Structural and Functional Dissection of the Abp1 ADFH Actin-binding Domain Reveals Versatile In Vivo Adapter Functions[□]

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Submitted January 24, 2005; Revised April 11, 2005; Accepted April 25, 2005
Monitoring Editor: Randy Schekman

Abp1 is a multidomain protein that regulates the Arp2/3 complex and links proteins involved in endocytosis to the actin cytoskeleton. All of the proposed cellular functions of Abp1 involve actin filament binding, yet the actin binding site(s) on Abp1 have not been identified, nor has the importance of actin binding for Abp1 localization and function in vivo been tested. Here, we report the crystal structure of the *Saccharomyces cerevisiae* Abp1 actin-binding actin depolymerizing factor homology (ADFH) domain and dissect its activities by mutagenesis. Abp1-ADFH domain and ADF/cofilin structures are similar, and they use conserved surfaces to bind actin; however, there are also key differences that help explain their differential effects on actin dynamics. Using point mutations, we demonstrate that actin binding is required for localization of Abp1 in vivo, the lethality caused by Abp1 overexpression, and the ability of Abp1 to activate Arp2/3 complex. Furthermore, we genetically uncouple ABP1 functions that overlap with SAC6, SLA1, and SLA2, showing they require distinct combinations of activities and interactions. Together, our data provide the first structural and functional view of the Abp1–actin interaction and show that Abp1 has distinct cellular roles as an adapter, linking different sets of ligands for each function.

INTRODUCTION

Abp1 is a highly conserved actin-binding protein that was first identified in *Saccharomyces cerevisiae* (Drubin *et al.*, 1988) and is thought to link functions of the actin cytoskeleton to endocytosis in yeast and mammals (Qualmann and Kessels, 2002; Engqvist-Goldstein and Drubin, 2003). A role for yeast Abp1 in regulating endocytosis is suggested by direct associations of its proline-rich and Src homology 3 (SH3) domains with endocytic machinery, including Sla1, Rvs167/amphiphysin, and actin-regulating kinases Ark1 and Prk1 (Lila and Drubin, 1997; Colwill *et al.*, 1999; Cope *et al.*, 1999; Warren *et al.*, 2002). Furthermore, Abp1 localizes to cortical actin patches (Drubin *et al.*, 1988), which recent studies show are sites of endocytosis (Kaksonen *et al.*, 2003; Huckaba *et al.*, 2004). The importance of mammalian Abp1 in regulating endocytosis is supported by dominant-negative and RNA interference knockdown studies and physical interactions with dynamin (Kessels *et al.*, 2001; Mise-Omata *et al.*, 2003). Although the gene encoding *S. cerevisiae* ABP1 is nonessential (Adams *et al.*, 1993), *abp1* null mutants are synthetic lethal (lethal in combination) with mutations in three other genes that facilitate endocytosis: SAC6/fimbrin, SLA1, and

SLA2/Hip1R (Holtzman *et al.*, 1993). Thus, Abp1 has a shared role in at least one essential function in vivo. However, it has been unclear which activities and domain–ligand interactions of Abp1 are required for this cellular function(s).

Abp1 is a multidomain protein consisting of an N-terminal actin depolymerizing factor homology (ADFH) domain, two centrally located acidic motifs, a proline-rich region, and a C-terminal SH3 domain (Figure 1B). Actin filament binding by Abp1 requires its ADFH domain (Kessels *et al.*, 2000; Goode *et al.*, 2001), a module found in two other conserved families of actin binding proteins, ADF/cofilins, and twinfilins (Lappalainen *et al.*, 1998). All three proteins use ADFH domains (~20% sequence identity) to interact with actin, yet each protein has highly distinct effects on actin dynamics. ADF/cofilins bind to actin filaments and monomers, and function primarily to promote filament severing and depolymerization (Carrier *et al.*, 1997; Blanchoin and Pollard, 1999). Twinfilins have no affinity for filaments, but they tightly sequester monomers (Goode *et al.*, 1998; Vartiainen *et al.*, 2000). Abp1 binds to filaments and not monomers, yet does not affect filament dynamics (Kessels *et al.*, 2000; Goode *et al.*, 2001). Thus, the ADFH domain is a versatile module that can be adapted to interact with actin in diverse ways and to perform a range of functions.

