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Self-association of the Spindle Pole Body-related Intermediate Filament Protein Fin1p and Its Phosphorylation-dependent Interaction with 14-3-3 Proteins in Yeast*

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The Fin1 protein of the yeast *Saccharomyces cerevisiae* forms filaments between the spindle pole bodies of dividing cells. In the two-hybrid system it binds to 14-3-3 proteins, which are highly conserved proteins involved in many cellular processes and which are capable of binding to more than 120 different proteins. Here, we describe the interaction of the Fin1 protein with the 14-3-3 proteins Bmh1p and Bmh2p in more detail. Purified Fin1p interacts with recombinant yeast 14-3-3 proteins. This interaction is strongly reduced after dephosphorylation of Fin1p. Surface plasmon resonance analysis showed that Fin1p has a higher affinity for Bmh2p than for Bmh1p (K_D 289 *versus* 585 nM). Sequences in both the central and C-terminal part of Fin1p are required for the interaction with Bmh2p in the two-hybrid system. In yeast strains lacking 14-3-3 proteins Fin1 filament formation was observed, indicating that the 14-3-3 proteins are not required for this process. Fin1 also interacts with itself in the two-hybrid system. For this interaction sequences at the C terminus, containing one of two putative coiled-coil regions, are sufficient. Fin1p-Fin1p interactions were demonstrated *in vivo* by fluorescent resonance energy transfer between cyan fluorescent protein-labeled Fin1p and yellow fluorescent protein-labeled Fin1p.

The *Saccharomyces cerevisiae FIN1* gene encodes a protein of 291 amino acids with a predicted pI of 10 that contains two putative coiled-coil regions at its C terminus. We showed recently (1) that the subcellular localization of the GFP[1]-Fin1 protein is highly cell cycle-dependent (1). In large budded cells the protein is visible as a filament between the two spindle pole bodies. In resting cells the protein is undetectable, and in small budded cells it is localized in the nucleus. During late mitosis it localizes on the spindle pole bodies. Purified His_6-tagged Fin1p self-assembles into filaments with a diameter of ~10 nm after dialysis against low salt buffers as is observed for other intermediate filament-forming proteins. The Fin1 protein is an interaction partner of the yeast 14-3-3 protein Bmh2 (1).

The 14-3-3 proteins form a family of highly conserved acidic dimeric proteins that are present, often in multiple isoforms, in all eukaryotic organisms (for review see Refs. 2–6). They bind to more than 120 different proteins and play a role in the regulation of many cellular processes, including signaling, cell cycle control, apoptosis, exocytosis, cytoskeletal rearrangements, transcription, and regulation of enzymes. Although the exact function of the 14-3-3 proteins is still not completely understood, three main mechanisms seem to be important. First, 14-3-3 proteins positively or negatively regulate the activity of enzymes; second, 14-3-3 proteins may act as localization anchors, controlling the subcellular localization of proteins; and third, 14-3-3 proteins can function as adaptor molecules or scaffolds, thus stimulating protein-protein interactions. Binding motifs have been identified in a number of proteins that bind to the 14-3-3 proteins. Many of these binding motifs consist of a phosphorylated serine residue, flanked by a proline and arginine residue (7–9). However, other 14-3-3 binding motifs have been identified that are seemingly unrelated and do not contain a phosphoserine (10, 11).

The yeast *S. cerevisiae* has two genes, *BMH1* and *BMH2*, encoding 14-3-3 proteins (12–15). A *bmh1 bmh2* disruption is lethal in most but not all laboratory strains, and the lethal *bmh1 bmh2* disruption can be complemented by at least four of the *Arabidopsis* isoforms and by a human and *Dictyostelium* isoform (16). As in higher eukaryotes, the *S. cerevisiae* 14-3-3 proteins are involved in many cellular processes, and many different binding partners have been identified (14), including the protein kinases Ste20p (17) and Yak1p (18) and the transcription factors Rtg3 (19), Msn2, and Msn4 (20). Recently, it has been shown (21) that the yeast 14-3-3 proteins bind to cruciform DNA.

Here, we describe the two-hybrid screen identifying the Fin1 protein as an interaction partner of the Bmh2 protein and detailed studies on the interaction between of the Fin1 protein and the yeast 14-3-3 proteins. In addition, the self-association of Fin1p was studied using both the yeast two-hybrid system and fluorescence resonance energy transfer in intact yeast cells.

MATERIALS AND METHODS

Strains, Culture Media, and Plasmids—S. cerevisiae HF7c (22) was used as reporter strain in two-hybrid screens. *S. cerevisiae* strain GG3100 is CEN-PK113-5D (*MATa ura3–52*; P. Kötter, Göttingen, Germany) containing plasmid pRUL182. The *bmh1 bmh2* strain RR1249 and the isogenic control strain F3α were obtained from Dr. G. R. Fink (MIT, Cambridge, MA) (17). GG3066 expressing both CFP-Fin1 and

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[1] The abbreviations used are: GFP, green fluorescent protein; ad, activating domain; bd, binding domain; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ELISA, enzyme-linked immunosorbent assay.

