The *Saccharomyces cerevisiae* 14-3-3 protein Bmh2 is required for regulation of the phosphorylation status of Fin1, a novel intermediate filament protein

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In order to identify proteins that interact with Bmh2, a yeast member of the 14-3-3 protein family, we performed a two-hybrid screening using LexA-Bmh2 as bait. We identified Fin1, a novel intermediate filament protein, as the protein that showed the highest degree of interaction. We also identified components of the vesicular transport machinery such as Gic2 and Msb3, proteins involved in transcriptional regulation such as Mbf1, Gcr2 and Reg2, and a variety of other different proteins (Ppt1, Lre1, Rps0A and Ylr177w). We studied the interaction between Bmh2 and Fin1 in more detail and found that Bmh2 only interacted with phosphorylated forms of Fin1. In addition, we showed that Glc7, the catalytic subunit of the protein phosphatase 1 complex, was also able to interact with Fin1.

Key words: Glc7 protein phosphatase, two-hybrid analysis.

INTRODUCTION

14-3-3 proteins form a group of highly conserved, acidic, dimeric proteins with a subunit molecular mass of around 30 kDa. They are involved in the signal transduction of different crucial cellular processes, including apoptosis and cell-cycle regulation, in which they participate in several ways: (i) they may alter the conformation of the target protein, modifying its enzymic activity; (ii) they may promote the assembly of oligomeric signalling complexes; (iii) they may act as attachable nuclear-export signals, promoting the transport of nuclear proteins to the cytoplasm or (iv) they may act as localization anchors in the cytosol, changing the subcellular localization of the binding partners (see [1–3] for reviews). 14-3-3 proteins usually bind to phosphorylated serine (pS) residues that are located in a typical consensus motifs RSXpSXP or RX(Y/F)XpSXP [4,5], although it has also been reported that 14-3-3 proteins may interact with unphosphorylated substrates [6].

Bmh1 and Bmh2 are the *Saccharomyces cerevisiae* members of the 14-3-3 protein family [1,2]. They share a great degree of homology (92% identity; differing by only 15 amino acids out of the first 256, from a total of 273 amnio acids), although Bmh2 contains a divergent C-terminus (a stretch of 17 glutamine residues) [7,8]. In *S. cerevisiae*, these proteins participate in the regulation of a variety of crucial physiological processes, such as exocytosis and vesicle transport [7,9], Ras/mitogen-activated protein kinase signalling cascade during pseudohyphal development [10], regulation of the subcellular localization of Msn2/4 [transcriptional activators of stress response element (STRE)-regulated genes] [11] and Yak1 (a serine/threonine protein kinase that negatively regulates cell growth [12,13]), regulation of rapamycin-sensitive signalling [through the target of rapamycin (TOR) kinase pathway] [14] and proteasome-regulated protein degradation [15].

To understand how 14-3-3 proteins participate in these processes, several strategies have been used to identify the components of the pathways that interact with them. In a large-scale two-hybrid screening [16], three genes were identified which encoded proteins that interacted with Bmh2 when fused to the Gal4 DNA-binding domain (GBD-Bmh2). These were *ECM13*, encoding a protein possibly involved in cell-wall structure and biogenesis [17], *KCS1*, which encodes an inositol (1,2,3,4,5,6)-hexophosphate kinase also involved in vesicular transport [18], and *BOP3*, encoding a protein of unknown function [19]. Additional strategies have defined the interaction of Bmh2 with other partners, such as Chc1 (clathrin heavy chain required for the internalization step of endocytosis [7]), Ste20 (upstream activator of the Ras/mitogen-activated protein kinase signalling pathway involved in pseudohyphal development [10]), Msn2, Msn4 and Yak1 (see above), among others.

In order to identify additional interacting proteins that may affect any of the processes described above, we performed a two-hybrid screening using LexA-Bmh2 as bait. We describe in this study the results of this screening and the characterization of the interaction of Bmh2 with Fin1, a novel intermediate filament (IF) protein.