The crystal structures of cofilin/ADF family members have been reported (Hatanaka *et al.*, 1996; Fedorov *et al.*, 1997; Leonard *et al.*, 1997; Bowman *et al.*, 2000; Pope *et al.*, 2004), and their actin-binding interfaces have been defined by mutagenesis, synchrotron footprinting, and NMR methods (Sutoh and Mabuchi, 1989; Lappalainen *et al.*, 1997;

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-01-0059>) on May 4, 2005.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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Figure 1. Crystal structure of the *S. cerevisiae* Abp1 ADFH domain and comparison to other ADFH family members. (A) Final $2F_o - F_c$ electron density map of the five-stranded mixed β -sheet contoured at 1.0σ and corresponding ribbon diagram. (B) Schematic of domain organization for the three major ADFH domain family members: Abp1, ADF/cofilin, and twinfilin. Lengths in amino acid residues are given for *S. cerevisiae* proteins. α , α -helix rich region; A, acidic motif (found in Arp2/3 nucleation-promoting factors); and P-rich, proline-rich region. (C) Comparison of the crystal structures of *S. cerevisiae* Abp1 ADFH domain (this study), *S. cerevisiae* cofilin (Fedorov *et al.*, 1997), and *Mus musculus* twinfilin N-terminal ADFH domain (Paavilainen *et al.*, 2002). Residues mutated in selected alleles of Abp1 (this study), cofilin (Lappalainen *et al.*, 1997), and twinfilin (Paavilainen *et al.*, 2002) are highlighted and color-coded; however, Paavilainen and coworkers did not assign allele numbers for twinfilin mutations. The same color is used for mutations residing at analogous positions in the three structures. The mutations shown on twinfilin disrupt G-actin binding, whereas those on cofilin and Abp1 have diverse effects on G-actin and/or F-actin binding (Table 1).

Moriyama and Yahara, 1999, 2002; Ono *et al.*, 2001; Guan *et al.*, 2002; Pope *et al.*, 2004). Electron microscopy and computational studies have led to the view that ADF/cofilins bind filamentous actin through two surfaces (the G- and F-sites) located on opposite faces of the protein (reviewed in Ono, 2003) (Figure 7). Mutations at the G-site disrupt binding to both actin monomers (G-actin) and actin filaments (F-actin). Mutations at the F-site only disrupt binding to F-actin. The structure of one twinfilin ADFH domain has been reported, and mutagenesis shows that conserved residues in the G-site are critical both for binding G-actin and localization and function of twinfilin in vivo (Paavilainen *et al.*, 2002). In

contrast, the structure of the Abp1 ADFH domain has been unknown, its actin-binding surface has not been mapped, and the importance of actin binding for Abp1 localization and function in vivo has not been tested.

Yeast Abp1 also regulates actin filament assembly through its direct effects on Arp2/3 complex (Goode *et al.*, 2001). The Arp2/3 complex is a highly conserved assemblage of seven proteins that, upon interaction with a nucleation-promoting factor (NPF), nucleates actin polymerization (Welch and Mullins, 2002). The most well studied NPFs are in the WASp/SCAR/WAVE family. Their mechanism of activation involves stabilizing Arp2/3 complex in a closed,

actin nucleation-primed conformation (Goley *et al.*, 2004; Rodal *et al.*, 2004) and binding to and presenting an actin monomer to the complex to stimulate nucleation (Welch and Mullins, 2002). Yeast Abp1 and mammalian cortactin define a novel second class of Arp2/3 NPFs, because they bind to F-actin as opposed to G-actin (Goode *et al.*, 2001; Uruno *et al.*, 2001; Weaver *et al.*, 2001). It has been postulated that they activate Arp2/3 complex by recruiting it to the sides of preexisting filaments, thereby promoting nucleation and/or stabilizing filament branches created by Arp2/3 complex. Consistent with this hypothesis, deletion of either the ADFH domain in Abp1 or the actin-binding domain in cortactin abolishes the stimulatory effects these NPFs have on Arp2/3 complex. However, the recruitment mechanism has not been tested using point mutations that disrupt actin binding without affecting other possible functions of these domains.