TABLE I
Plasmid constructions

Plasmid	Features/construction
pGAD-C1, -C2, and -C3	Two hybrid vectors for the expression of Ga14 activating domain fusion proteins (22)
pGBDK-C1, -C2, and -C3	Two-hybrid vectors containing a kanamycin resistance marker. Constructed from pGBD-C1, -C2, and -C3 (22), respectively, by replacing a *Aat*II-*Bgl*I fragment containing the larger part of the ampicillin resistance marker by a 1.2-kb *Aat*II-*Bgl*I fragment with the kanamycin resistance marker from plasmid pRUL531 using a linker consisting of oligonucleotides MH-1 and MH-2.
pMVHis	Plasmid allowing the galactose-inducible expression of His_6-tagged proteins in yeast. A synthetic DNA fragment consisting of oligonucleotides MH-5 and MH-6 was ligated in the *Bam*HI-*Hind*III sites of pYES2 (Invitrogen).
pUG36[*FIN1*]	Plasmid allowing the expression of a N-terminal GFP-Fin1 fusion protein under control of the *MET25* promoter (1).
pRUL182	Plasmid allowing the expression of a N-terminal His_6 Fin1p fusion protein in yeast. The 0.9-kb *Bam*HI-*Sal*I fragment from pRUL557, containing *FIN1,* was cloned in the *Bgl*II-*Xho*I sites of pMVHis.
pRUL348	Library plasmid isolated during a two-hybrid screen with *BMH2* as bait. Encodes a fusion between the *GAL4* activating domain and *FIN1,* lacking the first 30 bp.
pRUL360	pGAD plasmid with part of *FIN1* isolated during a two-hybrid screen with *FIN1.*
pRUL361	pGAD plasmid with part of *FIN1* isolated during a two-hybrid screen with *FIN1.*
pRUL362	pGAD plasmid with part of *FIN1* isolated during a two-hybrid screen with *FIN1.*
pRUL366	pGAD plasmid with part of *FIN1* constructed from pRUL348 by deleting a 1.3-kb *Xho*I-*Sal*I fragment.
pRUL367	pGAD plasmid with part of *FIN1* constructed from pRUL348 by deleting a 1.8-kb *Stu*I-*Msc*I fragment.
pRUL368	pGAD plasmid with part of *FIN1* constructed by insertion of a synthetic DNA fragment consisting of oligonucleotides MH-9 and MH-10 in the *Eco*RI-*Pst*I sites of pGAD-C1.
pRUL369	pGAD plasmid with part of *FIN1.* A synthetic DNA fragment consisting of oligonucleotides MH-9 and MH-10 was digested with TaqI and inserted in the *Eco*RI-*Cla*I sites of pGAD-C1.
pRUL370	pGAD plasmid with part of *FIN1.* Made by insertion of a linker consisting of oligonucleotides MH-11 and MH-12 in the *Eco*RI-*Pst*I sites of pGAD-C1.
pRUL371	pGAD plasmid with part of *FIN1.* A 1.3-kb *Xho*I-*Bgl*II fragment of pRUL348 was replaced by a fragment generated by PCR with primers MH-13 and MH-14 on pRUL348, followed by digestion with *Xho*I and *Bgl*II.
pRUL372	pGBDK plasmid with part of *FIN1.* A 102-bp *Eco*RI-*Bgl*II fragment from pRUL369 was cloned in pGBDK-C1.
pRUL526	Plasmid allowing the expression of a N-terminal His_6 Bmh1p fusion protein in *E. coli.* A 1-kb *Hin*CII-*Sal*I fragment with *BMH1* from plasmid pRS306[*BMH1*] (13) was ligated into the *Sma*I-*Sal*I sites of pQE-30 (Qiagen).
pRUL527	Plasmid allowing the expression of a N-terminal His_6-Bmh2p fusion protein in *E. coli.* The *BMH2*-containing *Bam*HI-*Sal*I fragment from plasmid pRUL532 was cloned in the *Bam*HI-*Sal*I sites of pQE-32 (Qiagen).
pRUL532	Plasmid with *BMH2* open reading frame. *Bam*HI and *Sal*I restriction sites were introduced at the ends of the *BMH2* open reading frame by PCR on plasmid pBlue[*BMH2*] (13) using primers MH-3 and MH-4. The resulting fragment was ligated into the *Bam*HI-*Sal*I sites of pUC18.
pRUL551	pGBDK plasmid with *BMH2.* The *BMH2*-containing *Bam*HI-*Sal*I fragment from pRUL532 was cloned in pGBDK-C2.
pRUL557	Plasmid with the *FIN1* open reading frame. A *Bam*HI site was introduced directly upstream of the *FIN1* start codon and a *Sal*I site 42 bp downstream of the stop codon by PCR on genomic DNA from *S. cerevisiae* strain S288c, using primers MH-7 and MH-8. This 0.9-kb PCR product was ligated into the *Bam*HI-*Sal*I sites of pUC18.
pRUL558	pGBDK plasmid with *FIN1.* The 0.9-kb *Bam*HI-*Sal*I fragment with *FIN1* from pRUL557 was cloned in pGBDK-C2.
pRUL1004	Plasmid suitable for expression of YFP fusion proteins. The GFP-containing *Xba*I-*Bam*HI fragment of plasmid pUG36 was replaced by a YFP-containing fragment. The latter fragment was made by PCR on plasmid pDH5 (D. Haley, Yeast Resource Center, University of Washington, Seattle, WA) as described previously (1).
pRUL1008	Plasmid encoding YFP-Fin1p. The *FIN1* open ready frame was cloned into pRUL1004 as described previously (1).
pRUL1072	pGAD plasmid with part of *FIN1* made by cloning a PCR fragment obtained by using the primers FIN1-K and FIN1-B into pGAD-C1 digested with *Cla*I and *Bgl*II.
pRUL1070	pGAD plasmid with part of *FIN1* made by cloning a PCR fragment obtained by using the primers FIN1-G and FIN1-B into pGAD-C1 digested with *Cla*I and *Bgl*II.

YFP-Fin1, was constructed by transformation of CENPK113–3B (*MATα ura3–52 his3*; P. Kötter, Göttingen, Germany) with the plasmids pRUL1005 (1) and pRUL1008. The control strain GG3087 expressing free CFP and free YFP was constructed by transformation of CENPK113–3B with the plasmids pRUL1001 (1) and pRUL1004. *Escherichia coli* (strain XL1-blue) and yeast were cultured as described before (13). The plasmids constructions are listed in Table I. The sequences of the oligonucleotides used are shown in Table II.

Nucleic Acid Manipulations—Nucleic acid manipulations were performed by standard techniques. The QIAprep mini spin kit (Qiagen) was used to isolate plasmid DNA from *E. coli*. The same kit was used to purify plasmid DNA from *S. cerevisiae*, after yeast cells were incubated with 1 mg/ml lyticase in buffer P1 (Qiagen) for 30 min. Subsequently, the isolated plasmid DNA was amplified in *E. coli* XLI-blue.

