Yeast Adaptor Protein, Nbp2p, Is Conserved Regulator of Fungal Ptc1p Phosphatases and Is Involved in Multiple Signaling Pathways

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Background: Nbp2p is a yeast SH3 domain-containing adaptor protein.

Results: We identified a highly conserved motif in Nbp2p required for binding the Ptc1p phosphatase. The Nbp2p-Ptc1 complex down-regulates the cell wall integrity MAPK pathway and other pathways.

Conclusion: A conserved role of Nbp2p is to recruit Ptc1p through SH3 domain-mediated interactions.

Significance: Knowledge of adaptor protein functions is extended.

Nbp2p is an Src homology 3 (SH3) domain-containing yeast protein that is involved in a variety of cellular processes. This small adaptor protein binds to a number of different proteins through its SH3 domain, and a region N-terminal to the SH3 domain binds to the protein phosphatase, Ptc1p. Despite its involvement in a large number of physical and genetic interactions, the only well characterized function of Nbp2p is to recruit Ptc1p to the high osmolarity glycerol pathway, which results in down-regulation of this pathway. In this study, we have discovered that Nbp2p orthologues exist in all Ascomycete and Basidiomycete fungal genomes and that all possess an SH3 domain and a conserved novel Ptc1p binding motif. The ubiquitous occurrence of these two features, which we have shown are both critical for Nbp2p function in Saccharomyces cerevisiae, implies that a conserved role of Nbp2p in all of these fungal species is the targeting of Ptc1p to proteins recognized by the SH3 domain. We also show that in a manner analogous to its role in the high osmolarity glycerol pathway, Nbp2p functions in the down-regulation of the cell wall integrity pathway through SH3 domain-mediated interaction with Bck1p, a component kinase of this pathway. Based on functional studies on the Schizosaccharomyces pombe and Neurospora crassa Nbp2p orthologues and the high conservation of the Nbp2p binding site in Bck1p orthologues, this function of Nbp2p appears to be conserved across Ascomycetes. Our results also clearly imply a function for the Nbp2p-Ptc1p complex other cellular processes.

Adaptor proteins play crucial roles in regulating signal transduction pathways. They are generally composed of multiple protein-protein interaction domains or motifs and lack enzymatic activities. Through their ability to bind multiple proteins, they bring together pathway components and may also provide a mechanism for the integration and/or regulation of diverse pathways within the cell. Thus, understanding the functions of adaptor proteins is a requirement for gaining a clear picture of the global regulation of signal transduction.

Yeast Nbp2p* (Nap1p-binding protein 2) is a 236-residue Src homology 3 (SH3) domain-containing adaptor protein that has been shown through high throughput screens to interact with many proteins carrying out diverse functions (1–3). Strains bearing NBP2 deletions display a large number of genetic interactions, implicating Nbp2p in the regulation of multiple cellular processes (4–6). Despite the apparently widespread role of Nbp2p, its only well characterized function is in the down-regulation of the high osmolarity glycerol (HOG) MAPK pathway (7). To carry out this role, Nbp2p binds to Pbs2p, the scaffold and MAPK kinase (MAPKK) of the pathway, and recruits the Ptc1p phosphatase, which results in the dephosphorylation of the Hog1p MAPK. Nbp2p in conjunction with Ptc1p also appears to regulate the cell wall integrity (CWI) MAPK pathway. Cells lacking Ptc1p or Nbp2p show constitutively high levels of the phosphorylated Slt2p, the MAPK of the CWI, which may cause some of the phenotypes associated with NBP2Δ strains, such as sensitivity to high temperature and cell wall stress (8, 9). Strains lacking Nbp2p also display defects in organelle inheritance and septin mislocalization (1, 10).

Characterization of protein interactions mediated through different regions of Nbp2p is crucial for understanding its function. The only recognized protein-protein interaction domain within Nbp2p is the SH3 domain. However, a region N-terminal to its SH3 domain has been shown to bind Ptc1p (7), and a region C-terminal to the SH3 domain has been shown to interact with Nap1p (1). The specific domains or motifs responsible for these interactions have not been identified. The binding of Nap1p to Nbp2p appears to bridge the interaction between Nbp2p and several kinases (including Gin4p, Kcc4p, and Hsl1p), but the functional importance of these interactions is not known (1). The Nbp2p SH3 domain recognizes peptides containing a ΨXXPRXPXP motif, where Ψ is a hydrophobic residue.
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### Table 1