MATERIALS AND METHODS

Strains and genetic methods

S. cerevisiae strain FY250 (*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52*) was a gift from Dr F. Winston (Harvard Medical School, Boston, MA, U.S.A.). Strains Σ1278 (10560-6b) (*MATα his3 leu2 trp1 ura3-52*) and Σ1278 *bmh1bmh2*Δ (a *bmh1*Δ::*HIS3 bmh2*Δ::*HIS3* derivative of Σ1278b) [10] were from Dr G. Fink (Whitehead Institute for Medical Research, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). Strain *glc7-1* was from Dr K. Tatchell (Louisiana State University Medical

Abbreviations used: GAD, Gal4-activating domain; GST, glutathione S-transferase; HA, haemagglutinin epitope; IF, intermediate filament; pS, phosphorylated serine residue; SC, synthetic complete.

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Center, Shreveport, LA, U.S.A.). Strains CTY10-5d (*MATa ade2 his3 leu2 trp1 gal4 gal80 URA3::lexAop-lacZ*) and TAT-7 (*MATa ade2 his3 leu2 trp1 gal4 gal80 LYS2::lexAop-HIS3 URA3::lexAop-lacZ*) were a gift from Dr R. Sternglanz (State University of New York, Stony Brook, NY, U.S.A.).

Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids [20], supplemented with different carbon sources.

Plasmids

Oligonucleotides BMH2-1 (5′-CCGGGATCCCAAAAAAA-AATGTCCCAAACTC-3′; the initial ATG is underlined) and BMH2-2 (5′-CCCCTCGAGTTTGTCGACGCTCTTATTTG-GTTGG-3′; the stop codon is underlined) were used to amplify by PCR the coding region of the *BMH2* gene using genomic DNA of FY250 as a template. The amplified fragment was digested with *Bam*HI and *Sal*I and subcloned into pBTM116 [21] to obtain pBTM-Bmh2 (expressing the LexA-Bmh2 fusion protein; TRP1 was the selection marker on the plasmid). The same fragment was subcloned into pEG-GST {a derivative of pEG202 [22] where the *LexA* gene has been substituted by the glutathione S-transferase (GST) gene from *Schistosoma japonicum*} to obtain pGST-Bmh2 (expressing GST-Bmh2; HIS3 selection marker).

Oligonucleotides FIN1-1 (5′-CAAGGATCCAAATTATGA-GCAATAAAAGCAACC-3′; the initial ATG is underlined) and FIN1-2 (5′-AAGGGTCGACATTACTTATGTTTCGGTATT-TCC-3′; the stop codon is underlined) were used to amplify by PCR the coding region of the *FIN1* gene using genomic DNA of FY250 as a template. The amplified fragment was digested with *Bam*HI and *Sal*I and subcloned into pEG202 [22] to obtain pEG202-Fin1 (expressing LexA-Fin1; HIS3 selection marker). The same fragment was introduced into pACTII [23] and pWS93 [24] to obtain pACTII-Fin1 (expressing GAD-Fin1; LEU2 selection marker; where GAD is Gal4-activating domain) and pWS-Fin1 (expressing HA-Fin1, URA3; where HA represents the haemagglutinin epitope) respectively.

To construct pGST-Glc7 (expressing GST-Glc7; HIS3 selection marker), a fragment from pHA-Glc7 [25] was introduced into pEG-GST (see above). In some experiments we also used pGAD-Glc7 [25].

Two-hybrid screening

A two-hybrid screening [26] for proteins that interacted with LexA-Bmh2 (pBTM-Bmh2, see above) was carried out in *S. cerevisiae* strain TAT-7 that contained two chromosomally located reporter genes, *lexAop-HIS3* and *lexAop-lacZ* [27]. The strain was transformed with a library of *S. cerevisiae* cDNAs fused to GAD (a generous gift from Dr S. Elledge, Baylor University, Waco, TX, U.S.A.; [28]). Transformants were selected in SC medium with 2% glucose plates containing 10 mM 3-amino-1,2,4-triazole and were subsequently screened for β-galactosidase activity using a filter lift assay [29]. Positive transformants were grown in plates containing increasing concentrations of 3-amino-1,2,4-triazole (from 20 to 120 mM) to give an estimation of the strength of interaction.