Two-hybrid Screen—Library Y2HL (22), consisting of 0.5–3-kb fragments of genomic DNA fused to the *GAL4*ad, was a gift of Philip James. Strain HF7c was first transformed (23) with pRUL551 and subsequently with 50 μg of each of the libraries Y2HL-C1, Y2HL-C2, and Y2HL-C3. Transformants with an activated *HIS3* reporter were selected on MY medium supplemented with adenine (20 μg/ml), lysine (30 μg/ml), and 20 mM 3-aminotriazole and subsequently assayed for β-galactosidase activity. Library plasmids were isolated from His^+ β-$galactosidase^+$ transformants by amplification in *E. coli* after selection for ampicillin resistance.

Purification of Bmh1p and Bmh2p—His_6-tagged recombinant Bmh1p and Bmh2p were purified from *E. coli* XLI containing pRUL526 and pRUL527, respectively. Cells were grown to an A_{620} of 0.25 in TB supplemented with ampicillin. After addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, the cells were grown for an additional 2.5 h to an A_{620} of 0.82. Two grams (wet weight) of cells were harvested by centrifugation, resuspended in 15 ml of 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl, 1 mg/ml lysozyme, 10 μg/ml RNase A, and 10 μg/ml DNase I and incubated for 30 min at 0 °C. Then Triton X-100 was added to a final concentration of 0.1%, and the cells were incubated on ice for an additional 10 min. The lysate was cleared by two centrifugation steps at 13,000 × *g* for 10 min at 4 °C. One ml of nickel-nitrilotriacetic acid-agarose suspension (Qiagen) was added to the lysate, and after a 90-min incubation at 4 °C under continuous stirring the mixture was poured into a column. The column was washed with 100 ml of 50 mM phosphate buffer, pH 7.8, 300 mM NaCl, at 20 ml/h, followed by washing with 55 ml of Buffer B (50 mM phosphate buffer, pH 6.5, 300 mM NaCl, 10% glycerol). The His_6-tagged proteins were eluted with a 40-ml 0–500 mM gradient of imidazole in Buffer B. Fractions containing the purified His_6-Bmh1p or His_6-Bmh2p were dialyzed against 50 mM phosphate buffer, pH 7.5, 50% glycerol, 300 mM NaCl. Protein concentrations were determined by Bradford analysis (24), and purity was estimated by SDS-PAGE followed by Coomassie and silver staining. The purified proteins were stored at −80 °C.

Purification of His_6-Fin1p—*S. cerevisiae* strain GG3100 was grown

TABLE II
Oligonucleotide sequences

MH-1	GTCAAGCTTCGAGTTC[a]
MH-2	CTCGAAGCTTGACTTC
MH-3	CGGGATCCAAATGTCCCAAACTCGT
MH-4	GCGTCGACCCCCTTGTATTTCTCAG
MH-5	AGCTAAAATGAGAGGTTCTCATCACCATCACCATCACAGATCTCACGTGTTGGCCATCCCGGG
MH-6	GATCCCCGGGATGGCCAACACGTGAGATCTGTGATGGTGATGGTGATGAGAACCTCTCATTTT
MH-7	CGGGATCCATGAGCAATAAAAGCAACCG
MH-8	ACGCGTCGACCTTGCTTACTTCCTGTGTAA
MH-9	AATTCGTGGAACTTAAAGAAATAAAGGACTTGCTACTACAAATGTTGAGAAGACAGCGAGAGA TTGAATCAAGATTATCCAATATCGAACTTCAACTCACGGAAATACCGAAACATAAGTAACTGCA
MH-10	GTTACTTATGTTTCGGTATTTCCGTGAGTTGAAGTTCGATATTGGATAATCTTGATTCAATCTC TCGCTGTCTTCTCAACATTTGTAGTAGCAAGTCCTTTATTTCTTTAAGTTCCACG
MH-11	AATTCATGTTGAGAAGACAGCGAGAGATTGAATCAAGATTATCCAATATCGAACTTTAAGCTTGTGCA
MH-12	CAAGCTTAAAGTTCGATATTGGATAATCTTGATTCAATCTCTCGCTGTCTTCTCAACATG
MH-13	GGTGATGGTTCGTTAACGAG
MH-14	GAAGATCTAAGCTTAGGTTTCTTCAGTCACTATATTAT
FIN1-B	GAAGATCTTTACTTATGTTTCGGTATTTCC
FIN1-G	CCATCGATGGAATGAAGCATAGTATA
FIN1-K	CCATCGATAAGACAGATGGAATGAAGCAT

[a] All sequences are given 5′ to 3′.

in 500 ml of MY medium to an A_{620} of 0.8. Cells were harvested, washed, and cultured for 16 h in 500 ml of MYZ medium supplemented with 1% galactose to induce the expression of the recombinant protein. Cells were harvested by centrifugation, and the pellet (3.8 g) was vortexed with 4 g of glass beads (600 μm; Sigma) in 40 ml of lysis buffer (8 M urea, 50 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml chymostatin, 1 mM ϵ-aminocaproic acid, 1 μg/ml E-64, 1 μg/ml leupeptin, 2 μg/ml aprotonin, and 1 mM Na_3VO_4. Cell debris was removed by centrifugation for 10 min at 13,000 × *g*, and the pH of the clear supernatant was adjusted to 8.0 with NaOH. The extract was incubated with 500 μl of nickel-nitrilotriacetic acid-agarose (Qiagen) for 16 h at room temperature. All washing and elution steps were performed in spin columns. The nickel-nitrilotriacetic acid-agarose was washed six times with 0.5 ml of lysis buffer, 10 times with 0.5 ml of lysis buffer containing 10 mM imidazole, and single times with 0.5 ml of lysis buffer containing 20, 30, and 40 mM imidazole. His_6-Fin1p was eluted with 0.5 ml of lysis buffer containing 400 mM imidazole. The eluted protein was dialyzed against 50 mM phosphate buffer, pH 7.5, 500 mM NaCl, 50% glycerol at 4 °C. Alternatively, the protein was stored at −20 °C in the 8 M urea-containing elution buffer. The protein concentration was determined with the Bradford assay, and purity was estimated by SDS-PAGE and Coomassie staining.