| Strain      | Genotype                                      | Source |
|-------------|-----------------------------------------------|--------|
| YCW362      | MATa ura3 leu2 his3 trp1 sak2Δ::LEU2 sak22Δ::LEU2 | [14]   |
| SY29*       | NBP2::3HA[TRP1]                               | This study |
| SY56*       | NBP2::nbp2 Δ::URA3                            | This study |
| SY58*       | NBP2::nbp2 Δ::URA3                            | This study |
| SY59*       | NBP2::nbp2 Δ::URA3                            | This study |
| SY69*       | NBP2::3HA[TRP1] BCK1::6His-10FLAG[ KanMX6]     | This study |
| SY84*       | BCK1::6His-10FLAG[ KanMX6]                    | This study |
| SY87*       | NBP2::3HA[TRP1] BCK1::bck1PXXPΔ::6His-10FLAG[ KanMX6] | This study |
| SY129*      | NBP2::3HA[TRP1] BCK1::bck1PXXPΔ::6His-10FLAG[ KanMX6] | This study |
| SY100*      | ptc1Δ::URA3                                   | This study |
| SY110*      | NBP2::3HA[TRP1] PTC1::6His-10FLAG[ KanMX6]    | This study |
| SY133*      | NBP2::nbp2XXPΔ::6His-10FLAG[ KanMX6]          | This study |
| SY136*      | NBP2::nbp2XXPΔ::6His-10FLAG[ KanMX6]          | This study |
| SY109*      | ptc1Δ::URA3                                   | This study |
| SY110*      | NBP2::3HA[TRP1] PTC1::6His-10FLAG[ KanMX6]    | This study |
| SY133*      | NBP2::nbp2XXPΔ::6His-10FLAG[ KanMX6]          | This study |
| SY110*      | NBP2::3HA[TRP1] PTC1::6His-10FLAG[ KanMX6]    | This study |
| SY109*      | ptc1Δ::URA3                                   | This study |
| SY133*      | NBP2::nbp2XXPΔ::6His-10FLAG[ KanMX6]          | This study |
| FY286       | h+ ade6-M10 ura4-D18 leu1−32                  | Bioneer |
| FY286       | ade6-M10 ura4-D18 leu1−32                     | (29)   |
| FGSC 9718   | mat a mus-51:bar                              | (29)   |
| FGSC 16416  | mat a mus-51:bar ncu03113::hph                | (29)   |
| FGSC 11321  | mat a mus-51:bar ncu03113::hph                | (29)   |

* YCW362 derivatives.

### Experimental Procedures

#### Protein Expression and Purification—The Nbp2p SH3 domain (residues 113–170) and the Skb5p SH3 domain (residues 80–140) were expressed from the pET-21d vector (Novagen) with a C-terminal His tag. The Bck1p peptide (residues 800–815, PELAPKREAPKPPANT) and Mkh1p peptide (residues 535–551, RNFVAHRDPPPPPTETS) were expressed in fusion with His$_6$-tagged bacteriophage CI repressor protein as described previously [13]. Proteins were purified using nickel affinity chromatography and dialyzed against 50 mM sodium phosphate, pH 7.0, 100 mM NaCl buffer. All subsequent assays were carried out in this buffer.

#### In Vitro Peptide Binding Assays—Peptide binding by SH3 domains was quantitated using Trp fluorescence as described previously [13]. An AVIV spectrofluorometer, model ATF105, was used to measure the binding affinity between SH3 domains and their target peptides. All experiments used an SH3 domain concentration of 1 μM whereas the target peptide was titrated to various concentrations by a Microlab 500 series automated titrator. The mixture was equilibrated for 1 min before the binding was assessed. Total tryptophan fluorescence was measured at 328 nm using an excitation wavelength of 295 nm.

#### Fungal Strains and Genetic Techniques—The fungal strains used in this study are listed in Table 1. All Saccharomyces cerevisiae strains used were derived from YCW362 [14]. Gene disruptions and modifications were achieved by homologous recombination at their chromosomal loci by standard PCR-based methods [15]. Deletion of genes was performed by substitution of the open reading frame with the URA3 gene amplified from pFA6a-URA3 [15]. Mutant strains were constructed by replacing the gene to be mutated with the URA3 gene, followed by a second homologous recombination event at the same locus to integrate linear DNA encoding the mutated gene of interest. Successful recombination events were selected on 5-fluoroorotic acid, and the mutation was confirmed by sequencing.

#### Immunoprecipitation—To prepare crude extracts for immunoprecipitation, 50-ml cultures were grown to $A_{600} 0.6–0.8$ and cells were harvested, washed in ice cold water, and flash.

Amino acid (11). This motif contains the canonical RXXPXXP motif recognized by SH3 domains and includes additional features that confer specificity for the Nbp2p SH3 domain. The interaction between Nbp2p and its target in the HOG pathway, Pbs2p, is mediated by binding of the Nbp2p SH3 domain to a $\Psi$XRXXAPXXP motif. The MAPKKK of the CWI pathway, Bck1p, also contains a $\Psi$XRXXAPXXP motif, and we have recently reported that the Nbp2p SH3 domain binds to this Bck1p motif with high affinity (11). Bacterially expressed Nbp2p has also been shown to interact with the N-terminal region of Bck1p expressed at endogenous levels in yeast, but not with the same fragment lacking the $\Psi$XRXXAPXXP motif (1). Considering that Nbp2p and Ptc1p are both involved in down-regulation of the CWI pathway (8, 9), the interaction between the Nbp2p SH3 domain and Bck1p motif likely functions to recruit Ptc1p to the CWI pathway; however, this has not been directly demonstrated. We and others have shown that the Nbp2p SH3 domain also binds to $\Psi$XRXXAPXXP motifs found in the Ste20p and Cla4p p21-activated kinases (1, 11, 12).

