 5, A and B).

We next asked whether the abilities of Bni1 and Bnr1 to nucleate filament assembly from profilin-bound actin monomers require their physical association with profilin. Profilin interacts via two separate binding surfaces with the polyproline motifs in FH1 domains and actin monomers (4, 12, 34–36). We used a profilin mutant (Pfy1–19) with at least 10-fold reduced affinity for polyproline (4, 35). Pfy1-19 is impaired in binding to the Bni1 FH1 domain and thus inhibits Bni1-induced actin assembly compared with wild type profilin (4).

Similarly, we found that Bnr1 is impaired for actin assembly in the presence of 10 μM Pfy1-19 compared with 10 μM Pfy1 (Fig. 5, C–E). Therefore, the interaction of profilin with polyproline regions of both Bnr1 and Bni1 facilitates assembly of filaments from profilin-bound monomers. Consistent with these data, Bni1 and Bnr1 each bound directly to Pfy1, but not Pfy1-19, in pull-down assays using purified proteins (Fig. 5F). These data and the elongation data in Fig. 3, B–E, show that profilin regulates Bni1 and Bnr1 similarly in vitro and is not likely to contribute to differential regulation of Bni1 and Bnr1 in vivo.

Bud6 Binds Specifically to Bni1 but Not Bnr1—We next compared regulation of Bni1 and Bnr1 by the cell polarity factor Bud6/Aip3. A number of studies have implicated Bud6 in the regulation of yeast formin-mediated actin assembly, but it has remained unclear whether the formin-related functions of Bud6 are linked to Bni1 and/or Bnr1. Independent studies have reported two-hybrid interactions for Bud6 with Bni1 (37) and Bnr1 (19), which has led to a commonly held view that Bud6 directly regulates both formins. However, these interactions have not been verified biochemically. We tested direct biochemical interactions of Bni1 and Bnr1 (immobilized on beads) with a purified soluble carboxyl-terminal fragment of Bud6 (C-Bud6; residues 489–788) that we previously showed stimulates Bni1 activity between Bud6 and Bni1 prompted us to map sequences in Bni1 required for Bud6 binding. Bni1 residues 1647–1953 are sufficient for Bud6 interaction by two-hybrid assay (37). These residues contain a small portion of the Bni1 FH2 domain and the complete flanking carboxyl-terminal extension (Fig. 1A), which has limited sequence homology with the carboxyl-terminal extension of Bnr1 (Fig. 6D). We expressed a set of Bni1 truncations as His6 fusion proteins in bacteria, immobilized them on Ni2+-NTA-agarose beads, and used pull-down assays to test their direct interactions with Bud6-(550–788). Bni1 constructs with the carboxyl-terminal boundaries extending beyond Arg1924 bound to C-Bud6 similarly to Bni1-(1750–1824), whereas a construct with its carboxyl terminus at Ala1813 failed to bind Bud6. This demonstrates that Bni1 residues 1816–1824 are required for interactions by two-hybrid assay (37). These residues contain a small portion of the Bni1 FH2 domain and the complete flanking carboxyl-terminal extension (Fig. 1A), which has limited sequence homology with the carboxyl-terminal extension of Bnr1 (Fig. 6D). We expressed a set of Bni1 truncations as His6 fusion proteins in bacteria, immobilized them on Ni2+-NTA-agarose beads, and used pull-down assays to test their direct interactions with Bud6-(550–788). Bni1 constructs with the carboxyl-terminal boundaries extending beyond Arg1924 bound to C-Bud6 similarly to Bni1-(1750–1824), whereas a construct with its carboxyl terminus at Ala1813 failed to bind Bud6.
Bud6 binding (Fig. 6, B and C). However, an alignment of this region shows that most of the residues in this nine-amino-acid stretch (underlined in Fig. 6D) are conserved in Bni1 and Bnr1. We noted the presence of two serine residues in Bni1 (Ser\textsuperscript{1819} and Ser\textsuperscript{1820}) that are not conserved in Bnr1. A double point mutation at this site (S1819A and S1820A) abolished binding of Bni1-(1750–1824) to Bud6-(550–788) (Fig. 6C), demonstrating that the two serine residues in Bni1 are required for Bud6 binding. We define this site (residues 1816–1824) as the Bud6-binding site (BBS). This does not exclude the possibility that sequences outside of the BBS also interact with Bud6.