Enzyme-linked Immunosorbent Assay (ELISA)—Microtiter plates (Flow Laboratories) were coated overnight at 4 °C with varying amounts of purified His_6-Fin1p or cytochrome *c* (Sigma) in 50 μl of PBS (50 mM phosphate buffer, pH 7.5, 150 mM NaCl). After washing with 200 μl of PBS, the wells were blocked with 200 μl of 1% blocking reagent (Roche Molecular Biochemicals) in PBS for 90 min at room temperature. When desired, bound Fin1p was dephosphorylated by incubation with 100 units of λ protein phosphatase (New England Biolabs) in 100 μl of λ protein phosphatase buffer for 60 min at 30 °C. Controls contained heat-inactivated protein phosphatase (10 min at 100 °C in the presence of 100 mM EDTA, pH 8.0). After five washes with 200 μl of PBST (PBS, 0.5% blocking reagent, 0.05% Tween 20), the wells were incubated with 50 μl of PBS containing the indicated amounts of purified His_6-Bmh1p or His_6-Bmh2p for 1 h at room temperature. The wells were washed five times with 200 μl of PBST, and 100 μl of anti-Bmh1 antiserum (13) (1:2000) in PBST was added followed by a 1-h incubation at room temperature. After washing with PBST, goat anti-rabbit IgG alkaline phosphatase conjugate (Promega) and nitrophenylphosphate were used to detect bound antiserum. Bound Fin1p was detected by a similar procedure except that anti-$RGSH_4$ antibody (Qiagen) and anti-mouse/rabbit IgG-peroxidase (Roche Molecular Biochemicals) were used.

Surface Plasmon Resonance Measurements—Surface plasmon resonance analysis was done with a BIAcore-3000. 2000 resonance units of Fin1p were coupled to a CM5 sensor chip (BIAcore) by the *N*-hydroxysuccinimide/*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (NHS/EDC) method (25). Fin1p was dialyzed against 2.5 mM phosphate buffer, pH 7.5, 25 mM NaCl. The reference channel was made by activating and deactivating the surface without coupling a protein. Measurements were done in HBS buffer (BIAcore) at 25 °C at a flow rate of 30 μl/min. Each run consisted of an association step of 5 min using either His_6-Bmh1p or His_6-Bmh2p dissolved in HBS, followed by a dissociation step with HBS buffer. Between runs the chip was regenerated with 10 mM glycine, pH 10. Analysis of kinetic data was performed with the BIAevaluate 3.0 software (BIAcore) using the 1:1 (Langmuir) binding fitting model. Simulations with experimentally found kinetic parameters were done with BIAsim (BIAcore) and Clamp software (26).

FRET Analysis—Yeast cells were cultured in MY medium, and exponentially growing cells were analyzed using a Zeiss Axiovert 135 TV microscope (Zeiss, Jena, Germany), equipped with a 100-watt mercury lamp and a ×100/NA 1.30 Plan Neofluar phase objective. YFP was detected with a filter set consisting of a 500/20-nm bandpass filter, a 515-nm dichroic mirror, and a 535/30-bandpass emission filter. For FRET images the emission filter of the YFP filter set was used in combination with the CFP excitation filter and dichroic mirror. CFP was detected with a filter set consisting of a 436/20-nm bandpass excitation filter, a 455-nm dichroic mirror, and a 480/20-nm bandpass emission filter (all from Chroma Technologies). Images were acquired using a cooled CCD camera (Princeton Instruments), in the sequence YFP-, FRET-, and CFP-image (27). Typical exposure times were 0.2 s. The set of three images was corrected for background and, when necessary, for pixel shift. Overlap from the CFP and YFP into the FRET image was corrected at each pixel. Images were further processed with in-house software. For calculation of relative FRET values the formula, $FRET_C = (I_{FRET} - a * I_{CFP} - b * I_{YFP})/I_{CFP}$, essentially as described (28), was used, where $FRET_C$ is the corrected FRET value, and I_{FRET}, I_{CFP}, and I_{YFP} are intensities measured through FRET, CFP, and YFP filter sets, respectively. *a* and *b* are bleed-through percentages measured in cells only expressing CFP or YFP. Calculated $FRET_C$ values are displayed in pseudocolor.

RESULTS

Identification of Fin1p as a Binding Partner for Bmh2p—To identify binding partners of the yeast 14-3-3 protein Bmh2 we made use of the two-hybrid system. To facilitate analysis of positive clones, we constructed "bait" and "prey" vectors with a different selection marker. Therefore, we replaced the ampicillin resistance marker of bait vectors pGBD-C1, pGBD-C2, and pGBD-C3 (22) by the kanamycin resistance marker. Then, we constructed plasmid pRUL551 encoding a fusion between Bmh2p and Gal4bd. This construct was able to complement a lethal *bmh1 bmh2* disruption, indicating that the fusion protein has retained (at least) its essential 14-3-3 function (data not shown). pRUL551 alone already activated the *HIS3* reporter of HF7c, but this could be suppressed by addition of 20 mM 3-aminotriazole. Of 22×10^6 transformants, five had both an active *HIS3* and β-galactosidase reporter.

We isolated plasmids from the His^+ β-galactosidase$^+$ transformants, and after transformation of *E. coli*, colonies were selected on medium supplemented with ampicillin. None of these *E. coli* colonies contained the bait plasmid pRUL551. In two other two-hybrid screens using bait plasmids with a kana-

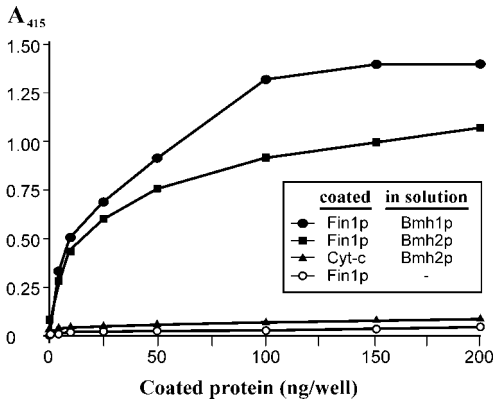

FIG. 1. **ELISA to study the binding of Bmh1p and Bmh2p to Fin1p.** Increasing amounts of purified His_6-Fin1p or cytochrome *c* (▲) were coated to the wells of a microtiter plate. After blocking and washing, the wells were incubated with a solution containing 2 μg Bmh2p (■ and ▲), 2 μg Bmh1p (●), or no protein (○). The amount of 14-3-3 protein retained in the wells after washing was determined immunologically (A_{415}).