Despite considerable information regarding protein interaction mediated by Nbp2p, the functional significance of most of these interactions remains unknown, and the role of Nbp2p in other pathways besides HOG pathway is poorly characterized. The goals of this study were to identify key functional domains and/or motifs within Nbp2p and characterize their functions. To this end, we aligned homologues of Nbp2p from diverse fungal species, which allowed us to identify the SH3 domain and a newly recognized Ptc1 binding motif as the defining functional components of these proteins. We also addressed whether the interaction between the Nbp2p SH3 domain and the Bck1p motif is required for the down-regulation of the CWI pathway and whether the role of Nbp2p in regulating this pathway can fully account for the phenotypes associated with Nbp2p deletion. Finally, to assess the conservation of Nbp2p function through fungal evolution, we investigated the role of the Schizosaccharomyces pombe and Neurospora crassa Nbp2p orthologues in regulating the CWI pathways in these organisms.
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SH3 Domain and Ptc1 Binding Motif Are Conserved Elements of Nbp2p Orthologues—To identify the conserved functions of Nbp2p, we constructed a sequence alignment of all Nbp2p homologues in available fungal genomes. By searching with PSI-BLAST using S. cerevisiae Nbp2p as a query, we were able to identify an obvious Nbp2p orthologue in all sequenced Ascomycete species (supplemental Table S1). Although they varied in length from 130 to >600 residues, Nbp2p orthologues were easily discerned due to the very high conservation of many unique features within the SH3 domain (e.g. Glu residues at SH3 domain positions 52 and 53, and Tyr residues at position 30; Fig. 1A). By aligning these orthologues, a second highly conserved motif (Nbp2 residues 61–75) was identified N-terminal to the SH3 domain (residues 112–170). This motif was found in all of the orthologues and was conserved even among the most diverged species (Fig. 1B; e.g. S. cerevisiae and S. pombe diverged >400 million years ago) (17). By performing PSI-BLAST searches with the N-terminal conserved motif as a query, we identified a group of proteins containing very close matches to this motif from the more distantly related Basidiomycete fungi (Fig. 1B and supplemental Table S1). Interestingly, these proteins all possess a C-terminal SH3 domain starting between 100 and 200 residues past the conserved motif, suggesting that these proteins may also be Nbp2p orthologues. However, these SH3 domains are distinct in sequence from those in the Ascomycete Nbp2p orthologues and are not as similar to each other as are the Ascomycete Nbp2p SH3 domains (Fig. 1A). We conclude that Nbp2p is conserved across the Ascomycete phylum and that the N-terminal motif found in the Ascomycete homologues is conserved in putative Basidiomycete Nbp2p homologues.

A previous study demonstrated that Ptc1p binds to a region of Nbp2p that is N-terminal to the SH3 domain; therefore, we hypothesized that the newly identified conserved N-terminal motif is involved in Ptc1p binding. To investigate this possibility, we tested two mutant versions of Nbp2p, in which the highly conserved KD and HY residue pairs in this motif were replaced with alamines (designated KD→AA, HY→AA) (Fig. 1B). To determine the effects of these substitutions on Ptc1p binding, HA-tagged versions of wild-type and mutant Nbp2p were immunoprecipitated, and co-precipitated FLAG-tagged Ptc1p was detected by Western blotting. Whereas a large amount of Ptc1p co-precipitated with wild-type Nbp2p, the level of Ptc1p co-precipitating with the mutant proteins was barely detectable (Fig. 1C). The failure of these mutants to bind Ptc1p was not due to low expression levels as demonstrated by Western blotting (Fig. 1D). These results demonstrate that the newly identified N-terminal motif is indeed involved in Ptc1p binding and that the highly conserved KD and HY residues of this Ptc1p Binding Motif (PBM) are crucial for mediating the interaction with Ptc1p.

SH3 Domain and PBM Motif Are Critical for Supporting Cell Growth under Cell Wall Stressing Conditions—

To assess the importance of the PBM motif for the function of Nbp2p we determined the effects of Nbp2p KD→AA and HY→AA mutants on cell fitness by testing yeast mutants expressing these proteins. Strains bearing an NBP2 deletion showed marked growth defects at high temperature and in the presence cell wall-stressing reagents (1, 6). Therefore, we evaluated Nbp2p function in the tested mutants by growing the cells at high temperature or in the presence of the anti-fungal drug, caspofungin, which disturbs cell wall integrity by inhibiting glucan synthesis (18). As can be seen in Fig. 2A, strains expressing Nbp2p bearing amino acid substitutions in the PBM displayed marked reductions in growth under restrictive conditions with the KD→AA mutant phenocopying the nbp2Δ and ptc1Δ strains. The severity of the phenotype observed with the Nbp2p KD→AA substitution shows that disruption of Nbp2p-Ptc1p interaction is equivalent to complete deletion of...
Nbp2p is a conserved adaptor for fungal Ptc1p phosphatases. The binding of Nbp2p to Ptc1p is absolutely required for the function of Nbp2p under the conditions tested. The milder phenotype caused by the Nbp2p HY → AA substitution could be interpreted to suggest that the PBM motif makes a functionally relevant interaction with another unknown protein and that the KD → AA substitution abrogates binding to this protein and to Ptc1p whereas the HY → AA affects only binding to Ptc1p. However, another explanation, which we feel is more likely, is that the HY → AA substitution does not completely abrogate the interaction with Ptc1p, but the residual binding activity of this mutant cannot be detected by the pulldown assay used. In studies on another yeast SH3 domain-containing protein, Abp1p, we found that partial reduction in binding affinity resulted in loss of detectable binding in pulldown assays, but biological activity could still be detected (19), and such a phenomenon was also observed in studies by another group (20). Supporting our hypothesis that the phenotype of the HY → AA mutant is the result of a partial reduction in binding affinity is the striking similarity of its phenotype (i.e. partial reduction of growth at 39 °C and only slight reduction of growth on caspofungin) to that of the Y8A SH3 domain mutant described below that partially reduces the affinity of the SH3 domain for its target sites. The less severe effect of the HY → AA substitution is also consistent with the lower degree of conservation of residues at these two positions as compared with the KD positions which display K/RD in all species examined (Fig. 1B).