β-Galactosidase assays

β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in [30].

λ-Phosphatase treatment

Preparation of crude extracts was essentially as described previously [31]. The extraction buffer was 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 10% glycerol and contained 2 mM PMSF and complete protease inhibitor cocktail (Boehringer Mannheim). Crude extracts (2 μg) in λ-phosphatase buffer containing 2 mM $MnCl_2$ were treated at 30 °C for 30 min with 50 or 100 units of λ-phosphatase (New England BioLabs) in the presence or absence of a mixture of phosphatase inhibitors (50 mM EDTA, 50 mM NaF and 100 mM sodium phosphate buffer, pH 8). The reactions were stopped by adding 1 vol. of Laemmli sample buffer and boiling for 3 min.

Pull-down assay

Crude extracts prepared as above were used in the pull-down assays and combined with GSH–agarose (Amersham Bioscience) as in [31]. Proteins in the pellet were analysed by Western blotting using anti-HA monoclonal antibodies.

Immunoblot analysis

Protein samples were separated by SDS/PAGE using 10% polyacrylamide gels and analysed by immunoblotting using either anti-GST (Amersham Bioscience) polyclonal or anti-HA (Boehringer Mannheim) monoclonal antibodies. Antibodies were detected by enhanced chemiluminescence with ECL® or ECL Plus® reagents (Amersham Bioscience).

RESULTS AND DISCUSSION

Identification of proteins that interacted with Bmh2

In *S. cerevisiae*, the members of the 14-3-3 protein family participate in the regulation of different physiological processes (see Introduction). In order to identify additional interacting proteins that may affect any of these processes, we performed a two-hybrid screening using LexA-Bmh2 as bait (see the Materials and methods section). After screening 10^6 transformants, we were able to recover more than 300 putative positive transformants that were able to grow in the absence of histidine and were blue in the β-galactosidase filter assay. In this study, we describe the characterization of the inserts contained in 50 of these transformants which showed the highest degree of interaction (measured as the capacity to grow in the presence of increasing concentrations of 3-amino-1,2,4-triazole). The plasmids contained in these selected transformants were recovered, the corresponding inserts sequenced and the identities of the respective genes determined by BLAST analysis [32].

As a result of this screening (Table 1) we identified components of the vesicular transport machinery, such as Gic2 (a GTPase-associated protein required for polarized cell growth [33]) and Msb3 (a GTPase-activating protein involved in bud emergence [34]), consistent with the participation of Bmh2 in this process [7]. Bmh2 was also able to interact with proteins involved in transcriptional regulation, such as Mbf1 (a transcriptional co-activator of Gcn4 [35]), Gcr2 (a transcriptional activator involved in the regulation of glycolytic gene expression [36]) and Reg2 (a Glc7 protein phosphatase regulatory subunit [37]), and with a variety of other proteins, such as Ppt1 (a Ser/Thr protein phosphatase of unknown function, a member of the PP5 phosphatase family [38]), Lre1 (a protein involved in cell-wall maintenance [39]), Rps0A (a ribosomal protein S0 [40]) and Ylr177w (a protein of unknown function [19]; Table 1). Bmh2 interacted also with itself and with Bmh1, the other *S. cerevisiae* member of

Table 1 Identity of proteins that interacted with LexA-Bmh2 in the two-hybrid screening

Two-hybrid interaction between pBTM-Bmh2 (LexA-Bmh2) and the corresponding partners was measured in TAT-7 cells as β-galactosidase activity. Codes in parentheses indicate the systematic *S. cerevisiae* open reading frame codes.