We noted that the BBS is adjacent to key functional residues in the diaphanous autoregulatory domain (DAD) (Fig. 6D, asterisks) (16). The mouse formin mDia1 is autoinhibited through direct interactions of its carboxyl-terminal DAD with its amino-
terminal diaphanous inhibitory domain. This inhibitory interaction can be at least partially disrupted by binding of Rho GTPases to the amino terminus of mDia1 (13, 16, 17, 38). The proximity of the BBS and DAD in Bni1 raises the possibility that Bud6 could help regulate Bni1 into or out of an autoinhibited state. However, autoinhibition has only been demonstrated biochemically for one formin protein, mDia1 (13), and it is not yet clear whether all formins are regulated similarly. For mDia1, amino-terminal fragments potently trans-inhibit the actin nucleation activities of carboxyl-terminal fragments at low nanomolar concentrations (13). To test trans-inhibition in Bni1, we expressed 12 different amino-terminal Bni1 fragments (using different tags fused to either the amino or carboxyl termini), but none of these proteins were soluble. Thus, we were unable to test Bni1 trans-inhibition. We note that since no interactions between the amino and carboxyl termini of Bni1 have been reported to date, it remains unclear whether Bni1 is regulated by an autoinhibition mechanism similar to mDia1.

Bud6 Stimulates Actin Assembly Nucleated by Bni1 but Not Bnr1—Previously, we determined that 200 nm C-Bud6 modestly stimulates Bni1(FH1-COOH) activity in actin assembly assays (6). We explored this activity in greater depth here by testing Bud6 effects on a wide range of Bni1 concentrations. This revealed a more pronounced stimulation of Bni1 by Bud6 (Fig. 7A). We attribute this in part to using lower Bni1 concentrations and in part to using gel-filtered actin monomers for all actin assembly assays (see “Materials and Methods”), which is critical for minimizing preformed actin nuclei. Consistent with our previous report, this more pronounced activity of Bud6 on Bni1 was additive with the modest stimulatory effects of 200 nm Pfy1 on Bni1 (Fig. 7A). These data show that Bud6 stimulates Bni1-mediated actin nucleation more strongly than profilin and point to a functional difference between these formin regulators. In contrast to Bni1, we could detect no stimulation of Bnr1 activity by C-Bud6 (Fig. 7B), which is consistent with our binding data (Fig. 6). This result was reproducible over a range of concentrations of Bnr1 and Bud6. Thus, all detectable binding and stimulation of formin activity by Bud6 is specific to Bni1 and not Bnr1.

Whereas 200 nm C-Bud6 stimulates Bni1-induced actin assembly, high concentrations of C-Bud6 sequester spontaneous filament assembly in the absence of Bni1 (6), similar to profilin (11). Thus, high concentrations of C-Bud6 and profilin both suppress actin nucleation in the absence of formins. Since both Bni1 and Bnr1 interact with profilin, and this interaction is required for formin-mediated actin assembly in high profilin concentrations, we considered that a similar mechanism might operate under high concentrations of C-Bud6. Therefore, we compared the abilities of Bni1 and Bnr1 to assemble 1 μM actin monomers in the presence of 5 μM C-Bud6, which abolishes all spontaneous actin nucleation. Under these conditions, Bni1 nucleated filament assembly, albeit less efficiently than in the absence of C-Bud6 (Fig. 7C). Thus, Bni1 can assemble filaments from C-Bud6-bound actin monomers. In contrast, Bnr1 did not assemble filaments in the presence of 5 μM C-Bud6, providing another line of evidence that Bud6 specifically regulates the activity of Bni1 and not Bnr1.