Here, we present the crystal structure of *S. cerevisiae* Abp1 ADFH domain and characterize its interaction with actin by mutagenesis. We show that actin binding is required for 1) Abp1 nucleation-promoting activity on Arp2/3 complex *in vitro*; 2) localization of Abp1 to actin patches *in vivo*; 3) lethality caused by Abp1 overexpression; and 4) genetic functions of *ABP1* that overlap with *SAC6* and *SLA2*, but not *SLA1*. Our data reveal that Abp1 has multiple biological functions that require distinct combinations of its activities and domain–ligand interactions.

MATERIALS AND METHODS

Crystallization, Data Collection, and Structure Solution

The ADFH domain (residues 1–141) of *S. cerevisiae* Abp1 was expressed in *Escherichia coli* and purified by conventional chromatography for crystallography. Crystals were obtained by hanging drop vapor diffusion. Protein at 10–15 mg/ml in 10 mM Tris, 50 mM NaCl, 1 mM dithiothreitol (DTT), pH 7.5, was equilibrated with 22–28% polyethylene glycol 4000, 0.2 M sodium citrate, pH 5.6, yielding crystals with typical dimensions of $0.3 \times 0.4 \times 1.0$ mm³ in a few days. Diffraction from these crystals is consistent with the monoclinic space group C2 ($a = 159.4$ Å, $b = 66.8$ Å, $c = 127.6$ Å, $\beta = 106.9^\circ$). Native and ethyl mercury phosphate (EMP) derivative data were collected at room temperature to 3.0 Å resolution on a SIEMENS X-1000 area detector coupled to a Rigaku RU-200 generator and reduced with XDS (Kabsch, 1988), resulting in R_{merge} of 7.2 and 8.4% for the native and EMP data sets, respectively. An additional high-resolution native data set to 2.1 Å was collected on beamline X9B at the National Synchrotron Light Source, Brookhaven National Laboratories (Upton, NY). Before synchrotron data collection, crystals were soaked in mother liquor containing 5% glycerol for 5–10 min and flash-cooled to 100 K by using an Oxford low-temperature system. These data were recorded on a MAR345 image plate by using a wavelength of 0.98 Å and processed with the HKL suite (Otwinowski and Minor, 1997), resulting in an R_{merge} of 6.9%. The large unit cell dimensions and relatively small size of the protein suggested that the asymmetric unit contained 6–10 copies of the ADFH domain. The heavy atom constellation was solved by isomorphous difference Patterson synthesis and heavy atom parameters were refined with MLPHARE (Otwinowski, 1991). Nonisomorphism limited the useful phase information to 6.0-Å resolution (figure of merit of 0.51), which precluded direct model building. The availability of a distant model (yeast cofilin with only ~20% sequence identity) allowed for successful structure solution but required the implementation of a novel six-dimensional search using the phased translation function (<http://russel.bioc.aecom.yu.edu/server/NYSGR.html>). The final model contained nine molecules in the asymmetric unit and was refined with XPLOR (Brünger, 1992) and CNS (Brünger *et al.*, 1998) to an R_{cryst} of 0.213 and R_{free} of 0.259 for all data between 2.0- and 2.1-Å resolution. The two N-terminal residues are not represented by electron density in any of the independent molecules. Coordinates have been deposited in the Protein Data Bank with code 1HQZ (Berman *et al.*, 2000). Full details of the structure solution and methodology will be presented elsewhere.