Name of partner	No. of isolates	β-Galactosidase activity (units)	Description [19]
FIN1 (YDR130c)	26	300–400	Intermediate filament protein
BMH1 (YER177w)	7	35–45	14-3-3 protein
BMH2 (YDR099w)	1	27	14-3-3 protein
GIC2 (YDR099w)	2	63–122	GTPase-associated protein required for polarized cell growth
MSB3 (YNL293w)	2	135–215	GTPase-activating protein involved in bud emergence
MBF1 (YOR298c)	1	23	Transcriptional co-activator of Gcn4
GCR2 (YNL199c)	1	127	Transcriptional activator involved in regulation of glycolytic gene expression
REG2 (YBR050c)	1	83	Possible regulatory subunit of Glc7
PPT1 (YGR123c)	1	13	Ser/Thr protein phosphatase, member of the PP5 phosphatase family
LRE1 (YCL051w)	1	98	Protein involved in cell-wall maintenance
RPS0A (YGR214w)	1	38	Ribosomal protein S0
YLR177w	2	31–146	Protein of unknown function
–	4	Not determined	Inserts were out of frame

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        .........10........20........30........40........50........60
AA      MSNKSNRRSLRDIGNTIGRNNIPSDKDNVFVRLSMSPLRTTSQKEFLKPPMRISPNKTDG
PHDsec          HHHHHH               EEEEEE       HHHHHH     EEE

        .........70........80........90........100.......110.......120
AA      MKHSIQVTPRRIMSPECLKGYVSKETQSLDRPQFKNSNKNVKIQNSDHITNIIFPTSPTK
PHDsec   EEEEE     EE HHH    EEEE               EEEE     EEEEE     E

        .........130.......140.......150.......160.......170.......180
AA      LTFSNENKIGGDGSLTRIRARFKNGLMSPERIQQQQQQHILPSDAKSNTDLCSNTELKDA
PHDsec  EEE          HHHHHHHHHHH     HHHHHHHHHHEEE

        .........190.......200.......210.......220.......230.......240
AA      PFENDLPRAKLKGKNLLVELKKEEEDVGNGIESLTKSNTKLNSMLANEGKIHKASFQKSV
PHDsec         HHH     EEEEE            EEE      HHHHHHH    EEEEEE EE

        .........250.......260.......270.......280.........291
AA      KFKLPDNIVTEETVELKEIKDLLLQMLRRQREIESRLSNIELQLTEIPKHK
PHDsec  EE            HHHHHHHHHHHHHHHHHHHHH    HHHHHH
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Figure 1 Secondary structure of the Fin1 protein

The secondary structure of the Fin1 protein was predicted using the PHDsec program [55]. H, helix; E, strand; AA, amino acid.

the 14-3-3 protein family, confirming the dimeric nature of these proteins and indicating that they may form both homo- and hetero-dimers (Table 1).

However, the clone that was recovered most frequently (26 isolates out of 50) corresponded to in-frame fusions of cDNAs of the *FIN1* gene that started at different positions either in the 5′ untranslated region or inside the coding region of the gene. In addition, transformants containing *FIN1* derivatives were the ones that presented the highest levels of interaction with Bmh2 (β-galactosidase activity; Table 1). For this reason we focused our attention on the study of the interaction between Bmh2 and Fin1.

Bmh2 interacts only with phosphorylated Fin1

FIN1 (systematic open reading frame code YDR130c) is a gene encoding a protein of 291 amino acids (33070 Da). Although the exact cellular role of Fin1 is still unknown, it has been described in the Yeast Proteome Database (YPD; [19]) and in the *Saccharomyces* Genome Database (SGD; [41]) as a novel IF protein that might play a role in cell-cycle regulation. In fact, it has been reported recently that the expression of *FIN1* is induced during the S/G_2 phase of the cell cycle [42], although its deletion has no observable phenotypes [43].