To further test the requirements for Bud6 stimulation of Bni1 activity, we purified and tested the activity of a Bni1(FH1FH2) fragment (residues 1227–1824; Fig. 8A). This fragment includes only those carboxyl-terminal sequences that are required and sufficient for Bud6 binding (i.e. this construct lacks residues 1825–1953). The actin assembly activity of this construct was not stimulated by C-Bud6 (Fig. 8B). Thus, whereas sequences carboxyl-terminal to the BBS are not required for Bud6 binding, they are required for Bud6 activity on Bni1. To probe the function of the Bni1 carboxyl terminus further, we fused it to Bnr1 to test whether it could confer Bud6 stimulation. The Bnr1-Bni1 chimera purified consists of the FH1 and FH2 domains of Bnr1 (residues 758–1289) fused to the BBS-containing carboxyl terminus of Bni1 (residues 1766–1953). Like Bnr1, the Bnr1-Bni1 chimera was significantly more potent than Bni1 in nucleating actin, suggesting that differences in the FH2 domains of Bnr1 and Bni1 account for their different affinities for F-actin. However, in contrast to Bnr1 (Fig. 7C), the Bnr1-Bni1 chimera was stimulated by 200 nm C-Bud6 (Fig. 8C). Thus, the Bni1 C terminus (residues 1766–1953) is sufficient to confer Bud6 stimulation to Bnr1.

Bud6 Protein Levels Peak during Bud Emergence and Growth—To further investigate the role of Bud6 in polarized cell growth, we tested whether its expression level changes throughout the cell cycle. We integrated a triple HA epitope tag
at the carboxyl terminus of the BUD6 gene. The BUD6-3HA fusion fully complemented function. A deletion of BUD6 causes substantial loss of visible actin cables (39) and is synthetic lethal with a pps1–4 mutation (6); BUD6-3HA showed none of these effects.2 Cells were arrested at early G1 (unbudded) by treatment with mating pheromone. Expression levels of Bud6-3HA were examined by immunoblotting whole cell extracts from synchronously dividing cells at different times following release from G1 arrest (Fig. 9A). 0–15 min after release, Bud6-3HA levels were low. Expression then increased dramatically 30 min after release, upon bud emergence. Bud6-3HA levels remained high until 90 min after release, shortly before cytokinesis. Note that Bud6-3HA migrates as two bands on immunoblots of whole cell extracts (Fig. 9A). The upper band appears to be phosphorylated, because only a single lower band remains after treatment of the precipitated proteins with λ-phosphatase (Fig. 9B). The functional significance of the Bud6 phosphorylation remains unclear, since there is no obvious change in the ratio of the upper to lower bands of Bud6-3HA during the cell cycle (Fig. 9A).

FIG. 9. Bud6 protein levels are cell cycle-regulated. A, BGY968 (BUD6-3HA) cells were arrested in G1 (>95% unbudded) by treatment with mating factor and then released to synchronize cell growth. Total protein samples were prepared at 15-min intervals after release, fractionated by SDS-PAGE, and immunoblotted with anti-HA antibodies (upper panel) and anti-tubulin antibodies (lower panel) as a loading control. Cell division peaked 105 min after release from G1. B, Bud6-3HA was immunoprecipitated from BGY968 cell lysates, treated with λ-phosphatase or mock-treated, fractionated by SDS-PAGE, and immunoblotted with anti-HA antibodies.

DISCUSSION

We have identified two differences in the biochemical activities of yeast formins Bni1 and Bnr1 that may contribute to their different functional roles during polarized cell growth. First, Bnr1 induces actin filament assembly >10-fold more potently than Bni1, possibly explained by a 10-fold higher affinity of Bnr1 for actin filament barbed ends. Second, Bnr1, but not Bni1, bundles actin filaments. Such activity differences may be tailored to different requirements for actin assembly at the bud tip and the bud neck, where Bni1 and Bnr1, respectively, localize throughout much of the cell cycle. Although this model requires further in vivo tests, the potent activity of Bnr1 may be necessary for driving robust actin cable assembly from the bud neck into the mother cell (larger than the bud compartment). It is also important to note that our comparisons are limited to the carboxyl-terminal fragments of formins, and ultimately it will be important to compare the activities of full-length Bni1 and Bnr1.