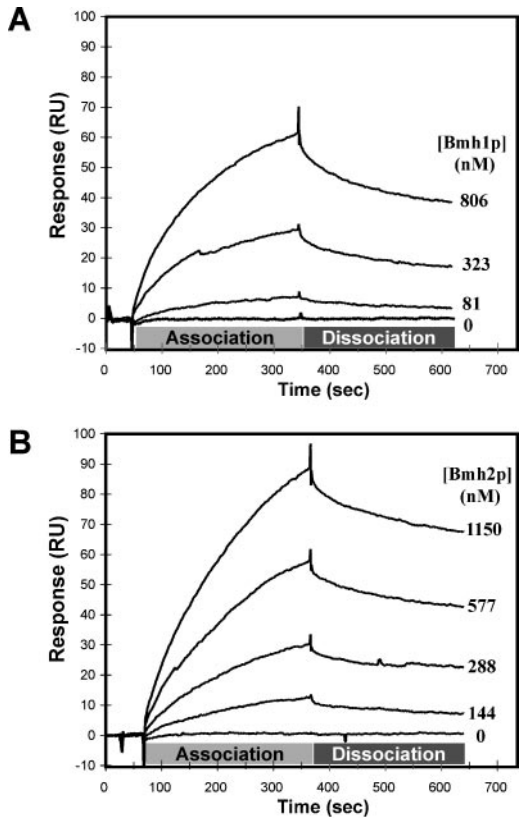

FIG. 2. **Overlay plots of sensograms of Bmh1p (*A*) or Bmh2p (*B*) binding to immobilized Fin1p during surface plasmon resonance analysis.** Two thousand resonance units of purified Fin1p were immobilized to a CM5 chip. Solutions containing the indicated concentrations of Bmh1p or Bmh2p were applied to allow binding (association). For dissociation buffer without protein was used.

mycin marker we did not isolate the bait plasmid either (data not shown), indicating the power of the use of different selection markers in the bait and prey plasmids. Cotransformation of HF7c with each of the five isolated library plasmids and pRUL551 resulted again in His^+ transformants with β-galactosidase activity. Cotransformation of HF7c with the isolated library plasmids and the empty vector pGBDK-C1 yielded His^- transformants without β-galactosidase activity. This demonstrates that the activation of the reporter genes of HF7c in these five positives is dependent on the presence of both pRUL551 and the library plasmid. Sequencing of the yeast DNA insert of one of the isolated plasmids revealed an in-frame fusion between the *GAL4*ad and the 3′-end of *GCR2*, encoding amino acids 223 to 534 of the Gcr2 protein. *GCR2* encodes a transcription factor required for full activation of glycolytic genes. Three other plasmids contain *SPT21*. However, for *SPT21* there is not an in-frame fusion with the *GAL4* activating domain. The last plasmid contains an in-frame fusion between the *GAL4*ad and *YDR130c*, lacking the first 30 base pairs. As the protein encoded by this open reading frame forms cell cycle-specific filaments, we named this open reading frame *FIN1* (filaments in between nuclei 1) (1). The reverse combination, *BMH2* fused to the *GAL4*ad and *FIN1* fused to *GAL4*bd, also reacts positively in the two-hybrid system (data not shown). We studied the interaction between Fin1p and the yeast 14-3-3 proteins in more detail.

In Vitro Interaction between the Yeast 14-3-3 Proteins and Fin1p—To confirm the interaction between the 14-3-3 proteins and Fin1p found in the two-hybrid system, we used a sandwich ELISA with purified proteins. To this end we isolated recombinant His_6-tagged Bmh1 and Bmh2 proteins from *E. coli* and His_6-tagged Fin1p from yeast (see "Materials and Methods"). Various amounts of purified Fin1p (0–200 ng) were immobilized to the wells of a microtiter plate. After blocking and washing, these wells were incubated with Bmh1p or Bmh2p, and the amount of Bmh protein bound was determined immunologically using our anti-14-3-3 protein antiserum (13). Fig. 1 shows that increasing the amount of immobilized Fin1p resulted in increased binding of Bmh1 and Bmh2p (● and ■, respectively). In the absence of a Bmh protein, no signal was observed (Fig. 1, ○). The apparent stronger binding of Bmh1p in comparison to Bmh2p is not caused by a higher affinity of Bmh1p for Fin1p (as will be discussed later) but is because of the higher affinity of the anti-14-3-3 antiserum for Bmh1p. As Fin1p is a highly basic protein, whereas the 14-3-3 proteins are acidic, we also performed an ELISA experiment in which we used another basic protein, cytochrome *c*, instead of Fin1p. As shown in Fig. 1 (▲), Bmh2p does not bind to cytochrome *c*. Similar results were obtained for Bmh1p (data not shown), demonstrating that there is a specific *in vitro* interaction between the yeast 14-3-3 proteins and Fin1p.

Surface Plasmon Resonance Analysis—The dissociation constants of the Fin1p-Bmh1p and Fin1p-Bmh2p interactions were determined by surface plasmon resonance analysis. To this end 2000 resonance units of Fin1p were covalently immobilized to the surface of a CM5 sensor chip, and solutions with various concentrations of Bmh1p or Bmh2p were injected over the chip. A relatively fast binding of Bmh1p or Bmh2p to Fin1p was observed, whereas dissociation was relatively slow (Fig. 2). Dissociation was not affected by addition of 2 M NaCl, 2 M KCl, or 2 M LiCl to the buffer, indicating that the Bmh-Fin1p interaction is resistant to high salt concentrations (data not shown). Using the 1:1 Langmuir binding model, we calculated for Bmh1p an association rate constant (k_a) of 1.85×10^3 M^{-1} s^{-1} and a dissociation rate constant (k_d) of 1.08×10^{-3} s^{-1} and for Bmh2p a k_a of 2.57×10^3 M^{-1} s^{-1} and a k_d of 7.44×10^{-4} s^{-1}. In these calculations we included our observation that the recombinant Bmh proteins form dimers (data not shown). These data resulted in a dissociation constant (K_D) of 585 nM for the Fin1p-Bmh1p interaction and of 289 nM for the Fin1p-Bmh2p interaction, indicating that Bmh2p has a higher affinity than Bmh1p for Fin1p. Simulated curves obtained by using the found dissociation constants matched with the experimental curves.