IF proteins are a family of heterogeneous proteins in terms of size and amino acid sequence that share a typical structural organization in common. All the IF proteins have a central α-helical rod domain flanked by non-helical N- and C-terminal domains that vary depending on the particular IF. The α-helical rod domain plays a central role in dimerization, where the two polypeptide chains are wound around each other in a coiled-coil structure. The dimers then associate in a staggered anti-parallel fashion to form tetramers, which can assemble end to end to form protofilaments [44,45]. Phosphorylation of the IF proteins is a major regulator of IF assembly; phosphorylation of IF results in their disassembly, whereas phosphatase treatment of phosphorylated subunits restores their competence for assembly (see [44] and [45] for a review).

Fin1 has the characteristic IF α-helical rod domain that might participate in dimerization (Figure 1). In fact, by two-hybrid

Table 2 Two-hybrid interaction between Bmh2 and Fin1

Two-hybrid interaction between pBTM-Bmh2 (LexA-Bmh2) and pACTII-Fin1 (GAD-Fin1) was measured in CTY10.5d cells. The interaction between pEG202-Fin1 (LexA-Fin1) and pACTII-Fin1 (GAD-Fin1) was measured in the same way. Values for cells growing exponentially in 2% glucose are mean β-galactosidase activities from four to six transformants, with S.D. values being lower than 15% in all the cases.

Fusion proteins	β-Galactosidase activity (units)
LexA-Bmh2, GAD-Fin1	478
LexA-Bmh2, GAD	11
LexA, GAD-Fin1	< 1
LexA-Fin1, GAD-Fin1	76
LexA-Fin1, GAD	< 1

Table 3 Two-hybrid interaction between Fin1 and Glc7

Two-hybrid interaction between pEG202-Fin1 (LexA-Fin1) and pGAD-Glc7 was measured in CTY10.5d cells. Values for cells growing exponentially in 2% glucose are mean β-galactosidase activities from four to six transformants, with S.D. values being lower than 15% in all cases. Please note that although the interaction between LexA-Fin1 and GAD-Glc7 was low (4 units), the difference between this and the controls was statistically significant, indicating a positive interaction between the proteins.

Fusion protein	β-Galactosidase activity (units)
LexA-Fin1, GAD-Glc7	4
LexA-Fin1, GAD	< 1
LexA, GAD-Glc7	< 1