We also evaluated the importance of the SH3 domain for Nbp2p function under the same conditions as described above. To weaken SH3 domain-mediated interactions, we substituted the Tyr8 and Phe54 residues in the SH3 domain with alanines (SH3 domain numbering is according to Ref. 21), which is expected to affect SH3 domain binding to all PXXP-containing targets (21). We have shown previously that Y8A and F54A substitutions reduce the binding of the Nbp2p SH3 domain to the Ste20p_PXXP peptide by 45-fold and 280-fold, respectively.
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Nbp2p Down-regulates CWI Pathway by Binding to Bck1p—Previous data suggested that Nbp2p is involved in down-regulation of the CWI pathway and that this regulation occurs through binding of Nbp2p SH3 domain to a PXXP-containing site in Bck1p, the MAPKKK of this pathway (1, 8, 9). To investigate the role of Nbp2p in the regulation of the CWI pathway, we established that Nbp2p interacts with Bck1p in yeast cells expressed at endogenous levels. The indicated protein constructs were co-expressed, HA-Nbp2p was precipitated with α-HA-agarose, and bound proteins were analyzed by SDS-PAGE. Immunoblotting was performed with α-HA and α-FLAG antibodies. Data shown are representative of three experiments.

by measuring the phosphorylation state of Slt2p, the MAPK of this pathway. Although little or no phosphorylated Slt2p was detected in wild-type cells under these conditions, a large amount was detected in strains bearing mutant versions of Bck1p that were unable to bind Nbp2p (Bck1PXXP P0A or Bck1PXXXΔ, in which the complete PXXP site is deleted; Fig. 4A). The deletion of NBP2 or expression of the Nbp2p SH3 domain Y8A or F54A mutants, which bind weakly to the Bck1PXXX motif, also caused elevated levels of phosphorylated Slt2p (Fig. 4B). The level of Slt2p phosphorylation was lower in the strain bearing the SH3 domain Y8A mutant than the SH3 domain F54A mutant, which is likely the result of the greater binding affinity of the Y8A mutant for the Bck1p binding site. The slightly lower level of Slt2p phosphorylation observed for the F54A mutant compared with the nbp2Δ strain is likely due to the residual PXXP-binding activity of this mutant. Because Bck1p is required for the activation of Slt2p, no phosphorylated Slt2p was detected in the bck1Δ strain. Importantly, we found that the down-regulation of CWI pathway by Nbp2p is dependent on its ability to bind Ptc1p. Strains expressing Nbp2p with amino acid substitutions in the PBM that abrogate Ptc1p binding displayed elevated levels of phosphorylated Slt2p, similar to

FIGURE 2. Effects of disrupting Nbp2p-mediated interactions on cells fitness under cell wall-stressing conditions. A and B, strains expressing mutants bearing the indicated substitutions in the Nbp2p SH3 (A) or SH3 domain (B) display severe growth defects under cell wall-stressing conditions. Substitutions were made to the chromosomal copy of NBP2. Cells were grown overnight, normalized to an A_(600nm) of 0.25, and spotted onto the designated media in a series of 5-fold dilutions. C, strains expressing mutants bearing the indicated Bck1P substitutions, display mild growth defects under cell wall-stressing conditions. The growth assays were performed as described in A.

(11), and we have confirmed that these substitutions also greatly reduce binding to the Bck1PXXP peptide (Fig. 3A). Strains expressing Nbp2p bearing the SH3 domain F54A substitution grew almost as poorly as the nbp2Δ strain (Fig. 2B), demonstrating the critical role of the peptide binding activity of the Nbp2p SH3 for in vivo function. Consistent with its less severe reduction of binding affinity compared with the F54A substitution, cells bearing the Nbp2p Y8A substitution displayed a milder growth defect, demonstrating that the ability of the Nbp2 SH3 domain to bind peptide correlates with its in vivo functional capacity.