Effect of Dephosphorylation on the Bmh2p-Fin1p Interaction—Western blot analysis with an antibody recognizing phos-

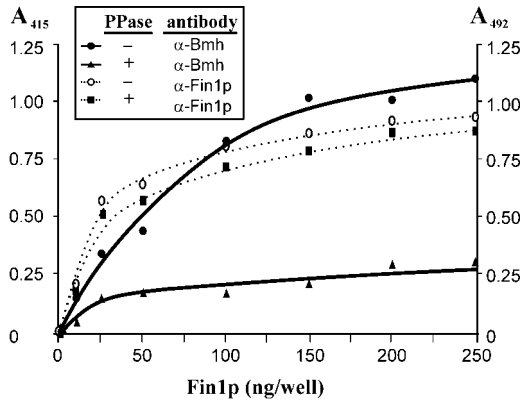

FIG. 3. **ELISA to study the effect of phosphatase treatment of Fin1p on the interaction with Bmh2p.** Increasing amounts of Fin1p were immobilized to the wells of a microtiter plate. Two series of wells were treated with either active (▲ and ■) or inactivated (● and ○) λ protein phosphatase. After blocking and washing, both series of wells were incubated with a solution containing 1 μg of Bmh2p. The amounts of Bmh2p bound to Fin1p (●) and to dephosphorylated Fin1p (▲) were determined immunologically using anti-14-3-3 protein antiserum (A_{415}). To determine the effect of phosphatase treatment on the amount of Fin1p present in the well, two other series of wells containing immobilized Fin1p were treated with inactivated (○) and active (■) protein phosphatase. Subsequently the amount of Fin1p was determined immunologically using anti-$RGSH_4$ antibody (A_{492}).

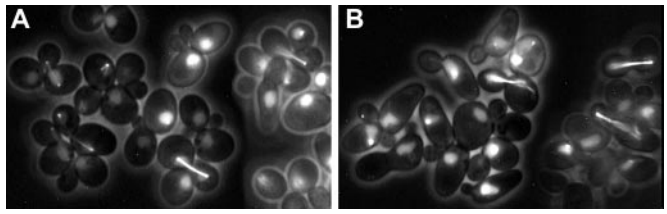

FIG. 4. **Fluorescence microscopy of F3α (*BMH1 BMH2*) (*A*) and RR1249 (*bmh1 bmh2*) (*B*) cells containing plasmid pUG36[*FIN1*] expressing GFP-Fin1p.**

phorylated serine, threonine, and tyrosine residues suggested that Fin1p is a (partly) phosphorylated protein (data not shown). To study the role of Fin1p phosphorylation in the interaction with the 14-3-3 proteins, increasing amounts of Fin1p (0–250 ng) were immobilized to the wells of a microtiter plate, and the immobilized Fin1p in one series of wells was treated with the nonspecific λ protein phosphatase. As a control, the other series of wells was incubated with inactivated protein phosphatase. As shown in Fig. 3 (●), Bmh2p binds to Fin1p treated with inactive phosphatase in a similar way as untreated Fin1p. However, treatment of Fin1p with protein phosphatase resulted in a strong reduction in Bmh2p binding (Fig. 3, ▲). The reduced binding of Bmh2p to Fin1p after dephosphorylation was not caused by a decrease in the amount of immobilized Fin1p, as the amount of immobilized Fin1p was not affected by the protein phosphatase treatment (Fig. 3, ○ and ■). Similar results were obtained with Bmh1p (data not shown). In addition, during surface plasmon resonance analysis dephosphorylation of Fin1p resulted in a strong reduction (~60%) of the Bmh2p binding (data not shown).

Fin1p Filament Formation in Cells Deficient in 14-3-3 Proteins—The Fin1 protein forms cell cycle-specific filaments between the spindle pole bodies of dividing yeast cells. To study the role of the 14-3-3 proteins in Fin1p filament formation we made use of the observation that in Σ1288-derived strains the 14-3-3 proteins are not essential (17). To this end the *bmh1 bmh2* strain RR1249 and the isogenic control strain F3α were transformed with plasmid pUG36[*FIN1*] encoding an N-terminal GFP fusion of Fin1p. As shown in Fig. 4, in both strains fluorescent filaments can be seen. In theory it is possible that in the Σ1288-derived strains the loss of 14-3-3 proteins is compensated by an unknown activity that replaces the 14-3-3 proteins during Fin1p filament formation. Therefore, we also looked at Fin1p filament formation in a strain in which *BMH1* and *BMH2* are replaced by a temperature-sensitive *bmh2* allele (19). Both at the permissive and restrictive temperature, filaments formed by GFP-Fin1 were observed (data not shown). Also in a strain in which both *BMH1* and *BMH2* are replaced by cDNA encoding a plant 14-3-3 protein isoform under control of the *GAL1* promoter (13), GFP-Fin1 filaments were observed after growth on medium with glucose as sole carbon source resulting in undetectable levels of 14-3-3 proteins (data not shown). These results indicate that the 14-3-3 proteins are not essential for Fin1p filament formation *in vivo*, in agreement with our previous observation that purified Fin1p forms filaments *in vitro* in the absence of other proteins.