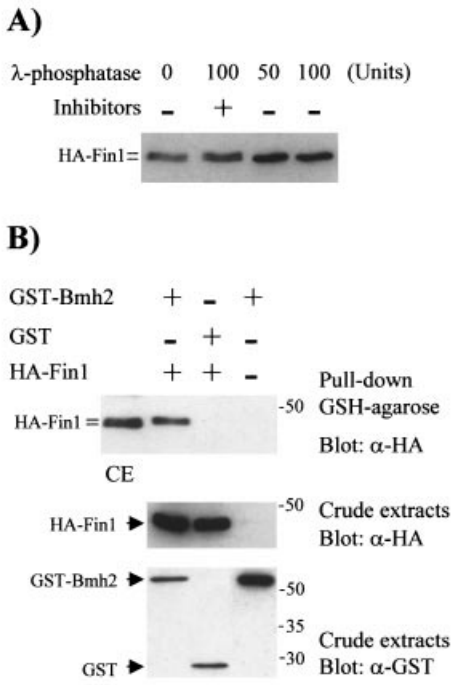

Figure 2 Phosphorylation status of Fin1 affects Bmh2 binding

(**A**) Phosphorylation status of Fin1. Wild-type FY250 cells expressing HA-Fin1 were grown in selective SC + 2% glucose medium. Crude extracts (2 μg) were treated with λ-phosphatase (50 or 100 units) in the presence or absence of phosphatase inhibitors (see the Materials and methods section). HA-Fin1 was immunodetected with anti-HA monoclonal antibody. (**B**) Pull-down assay of GST-Bmh2 and HA-Fin1. Crude extracts (250 μg) were prepared from FY250 glucose-growing cells expressing GST-Bmh2 and HA-Fin1 or containing the corresponding empty vectors. GST-Bmh2 fusion proteins were pulled-down with GSH–agarose. Proteins in the pellet were analysed by SDS/PAGE and immunodetected with anti-HA monoclonal antibodies (upper panel). Proteins in the crude extracts (5 μg) were also immunodetected with either anti-HA (middle panel) or anti-GST (lower panel) antibodies. CE, crude extract (1 μg) of cells containing GST-Bmh2 and HA-Fin1. Size standards are indicated in kDa.

analysis we detected that Fin1 (LexA-Fin1) was able to interact with itself (GAD-Fin1), forming stable complexes (Table 2). In addition, we observed that Fin1 was present in the crude extracts as a mixture of phosphorylated and unphosphorylated forms; an N-terminal HA-tag derivative of Fin1 (HA-Fin1; see the Materials and methods section) appeared in the crude extracts as a doublet, the upper band being sensitive to treatment with exogenous λ-phosphatase (Figure 2A).

In order to study the interaction between Bmh2 and Fin1, we amplified by PCR the coding region of *FIN1*, which was sequenced to confirm that no modifications had been introduced by the *Taq* polymerase. This amplified fragment was used to construct LexA-Fin1 and GAD-Fin1 derivatives (see the Materials and methods section). As shown in Table 2, LexA-Bmh2 interacted strongly with GAD-Fin1. The physical interaction between Bmh2 and Fin1 was confirmed by pull-down assays. In wild-type cells expressing the GST-Bmh2 and HA-Fin1 fusion proteins, we observed a specific interaction between Bmh2 and Fin1 (Figure 2B). This interaction was only observed with the upper phosphorylated forms of HA-Fin1, in agreement with the fact that 14-3-3 proteins usually interact with phosphoproteins [4,5]. As we did not find the consensus 14-3-3-binding motif (RSXpSXP or RX[Y/F]XpSXP [4,5]) in the Fin1 protein sequence, we suggest that alternative phosphorylated serine or threonine residues must be responsible for binding.

The interaction between IF proteins and 14-3-3 proteins has also been described in mammalian cells. 14-3-3 proteins associated with phosphorylated epithelial keratins during cell-cycle progression, acting as solubility cofactors [46,47]. Also, in a survey to identify cellular polypeptides that interacted with the 14-3-3ζ protein *in vivo*, vimentin (a member of the IF family) was found to be the most abundant protein that interacted with the 14-3-3 protein, and its association depended on vimentin phosphorylation [48]. The same authors suggested that phosphovimentin, by sequestering 14-3-3 proteins, may limit the availability of this protein to other targets, affecting intracellular signalling processes that required 14-3-3 proteins [48]. Perhaps Bmh2 could also act in the same way, acting as solubility factor of the Fin1 IF.

Protein phosphatase PP1 interacts physically with Fin1

It has been described recently by high-throughput two-hybrid analysis [16] that Fin1, fused to the Gal4 DNA-binding domain (GBD-Fin1), interacted with Glc7, the catalytic subunit of the protein phosphatase PP1 complex. To confirm these results we repeated the two-hybrid analysis between LexA-Fin1 and GAD-Glc7 (Table 3) and confirmed the interaction by pull-down assays in cells expressing GST-Glc7 and HA-Fin1 as fusion proteins (Figure 3). We observed that the interaction occurred only between Glc7 and the upper, phosphorylated forms of HA-Fin1. These results might suggest that Glc7 was involved in the regulation of the phosphorylation status of Fin1, either directly or indirectly. A similar situation occurs in mammalian cells where it has been reported that protein phosphatase PP1 modulates the turnover of phosphorylation and dynamics of desmin/vimentin filaments [49].