Nbp2p Down-regulates CWI Pathway by Binding to Bck1p—Previous data suggested that Nbp2p is involved in down-regulation of the CWI pathway and that this regulation occurs through binding of Nbp2p SH3 domain to a PXXP-containing site in Bck1p, the MAPKKK of this pathway (1, 8, 9). To investigate the role of Nbp2p in the regulation of the CWI pathway, we established that Nbp2p interacts with Bck1p in yeast cells expressed at endogenous levels of expression. Chromosomally expressed FLAG-tagged Bck1p was shown to co-precipitate with chromosomally expressed HA-tagged Nbp2p (Fig. 3B). This co-precipitation of Bck1p with Nbp2p demonstrated for the first time that full-length Nbp2p and Bck1p interact when each is expressed at endogenous levels. To assess the importance of the previously identified PXXP-containing site in Bck1p for mediating this interaction, we expressed full-length Bck1p bearing a mutant version of the site in which the first Pro residue of the PXXP motif was substituted with Ala (Bck1PXXP P0A substitution). This substitution was shown to completely abrogate binding in vitro (Fig. 3A). As shown in Fig. 3B, this substitution also drastically reduced the level of Bck1p-Nbp2p co-precipitation, implying that the PXXP site within Bck1p is crucial for binding to Nbp2p in vivo.

We next assessed the effect of disrupting the interaction between Nbp2p and Bck1p on the activity of the CWI pathway by measuring the phosphorylation state of Slt2p, the MAPK of this pathway. Although little or no phosphorylated Slt2p was detected in wild-type cells under these conditions, a large amount was detected in strains bearing mutant versions of Bck1p that were unable to bind Nbp2p (Bck1PXXP P0A or Bck1PXXXΔ, in which the complete PXXP site is deleted; Fig. 4A). The deletion of NBP2 or expression of the Nbp2p SH3 domain Y8A or F54A mutants, which bind weakly to the Bck1PXXX motif, also caused elevated levels of phosphorylated Slt2p (Fig. 4B). The level of Slt2p phosphorylation was lower in the strain bearing the SH3 domain Y8A mutant than the SH3 domain F54A mutant, which is likely the result of the greater binding affinity of the Y8A mutant for the Bck1p binding site. The slightly lower level of Slt2p phosphorylation observed for the F54A mutant compared with the nbp2Δ strain is likely due to the residual PXXP-binding activity of this mutant. Because Bck1p is required for the activation of Slt2p, no phosphorylated Slt2p was detected in the bck1Δ strain. Importantly, we found that the down-regulation of CWI pathway by Nbp2p is dependent on its ability to bind Ptc1p. Strains expressing Nbp2p with amino acid substitutions in the PBM that abrogate Ptc1p binding displayed elevated levels of phosphorylated Slt2p, similar to

FIGURE 3. Interaction between the Nbp2p SH3 domain and Bck1p. A, binding affinities between purified SH3 domains and target peptides investigated in this study. Binding was detected by monitoring SH3 domain intrinsic Trp fluorescence, which changes significantly upon peptide binding. The K_d values are represented as mean ± S.D., where each measurement was repeated at least twice. N.D.B. (no detectable binding) indicates absence of fluorescence shift upon addition of the peptide at 50 μM concentration. Values marked by asterisks were determined previously (11). B, interaction between Nbp2p and Bck1p expressed at endogenous levels. The indicated protein constructs were co-expressed, HA-Nbp2p was precipitated with α-HA-agarose, and bound proteins were analyzed by SDS-PAGE. Immunoblotting was performed with α-HA and α-FLAG antibodies. Data shown are representative of three experiments.

| Nbp2p SH3 | Peptide | K_d (μM) |
|-----------|---------|----------|
| WT        | Bck1    | 1 ± 0.2  |
| WT        | Bck1 P0A| N. D. B. |
| Y8A       | Bck1    | 18 ± 9   |
| F54A      | Bck1    | 71 ± 9   |
| WT        | Ste20   | 0.2 ± 0.1*|
| WT        | Cla4    | 0.65 ± 0.09*|
| WT        | Pbs2    | 3.0 ± 0.3*|
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those observed in the \( \text{nbp2} \Delta \) strain (Fig. 4C). Taken together, these results demonstrate that both Nbp2p-Ptc1p and Nbp2p-Bck1p interactions are crucial for the down-regulation of Slt2p phosphorylation, consistent with the proposed role of Nbp2p as a Ptc1p adaptor in the CWI pathway.

To assess whether CWI pathway hyperactivation is sufficient to account for the growth defects observed for \( \text{NBP2} \) mutant strains (Fig. 2, A and B), we tested the growth of strains expressing Bck1p mutants that are unable to interact with Nbp2p. Although strains expressing the Bck1p\( \Delta \) or Bck1p\( \text{pXXP} \) P0A mutant proteins displayed marked growth defects relative to the wild-type, these growth defects were much less pronounced than those observed with Nbp2p PBM or SH3 domain mutations (Fig. 2C). These results demonstrate that CWI pathway hyperactivation, which occurs in the strains bearing the Bck1p\( \text{pXXP} \) substitutions, is only one of the factors that contributes to the growth defects of \( \text{NBP2} \) mutant strains, implying that Nbp2p must function in the regulation of other cellular processes. This conclusion is also supported by the behavior of the strain bearing the HY\( \rightarrow \)AA mutant of the PBM motif, which displays apparently equal hyperactivation of the CWI pathway as the KD\( \rightarrow \)AA mutant, yet displays a less severe phenotype (Fig. 2B). As described above, we hypothesize that the HY\( \rightarrow \)AA mutant possesses residual Ptc1p binding, and this level of binding appears to be sufficient to mediate partial function in the other pathway(s) in which Nbp2p is involved.