Yeast Strains, Cell Growth, and Plasmid Construction

Standard methods were used for DNA manipulations, growth, sporulation, tetrad dissections, and transformation of yeast (Rose *et al.*, 1989). For details on yeast strains and plasmids, see supplementary data (Tables SI and SII, respectively). A PCR-based site-directed mutagenesis approach was used to generate Abp1 mutants. The integration plasmid pABP1⁺ (*HIS3*-marked) (Lila and Drubin, 1997) was used as the PCR template to produce Lys21(AAG) to Ala(GCG) and Arg24(AGA) to Ala(GCA) for

abp1-1, Lys80(AAG) to Ala(GCG) for *abp1-2*, Lys94(AAG) to Ala(GCG) and Arg96(AGG) to Ala(GCG) for *abp1-3*, Asp122(GAC) to Ala(GCC) and Asp125(GAT) to Ala(GCT) for *abp1-4*, and Lys134(AAA) to Ala(GCA) for *abp1-5*. Each allele introduces a diagnostic restriction site, and all open reading frames were sequenced.

Plasmids for the galactose-inducible overexpression of wild-type and mutant Abp1 proteins in yeast were constructed by the “gap repair” homologous recombination method. The coding sequences of wild-type and mutagenized pABP1⁺ were amplified by PCR by using primers D (GTAAATATACCTC-TATACTTTAACGTCAGGAGAAAAAAGTATAGGATCCA) and E (TTAATTAACCGGGAGATCTACCTTGAAAAATACAAATTTCCGCGGCCG-CCTAGTTGCCCAAGACACAT) and then cotransformed with *Bam*HI- and *Not*I-digested pRS426-GAL (*URA3* marked) into an *abp1Δ* strain. The repaired plasmids pGalABP1, pGalABP1-1, pGalABP1-2, pGalABP1-3, pGalABP1-4, and pGalABP1-5 were rescued from yeast and amplified in *Escherichia coli*. The coding sequence of the *abp1ΔADFH* allele was amplified by PCR from pABP1⁺ by using primers E and F (GCGCGGGATCCATGATTCAGACTTC-CTCCAAGC). The PCR product was subcloned into the *Not*I and *Bam*HI sites of pRS426-GAL to generate pGalABP1ΔADFH. The open reading frames of all plasmids were sequenced.

HIS3 marked *abp1* alleles were integrated into the haploid yeast strain BGY022 by methods described previously (Wertman *et al.*, 1992). The insertion cassettes were obtained from wild-type and mutagenized integration plasmids (above) by digestion with *Eco*RI and transformed into BGY022 (*abp1Δ::LEU2*). Transformants were selected for growth on His[−] media and loss of ability to grow on Leu[−] media. Integration was verified by PCR analysis. Genomic DNA was isolated, and the *ABP1* locus was PCR amplified using primers A (CGCGCGGATCCTAGTTGCCCAAGACACATA) and B (GCTGCTAGTCCACTCATCT GC), flanking the integration site. The presence of the correct mutation was verified by restriction analysis of PCR products. Plasmids for expressing N-terminal green fluorescent protein (GFP)-fusions of wild-type and mutant Abp1 proteins were constructed by gap repair. Coding sequences of mutagenized pABP1⁺ were amplified by PCR by using primers A and H (TAACAGCTGCTGGGATTACACATG-CGATGGATGAAGTATACAAAGGATCCATGGCTTTGGAACCTATTG). For constructing GFP-*abp1ΔADFH*, primers A and I (TAACAGCT-GCTGGGATTACACATGGCATGGATGAAGTATACAAAGGATCCA-TTCAGACTTCCTCCAAGC) were used. The PCR products were cotransformed with *URA3*-marked pRB2139 (Doyle and Botstein, 1996) gapped by digestion with *Bam*HI and *Hind*III and selected on Ura[−] media. Plasmids were rescued, amplified in *E. coli*, and verified by restriction analysis.