Self-association of Fin1p—Transformation of reporter strain HF7c with plasmids pRUL348 and pRUL558 encoding fusions between of Fin1p (amino acids 11–291) with Gal4ad and Gal4bd, respectively, yielded transformants that were able to grow on medium without histidine and that displayed β-galactosidase activity (data not shown). In addition, during a two-hybrid screen for interaction partners of Fin1p, we isolated several clones containing a *GAL4*ad-*FIN1* fusion (1). Therefore, Fin1p is able to interact with itself in the two-hybrid system. This observation is in agreement with our previous observation (1) that purified His_6-Fin1p self-assembles into 10-nm filaments after dialysis against low salt buffers.

Mapping of the Fin1p Domains Responsible for Self-association and Bmh2p Binding—To map the regions of Fin1p that are involved in self-association and in the binding to the 14-3-3 proteins, plasmids were constructed that expressed in-frame fusions of *GAL4*ad with various parts of *FIN1* (Fig. 5). The N terminus of Fin1p up to amino acid 253 could be deleted without affecting the interaction with Gal4bd-Fin1p (Fig. 5). On the other hand, deletion of the C-terminal part of Fin1p completely abolished the interaction with Gal4bd-Fin1p (Fig. 5). A fusion containing amino acids 266–282 was unable to interact with Gal4bd-Fin1p. In contrast, a Gal4ad fusion protein containing amino acids 254–280 of Fin1p (pRUL369) was able to interact with both Gal4bd-Fin1p and with Gal4bd fused to amino acids 254–280 of Fin1p. These data indicate that amino acids 254–280 of the C-terminal part of Fin1p are required for self-association and that this region alone is sufficient for this self-association. This region contains one of the two putative coiled-coil regions present in the Fin1 protein, suggesting that this coiled-coil region is involved in the self-association. The interaction with Bmh2p was also completely abolished after deletion of the C-terminal sequences of Fin1p. The interaction was not disturbed after deletion of the first 56 amino acids from the N terminus of Fin1p but was abolished after deletion of the first 58 amino acids from the N terminus, indicating that sequences in this part of the Fin1 protein are involved in 14-3-3 binding. This part of the protein does not contain sequences resembling known 14-3-3 binding sequences. As 14-3-3 binding to Fin1p was affected by dephosphorylation, we replaced the threonine residue at position 58 by a valine residue. However, binding to 14-3-3 still occurred (data not shown). Our results suggest that both sequences around residue 58 and the C-terminal part of Fin1p are required for association with Bmh2p. Alternatively, association with Bmh2p might require a dimeric Fin1p. In that case, deletion of the C terminus disrupts the Fin1p self-association, and deletion of sequences around residue 58 might remove the Bmh2p-binding domain.

FRET between CFP- and YFP-tagged Fin1 Proteins—FRET

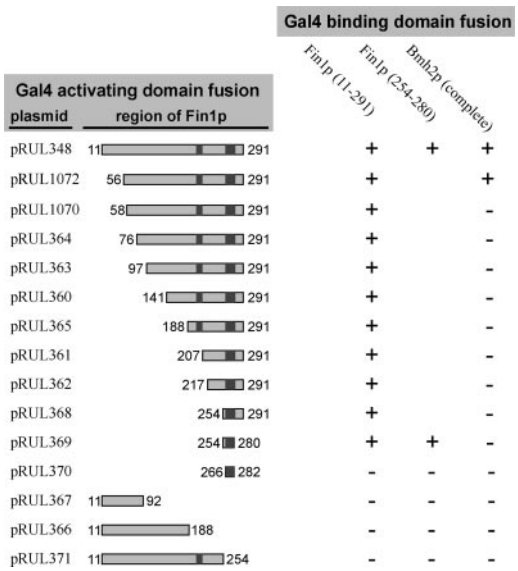

FIG. 5. **Mapping of the Fin1p domains responsible for self-association and interaction with Bmh2p using the two-hybrid system.** Various parts of Fin1p were expressed as Gal4ad fusion proteins. These were tested for interaction with Gal4bd fusions containing amino acids 11–291 of Fin1p (plasmid pRUL558), amino acids 254–280 of Fin1p (pRUL372), or full-length Bmh2p (pRUL551). A positive interaction, *i.e.* when a combination of bait and prey plasmid activated both the *HIS3* and β-galactosidase reporter genes in strain HF7c, is indicated with a plus sign (+). Double transformants negative for both reporter genes are indicated with a minus sign (−). *Dark boxes* in Fin1p indicate predicted coiled-coil regions.

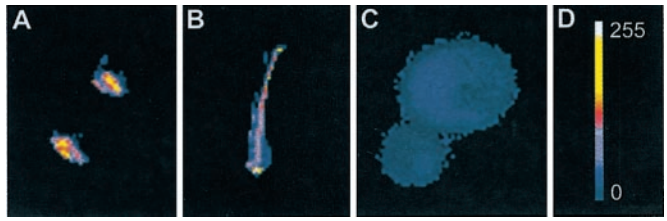

FIG. 6. **FRET between CFP-Fin1p and YFP-Fin1p in intact yeast cells.** Corrected FRET values (as described under "Materials and Methods") are displayed in pseudocolor. *A*, GG3036 cells, expressing CFP-Fin1p and YFP-Fin1p, both localized in the spindle pole bodies; *B*, GG3036 cells, expressing CFP-Fin1p and YFP-Fin1p, both localized in a filament between the spindle pole bodies; *C*, GG3087 cells, expressing free CFP and free YFP, both localized mainly in the cytoplasm; *D*, relationship between color and pixel value.

TABLE III
Dissociation constants (K_D) for the interactions between 14-3-3 proteins and a number of binding partners

Binding partner	K_D	Reference
	nM	
Tyr-hydroxylase complete	3	42
H^+-ATPase AHA2 peptide	88	43
MEKK3 N-terminus	90	44
Raf-based peptides	122–145	7
Interleukin-3 β chain peptide	152	45
Fin1p-Bmh2p	289	This study
Trp-hydroxylase	450	46
Fin1p-Bmh1p	585	This study
Tyr-hydroxylase peptide	81000	42

measurements were used to study the self-association of Fin1p in the intact yeast cell. To this end strain GG3066 was constructed expressing both CFP-tagged Fin1p and YFP-tagged Fin1p. Both labeled Fin1 proteins behaved similarly as we reported for GFP-Fin1p (1). In the dividing cell shown in Fig. 6*A*, both CFP-Fin1p as YFP-Fin1p were present in the spindle pole bodies. After correction for the spectral overlap of CFP and YFP, a clear energy transfer from the CFP-tagged Fin1p to the YFP-tagged Fin1p could be observed in the spindle pole bodies. In the dividing cell shown in Fig. 6*B*, both CFP-Fin1p and YFP-Fin1p were present in a filament between the spindle bole bodies. Again energy transfer was observed, although less than in the spindle pole bodies. No significant energy transfer was observed in cells expressing both free CFP and YFP (Fig. 6*C*).