Function of Nbp2p as Negative Regulator of CWI Pathway Is Conserved in \( \text{S. pombe} \) and \( \text{N. crassa} \)—Given the high degree of conservation among the Ascomycete Nbp2p SH3 domains, we hypothesized that the binding sites of these domains might also be conserved. Supporting this idea, we were able to identify an obvious PXXP-containing binding site for the Nbp2p SH3 domain in all orthologues of Bck1 in diverse Ascomycete species (Fig. 5A). These sites displayed strong conservation at several positions outside of the RXXPXXP canonical Class I SH3 domain binding site that we have shown to be important for Nbp2p binding (30), implying that the Nbp2p SH3 domain binds to Bck1p in all of these species.

It was surprising that the specificity of the Nbp2p SH3 domain appeared to be conserved even in the distantly related \( \text{S. pombe} \) species because the Nbp2p SH3 domain from this species showed considerable divergence from the other Nbp2p orthologues (Fig. 1A). To confirm this conservation of specificity, we measured the in vitro binding affinity of the SH3 domains from the \( \text{S. pombe} \) Nbp2 orthologue, Skb5p, for its putative binding site in the \( \text{S. pombe} \) Bck1p orthologue, Mkh1p. Strikingly, we found that the purified Skb5p SH3 domain bound to the Mkh1p\( \text{pXXP} \) peptide with an affinity of 1 \( \mu \text{M} \), which is identical to the affinity of Nbp2p SH3-Bck1p\( \text{pXXP} \) peptide interaction in \( \text{S. cerevisiae} \) (Fig. 5B). Furthermore, the Nbp2p SH3 domain bound to the Mkh1p\( \text{pXXP} \) peptide, and the Skb5p SH3 domain bound to the Bck1p\( \text{pXXP} \) peptide with similar affinities of 0.5 \( \mu \text{M} \) and 1.5 \( \mu \text{M} \), respectively (Fig. 5B). These results show that the Nbp2p SH3 domain binding specificity and its interaction with the Bck1p\( \text{pXXP} \) peptide are conserved in \( \text{S. pombe} \).

Because Skb5p binds to a site in the \( \text{S. pombe} \) Bck1p homologue and contains a well conserved PB M (Fig. 1B), we predicted that Skb5p would also play a role in regulating the CWI pathway in this species. To determine whether Skb5p functions in the down-regulation of the \( \text{S. pombe} \) CWI pathway, we compared the levels of Pmk1p (\( \text{S. pombe} \) Slt2p) phosphorylation in wild-type and \( \text{skb5} \Delta \) \( \text{S. pombe} \) strains. Similar to the \( \text{nbp2} \Delta \) strain, the \( \text{skb5} \Delta \) strain displayed a marked increase in Pmk1p phosphorylation levels in unstressed conditions (Fig. 5C). The \( \text{skb5} \Delta \) strain also displayed a slight increase in Pmk1p phosphorylation levels relative to wild-type upon activation of the pathway through hypertonic conditions (Fig. 5C). Because previous work has demonstrated a role for Ptc1p in down-regulating the CWI pathway in \( \text{S. pombe} \) (22), our results suggest that Skb5p is responsible for recruiting Ptc1p to the pathway in a manner identical to that demonstrated for Nbp2p in \( \text{S. cerevisiae} \).

To further validate the role of Nbp2p as a conserved negative regulator of the fungal CWI pathways we tested whether it is also involved in the down-regulation of the CWI pathway in \( \text{N. crassa} \), a filamentous fungal species of the Pezizomycotina subphylum, which is distantly related to both \( \text{S. cerevisiae} \) (Saccharomyces cerevisiae subphylum) and \( \text{S. pombe} \) (Taphrinomycotina subphylum). We first compared the Mak-1 (\( \text{N. crassa} \) Slt2p) phosphorylation levels in a wild-type strain and strain bearing a
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FIGURE 5. Nbp2p functions in down-regulation of S. pombe and N. crassa CWI pathways. A, Bck1PXXP motif is conserved in Ascomycete fungal species. Positions that are conserved in all the sites are shaded dark gray, and the positions that are partially conserved are shaded light gray. Position numbering is according to standard convention (28). B, interactions of purified Skb5p SH3 domain (Nbp2p homologue) and Mkh1XXP peptide (Bck1p homologue) are shown. The $K_d$ values of tested interactions were derived and are represented as described in Fig. 3A. C, activity of the CWI pathway in the N. crassa strain containing deletion of SKBS (Nbp2p homologue) is shown. The strains were cultured in liquid Vogel’s minimal medium (VM) for 16 h or liquid synthetic crossing medium (SCM) for 18 h, and cell lysates were prepared as described under "Experimental Procedures." Activation of the CWI pathway was detected using anti-phospho-p44/p42 antibody which also recognizes phosphorylated Pmk1p (MAPK of the S. pombe CWI pathway). D, activity of the CWI pathway in the N. crassa strain containing deletion of NCU03113 (Nbp2p homologue) is shown. The strains were cultured in liquid Vogel’s minimal medium (VM) for 16 h or liquid synthetic crossing medium (SCM) for 18 h, and cell lysates were prepared as described under "Experimental Procedures." Activation of the CWI pathway was detected using anti-phospho-p44/p42 antibody which recognizes Mak-1 (MAPK of the N. crassa CWI pathway, ~50 kDa) and Mak-2 (MAPK of the N. crassa mating pathway, ~43 kDa) (23). It should be noted that Mak-2 is hyperphosphorylated in the mak-2Δ strain, which is consistent with results of a previous study (23).