We also demonstrated specific and direct binding of the cell polarity factor Bud6 to Bni1 but not Bnr1. At a first glance, this appears to be at odds with previous two-hybrid and blot overlay interactions reported for Bud6-Bnr1 (19). These interactions were mapped to sequences included in our C-Bud6 and Bnr1(FH1-COOH) fragments. However, two-hybrid assays can detect low affinity interactions. Therefore, Bnr1-Bud6 interactions could be very weak or transient and thus not detected in our binding and kinetics assays. Perhaps most relevant are the functional data showing that Bud6 stimulates Bni1- but not Bnr1-mediated actin assembly and that this Bud6 stimulation requires the carboxyl extension of Bni1 absent in Bnr1 (Fig. 6D). Thus, even if weak binding does occur between Bnr1 and Bud6 in vivo, it probably does not stimulate Bnr1-mediated actin assembly. It will be important to test whether this differ-
tential regulation holds in the context of full-length formins; therefore, the fragments we used for our biochemical assays contain the sequences previously implicated in Bnr1-Bud6 and Bni1-Bud6 two-hybrid interactions. Therefore, our data support the view that Bud6 differentially stimulates Bni1 and not Bnr1.

Several lines of in vivo data also are consistent with the model that Bud6 regulates specifically Bni1 and not Bnr1. First, Bud6 and Bni1 have similar localization patterns, residing at the nascent and growing bud tip and then moving to the bud neck (first Bud6 and then Bnr1) late in the cell cycle prior to cytokinesis (18, 22, 37, 39). In contrast, Bnr1 localizes to the bud neck throughout the cell cycle (19, 22). Second, defects in bud6Δ cells more closely resemble those of bni1Δ cells than bnr1Δ cells. Deletion of either Bud6 or Bni1 disrupts the bipolar budding pattern of diploid cells (39, 40) and causes cell rounding (39, 41), indicative of cell polarity defects, whereas bnr1Δ cells do not adopt this rounded morphology (42). Further, in wild type and bnr1Δ cells, a key marker of polarized vesicular transport, Sec4, localizes normally to the bud tip (22, 42). However, Sec4 is diffusely spread at the bud neck in bnr1Δ and bud6Δ cells (22, 42).

A Model for Bud6-Bni1 Interactions Promoting Polarized Cell Growth—Based on our biochemical data and data from the above-mentioned studies, we propose a model in which Bud6-Bni1 interactions promote polarized cell growth. During early bud development, Bni1 nucleates the assembly of bud-specific actin cables, which serve as tracks for myosin-based delivery of secretory vesicles and other cargo to the bud tip (22, 43, 44). Delivery of Bud6 to the bud tip along cables through the secretory pathway (45) enhances Bni1-mediated actin cable formation, which in turn delivers more Bud6 to the bud tip in a positive feedback loop. This mechanism would explain why the loss of Bud6 leads to diminished actin cable staining and partial cell rounding (39). This model also is supported by our data showing that Bud6 expression peaks at bud emergence and growth, when Bni1 is located at the bud tip. Further, one recent study showed that trafficking of Bud6 to the bud tip contributes to sustained cable assembly by Bni1 in the bud (22). This sustained cable assembly required cable-dependent transport of the Rho-GTPases Cdc42 and Rho1 (22), suggesting that multiple positive feedback loops may contribute to sustained Bni1-mediated cable assembly.

Later in the cell cycle near the end of bud growth, Bud6 and Bni1 leave the bud tip to join Bnr1 at the bud neck (first Bud6 and then Bnr1). At the neck, we propose that Bud6 regulates Bni1, but not Bnr1, specifically stimulating Bni1-mediated formation of actin cables and/or the actin cytoskeletal ring. These two actin structures have complementary roles in promoting cytokinesis, and having two differentially regulated formins positioned at the bud neck to build these actin structures may help ensure proper completion of cell division.

We have shown here that profilin regulates Bni1 and Bnr1 similarly but that Bud6 stimulates the activity of Bni1 and not Bnr1. It seems likely that additional in vivo mechanisms must contribute to the differential regulation of yeast formins. Our observation that Bni1 purified from yeast is phosphorylated, whereas Bnr1 is not, hints at a second possible mechanism. In this regard, two important questions for future investigation are the following. 1) What kinase(s) phosphorylate the carboxy-terminal half of Bni1? Ste20 and Fus3 kinases are implicated in Bni1 phosphorylation (46, 47) and thus represent potential candidates, but many others are possible. 2) What function(s) of Bni1 are regulated by its phosphorylation? Phosphorylation did not affect the actin nucleation activity of the carboxy-terminal half of Bni1 or its stimulation by C-Bud6.
Bud6 Differential Regulation of Yeast Formins Bni1 and Bnr1

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