DISCUSSION

In this study we performed a two-hybrid screen to identify interaction partners of the Bmh2 protein. One of the binding partners was the Fin1 protein. While the manuscript was in preparation interaction between Fin1p and the yeast 14-3-3 proteins has also been reported by others (29). Fin1p is a 291-amino acid protein of unknown function with a pI of 10 that contains two putative coiled-coil regions in the C terminus. The protein is ~45% similar to myosins and tropomyosins from various organisms. In addition, it shares ~45% similar residues with parts of the kinesin-like proteins Kar3p from *S. cerevisiae* and human CENP-E and CENP-F. We showed recently (1) that Fin1p form cell cycle-specific filaments between the spindle poles of dividing cells. *In vitro*, the protein can form 10-nm filamentous structures resembling the filaments formed by other intermediate filament proteins. Here, we showed that in the two-hybrid system sequences at the C terminus of the Fin1 protein containing one of the two putative coiled-coil regions are sufficient for self-association (Fig. 5). In addition, we showed FRET between CFP-Fin1p and YFP-Fin1p in intact in yeast cells (Fig. 6). The function of the Fin1p filaments is still unknown. However, in the two-hybrid assay Fin1p interacts not only with the 14-3-3 proteins and Fin1p itself but also with Reb1p, Fir1p, and Wss1p (1). Both the Reb1 and Fir1 proteins are involved in RNA metabolism. The Reb1 protein is a DNA-binding protein required for termination of RNA polymerase I transcription (30, 31), and the Fir1 protein (32) has been identified as a factor interacting with the Ref2 protein, which is involved in 3′ mRNA processing (33). Wss1p is a weak suppressor of a *SMT3* mutation, a gene that may be involved in the function and/or structure of the kinetochore (34). In a large scale search for protein-protein interactions the Fin1 protein was found to interact with the Glc7 protein phosphatase (35).

The yeast *S. cerevisiae* is widely used as model organism for studies on cellular processes. We use this yeast to elucidate the function of the 14-3-3 proteins. 14-3-3 proteins are present in all eukaryotic organisms investigated, and more than 120 interaction partners have been identified (for review see Refs. 2–6). These interaction partners belong to many different classes of proteins, such as protein kinases, protein phosphatases, receptors, transcription factors, filament-forming proteins, and enzymes. In this study we identified the filament-forming Fin1 protein as an interaction partner of the yeast 14-3-3 proteins. Previously, the protein kinase Ste20p and Yak1p and the transcription factors Msn2p, Msn4p, and Rtg3p have been identified as interaction partners for the yeast 14-3-3 proteins, whereas a large scale analysis of yeast protein-protein interactions (35) revealed interactions with the extracellular membrane protein Ecm13p, with the inositolhexaphosphate kinase Kcs1p and with Bop3p, overexpression of which can bypass mutations in the phosphatase suppressor gene *PAM1*. Thus also in the relatively simple yeast cell the 14-3-3 proteins have different interaction partners, belonging to different classes of proteins.

In a large number of proteins interacting with 14-3-3 proteins specific binding motifs have been identified. Many of these motifs contain a phosphorylated serine residue flanked by a proline and arginine residue (7–9). The Fin1 protein does

not contain any of these phosphoserine-containing motifs, nor does it contain any of the previously defined 14-3-3 binding motifs lacking a phosphorylated serine residue (10, 11). However, phosphorylation of the Fin1 protein is involved in 14-3-3 binding. Deletion studies suggested that both sequences around residue 58 and in the C-terminal part of Fin1p are required for association with Bmh2p. These results may imply that multiple sites are responsible for 14-3-3 binding, that a dimeric Fin1 protein is required, or that a truncated Fin1 protein does not fold correctly.

Using surface plasmon resonance analysis we have determined the dissociation constants of the Fin1p-Bmh1p and Fin1p-Bmh2 interactions. These dissociation constants are in the same range as those published for other 14-3-3 binding partners (Table III). We showed that Bmh2p has a higher affinity than Bmh1p for Fin1p. However, Bmh1p and Bmh2p are for 97% identical over the first 250 amino acids. Therefore, any isoform specificity probably resides in the divergent C termini. The physiological significance of this difference in affinity remains to be elucidated. In the yeast cell the difference in affinities might be counterbalanced by an ~5-fold higher Bmh1p concentration than Bmh2p concentration.[2] In literature mainly qualitative examples of isoform specificity can be found. Muslin *et al.* (7) found little differences in the affinity constants for the interaction between a number of 14-3-3 isoforms (ζ, η, β, and τ) and a Raf-based peptide. The latter study was performed with short peptides, and we used a complete protein. Besides a 14-3-3 binding motif, other parts of the protein might be involved in modulating the interaction (36).

In this study we showed that the yeast 14-3-3 proteins interact with the intermediate filament protein Fin1p. Interactions between the 14-3-3 proteins and other intermediate filament or cytoskeleton proteins have been reported (37–39). In keratinocytes the keratin K8/K18 forms intermediate filaments in a cell cycle-regulated way (40, 41). The 14-3-3 proteins keep the keratin in a soluble form. The yeast 14-3-3 proteins may have a similar function in Fin1p filament formation. As in Σ1288-derived strains filament formation was not affected by the absence of 14-3-3 proteins, and the 14-3-3 proteins are not essential for filament formation (Fig. 4).

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Self-association of the Spindle Pole Body-related Intermediate Filament Protein Fin1p and Its Phosphorylation-dependent Interaction with 14-3-3 Proteins in Yeast

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