deletion of NCU03113 (N. crassa NBP2, henceforth referred to as Nc NBP2) grown under standard conditions (Vogel’s minimal medium-submerged hyphal cultures). As observed previously (23) Mak-1 is strongly phosphorylated in these conditions, and we detected no difference in Mak-1 phosphorylation levels between wild-type and Nc nbp2Δ strains (Fig. 5D). Because strong activation of the CWI pathway under tested conditions could potentially mask the effect of Nc NBP2 deletion, we next tested Mak-1 phosphorylation in nitrogen-starved synthetic medium cultures that were previously shown to have low levels of phosphorylated Mak-1(23). As observed previously, the level of phosphorylated Mak-1 was barely detectable in the wild-type strain under these conditions (Fig. 5D). Significantly, the phosphorylation of Mak-1 was considerably higher in the Nc nbp2Δ strain, suggesting that similar to its role in S. cerevisiae and S. pombe, Nbp2p also functions in the down-regulation of the CWI pathway in N. crassa.

DISCUSSION

In this study, we have shown that the PBM and SH3 domain of S. cerevisiae Nbp2p are both required for its function. Our discovery of the highly conserved PBM, which is found in both Ascomycete and Basidiomycete Nbp2p orthologues (i.e. it is conserved over at least 500 million years of evolution), implies that Ptc1p binding is a universally conserved feature of the Nbp2p family of proteins. Ptc1p is also conserved across all of these species. The presence of an SH3 domain in all Nbp2p orthologues implies that they serve as adaptors to recruit Ptc1p to proteins containing target sequences recognized by the SH3 domain.

Our experiments have clearly elucidated the role of Nbp2p in down-regulating the CWI pathway. Although implied in previous studies (8, 9), we have provided the first direct evidence that Nbp2p functions in the CWI pathway by binding to Ptc1p and a specific site in Bck1p. We have shown that full-length Nbp2p and Bck1p interact at endogenous levels of expression and that this interaction is abrogated by amino acid substitutions in the Bck1PXXP motif. Amino acid substitutions disrupting either the Nbp2p-Bck1p interaction (Fig. 3, A and B) or the Nbp2p-Ptc1p interaction (Fig. 1C) resulted in hyperactivation of the CWI pathway (Fig. 4), confirming that direct interactions between Ptc1p, Nbp2p, and Bck1p are essential for regulating the CWI pathway. The down-regulation of the CWI pathway by Nbp2p parallels its role in the HOG MAPK pathway where it binds Pbs2p, the MAPKK of the pathway, and results in reduced phosphorylation of Hog1p. Interestingly, to regulate the CWI, Nbp2p binds to Bck1, which is the MAPKKK of this pathway, indicating that either Ptc1p targets different steps in these two pathways or that the presence of multiprotein complexes allows Ptc1p to dephosphorylate analogous pathway components regardless of which upstream component it binds. At this point, these possibilities cannot be distinguished because the substrates of the Ptc1p in these pathways are not known.

The function of Nbp2p in the CWI pathway appears to be conserved across the Ascomycete species. This is supported by the high conservation of the Bck1PXXP site in all Ascomycete species examined (Fig. 5A). We demonstrated that the SH3 domain of Skb5p (S. pombe orthologue of Nbp2p) binds strongly to the PXXP motif from Mkh1p (S. pombe orthologue of Bck1p) (Fig. 5B) and that deletion of SKBS causes hyperactivation of the S. pombe CWI pathway (Fig. 5C). These data suggest that the role of Nbp2p in the CWI is conserved in S. pombe, which is the most distant relative of S. cerevisiae among Ascomycetes (17). Additionally, our results suggest that Nbp2p also impinges on the regulation of CWI pathway in the filamentous fungus N. crassa, where deletion of Nc NBP2 resulted in the hyperactivation of the pathway under certain conditions (Fig. 5D). Although a CWI pathway exists in Basidiomycetes (24), the role of the Basidiomycete Nbp2p homologues in regulating
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this pathway is not clear. None of the Bck1p homologues that we identified in these species (supplemental Table S1) contains a close match to the consensus PXXP-containing motif recognized by the Nbp2p SH3 domain. However, the Basidiomycete Nbp2p SH3 domains may bind to different sequences because they are quite highly diverged from the Ascomycete domains and also display considerably less conservation among themselves (Fig. 1A). We were able to identify PXXP motifs matching the consensus sequence recognized by the Nbp2p SH3 domain in the Ste20p and Cla4p kinases of the Basidiomycete species (supplemental Fig. S1). However, the PXXP motifs in these kinases in S. cerevisiae are also bound strongly by the Bem1p SH3 domain (12, 25), which is conserved in the Basidiomycete species. Therefore, these sites may be conserved in Basidiomycete species to bind Bem1p and not Nbp2p. In contrast with the Ste20p and Cla4p PXXP sites, the Bck1p SH3 domain only binds to the Nbp2p SH3 domain with high affinity, and the Bem1p SH3 domain does not bind this site strongly enough to impart full biological function (11). For this reason, we conclude that the high conservation of the Bck1p SH3 site is a result of its role in Nbp2p function.