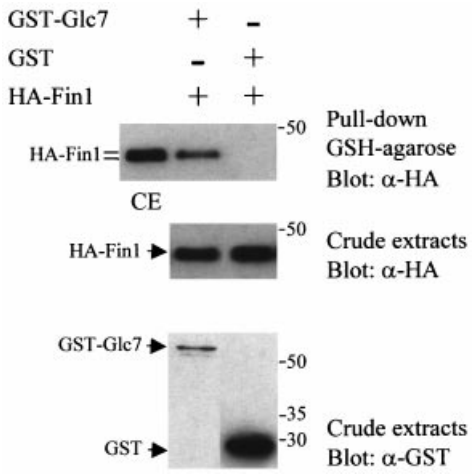

Figure 3 Pull-down assay of GST-Glc7 and HA-Fin1

Crude extracts (250 μg) were prepared from FY250 glucose-growing cells expressing GST-Glc7 and HA-Fin1 or containing the corresponding empty vectors. GST-Glc7 fusion proteins were pulled-down with GSH–agarose. Proteins in the pellet were analysed by SDS/PAGE and immunodetected with anti-HA monoclonal antibodies (upper panel). Proteins in the crude extracts (5 μg) were also immunodetected with either anti-HA (middle panel) or anti-GST (lower panel) antibodies. CE, crude extract (1 μg) of cells containing GST-Glc7 and HA-Fin1. Size standards are indicated in kDa.

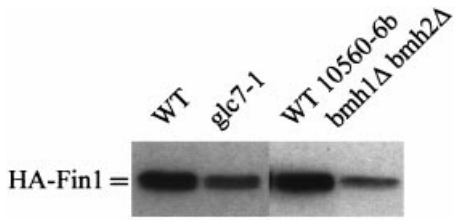

Figure 4 Phosphorylation status of Fin1 in *glc7-1* and double *bmh1Δ bmh2Δ* mutants

Crude extracts (2 μg) from different strains expressing HA-Fin1 were immunodetected with anti-HA antibodies. WT, wild type.

It is known that Glc7 participates in the regulation of different processes by binding to specific regulatory subunits that target the catalytic subunit to the corresponding substrates [25,37,50,51]. As we recovered Reg2 (a regulatory subunit of Glc7) as a Bmh2-interacting protein (see Table 1), we tested whether the interaction between Fin1 and Glc7 could be performed in the absence of Reg2. Pull-down assays using a double *reg1*Δ *reg2*Δ mutant (Reg1 is another Glc7 regulatory subunit involved in glucose repression [25]) showed that the interaction between Fin1 and Glc7 was similar to that between wild-type proteins (results not shown). We also studied the phosphorylation status of Fin1 in a partial *glc7-1* mutant (the deletion of *GLC7* is lethal [52]), which cannot interact with Gac1, another Glc7 regulatory subunit involved in glycogen metabolism [53,54]. As shown in Figure 4, HA-Fin1 showed the same doublet in the mutant as in the wild type. All these results may indicate that none of these regulatory subunits (Reg1, Reg2 or Gac1) was involved in the binding of Glc7 to Fin1.

Alternatively, Glc7 could interact with Fin1 either directly or via Bmh2. To distinguish between these possibilities we used a *bmh1*Δ *bmh2*Δ mutant. It is known that this double mutant is lethal in most genetic backgrounds but that it is still viable in the Σ1278b background [10]. We tried to repeat the pull-down experiments in the double mutant but were unable to recover any viable transformant expressing the GST-Glc7 fusion protein. In an alternative approach to assess the involvement of the 14-3-3 proteins in the regulation of the interaction between Glc7 and Fin1, we decided to analyse the phosphorylation status of Fin1 in the double *bmh1bmh2*Δ mutant by expressing the HA-Fin1 derivative in these cells. We observed that only the upper, phosphorylated forms of HA-Fin1 were present in the double mutant (Figure 4), indicating that in the absence of 14-3-3 proteins Fin1 could no longer be dephosphorylated.

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