Although Nbp2p clearly plays an important and conserved role in regulating the CWI pathway, this function alone cannot fully account for the severity of the phenotypes resulting from Nbp2 mutations. This point is illustrated by our observation that strains expressing the mutant forms of Bck1p that do not bind Nbp2p (Bck1pXXPΔ and Bck1pXXP P0A) display significantly milder growth defects (Fig. 2C) relative to strains expressing Nbp2p mutants that cannot bind Ptc1p (e.g. the KD → AA mutant; Fig. 2A) or SH3 domain mutants with reduced peptide binding activity (Y8A and F54A substitutions, Fig. 2B). This result shows that hyperactivation of the CWI pathway caused by the loss of Nbp2p SH3 domain interaction with Bck1p causes a relatively small effect on cell viability. By contrast, a strain bearing the Nbp2p SH3 domain Y8A mutant displays more severe growth defects than strains bearing the Bck1pXXP mutants (Fig. 2, B and C) even though the CWI pathway is only partially hyperactivated in the Y8A strain (Fig. 4B). Because HOG pathway hyperactivation, which also occurs in NB2P mutants, does not affect growth under the stress conditions used here (9), the more severe growth defects of NB2P mutants compared with the bck1pXXP mutants, can only be accounted for by the involvement of Nbp2p in other cellular processes.

At this point, we can only speculate about other functions of Nbp2p that, when abrogated, lead to the growth defects observed here. One possibility pertains to the demonstrated role of Ptc1p and Nbp2p in the inheritance of vacuoles (10). The Ste20p and Cla4p kinases, which both contain PXXP-containing sequences that bind the Nbp2p SH3 domain (1, 11), are also involved in this process (26). Overexpression of these p21-activated kinases or deletion of the NB2P or PTC1 genes results in a decrease in vacuole receptor protein (Vac17p) levels, suggesting that the Nbp2p-Ptc1p complex and the p21-activated kinases impinge on the same facet of the vacuole inheritance process. We have previously demonstrated that the Nbp2p SH3 domain binds to peptides derived from Ste20p and Cla4p more strongly (Kd values equal 0.2 and 0.5 μM, respectively) (11) than to peptides derived from the proven biological targets, Pbs2 (Kd = 3.2 μM) and Bck1 (Kd = 1.0 μM) (Fig. 3A). Thus, the interaction between Nbp2p and Cla4p or Ste20p may be required for regulation of organelle inheritance by Ptc1p. A complication in studying the functional significance of Nbp2p binding to Ste20p or Cla4p is that the second SH3 domain of Bem1p also binds strongly to these sites as mentioned above (12, 25). Nbp2p may also be involved in the regulation of other cellular pathways in which Ptc1p has been shown to play a role, including the TOR pathway (9) the calcineurin pathway (9), and the mating pathway (27). Our identification of the Nbp2p PBM KD → AA mutation which specifically disrupts the interaction between Nbp2p and Ptc1p will be a useful tool to assess the involvement of Nbp2p in other Ptc1p-associated processes.

Whereas all orthologues of Nbp2p possess a PBM and an SH3 domain, suggesting that a conserved role of Nbp2p is the recruitment of Ptc1p to its targets through SH3 domain-mediated interactions, some groups of closely related Nbp2p orthologues are likely to have unique functional roles not shared by other Nbp2p orthologues. For example, the role of Nbp2p in the HOG pathway appears to be conserved only in close relatives of S. cerevisiae (Saccharomycetaceae family), as the Pbs2pXXP motif that binds Nbp2p is only present in these species (supplemental Fig. S2). Similarly, interaction of the Nbp2p C terminus with Nap1p may also be a feature of homologues from only a subset of fungal species. We were able to identify a conserved C-terminal motif, which we suspect mediates binding to Nap1p, only in Nbp2p homologues from the Saccharomycotina subphylum (supplemental Fig. S3). This motif was not found in homologues of other species and some homologues (e.g. those from S. pombe and the Basidiomycete species) terminate at the end of the SH3 domain. Also supporting distinct functions for some Nbp2p orthologues, those from the Pezizomycotina subphylum and Basidiomycete phylum are considerably longer than other orthologues, and some of these subfamilies clearly display unique conserved motifs.

In conclusion, our studies show that the Nbp2p family is conserved in diverse fungal species and is involved in multiple functions, many of which are yet to be identified. Importantly, we show that the universally conserved features of Nbp2p orthologues are the SH3 domain and PBM. This conservation implies that the specific recruitment of Ptc1p phosphatase by Nbp2p is a key feature of many fungal signaling pathways. The involvement of adaptor proteins like Nbp2p in regulating multiple pathways likely provides an important means to coordinate the activities of these otherwise separate pathways, and further studies on these proteins will provide key insights into the global control of intracellular signaling.

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