Protein Purification

Yeast Arp2/3 complex (Goode *et al.*, 2001) and yeast actin (Goode, 2002) was purified as described. Rabbit skeletal muscle actin (unlabeled and pyrene labeled) was purchased from Cytoskeleton (Denver, CO). To isolate wild-type and mutant Abp1 proteins, the protease minus strain BJ2168 (Jones, 2002) was transformed with pGalABP1, pGalABP11, pGalABP12, pGalABP13, pGalABP14, pGalABP15, and pGalABP1ΔADFH, which overexpress different Abp1 proteins under control of the galactose-inducible promoter. Cultures were grown, induced, harvested, washed, and flash-frozen as described previously (Rodal *et al.*, 2002). Cells were lysed by liquid nitrogen and mechanical shearing in a blender, and a high-speed supernatant in HEK buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, and 50 mM KCl) was generated as described previously (Goode, 2002). The supernatant was applied to a Hi-trap Q fast flow 5-ml anion exchange column (AP Biotech, Piscataway, NJ). Proteins were eluted using a linear gradient of KCl (0.2–0.6 M) in HEK5 buffer (HEK plus 5% glycerol). Peak fractions containing Abp1 (eluting at 0.35–0.45 M KCl) were pooled, diluted to 0.1 M KCl in HEK buffer, and loaded onto a Mono Q (5/5) ion exchange column (Applied Biosystems, Foster City, CA). Proteins were eluted using a linear gradient of KCl (0.25–0.60 M) in HEK5. Peak fractions (eluting at 0.35–0.45 M KCl) were concentrated to 300 μl and then further purified on a Superose 12 (10/30) gel filtration column (Applied Biosystems) equilibrated in HEK5. Peak fractions were concentrated to 15–75 μM and flash frozen in liquid nitrogen.

Actin Filament Cosedimentation

Two different experiments were performed to compare the abilities of wild-type and mutant Abp1 proteins to cosediment with F-actin. In the first experiment (Figure 2C), 1 μM Abp1 was added to 3.5 μM preassembled yeast actin in F-buffer (10 mM Tris, pH 7.5, 0.7 mM ATP, 0.2 mM CaCl₂, 2 mM MgCl₂, 50 mM KCl, and 0.2 mM DTT). Reactions were incubated for 10 min at 25°C and then centrifuged for 20 min at 90,000 rpm in a TLA100 rotor (Beckman Coulter, Fullerton, CA). Pellets and supernatants were fractionated on gels, stained with Coomassie Blue, and the band intensities were quantified by densitometry. In the second experiment (Figure 2, D and E), we measured the dose-responsive binding affinities (K_d) of wild-type and mutant Abp1 proteins for F-actin. Yeast actin was polymerized as described above, stabilized with equimolar phalloidin, diluted (final reaction concentrations 0.05–4 μM F-actin), and incubated with 0.09 μM Abp1. Reactions were centrifuged as described above. Pellets and supernatants were immunoblotted using a vacuum manifold (Schleicher & Schuell, Keene, NH) and

Figure 2. Purification and actin binding affinities of mutant Abp1 proteins. (A) Alignment of the primary sequences of *S. cerevisiae* cofilin and *S. cerevisiae* Abp1 ADFH domain. Asterisks show positions of charge-to-alanine substitutions introduced in each *abp1* allele (this study) and the corresponding mutations in *cof1* alleles (Lappalainen *et al.*, 1997). (B) Coomassie-stained SDS-PAGE loaded with 300 ng each of purified mutant and wild-type Abp1 proteins. (C) Purified yeast actin was assembled into actin filaments (3 μ M), incubated with purified wild-type or mutant Abp1 protein (1 μ M), and centrifuged at high speed to pellet F-actin. Pellets and supernatants were fractionated on gels, Coomassie stained, and bands quantified by densitometry. The results were averaged from reactions performed in triplicate (error bars shown). Abp1 Δ ADFH exhibited a low level of nonspecific binding, which was subtracted from the values shown for all Abp1 proteins (wild-type and mutant). Under these reaction conditions, 73% of wild-type Abp1 copelleted with F-actin. This value was set as 100% binding for comparison to mutants. Each mutant was graphed as a percent of wild-type Abp1 binding. (D) Dose-responsive binding curves for the interactions of wild-type and select mutant Abp1 proteins with F-actin (see *Materials and Methods*): wild-type Abp1 ($\blacktriangle$), Abp1-5 ($\circ$), Abp1-2 ($\triangle$), Abp1 Δ ADFH ($\square$). (E) Dissociation constants (K_d) were derived from the binding data in D (see *Materials and Methods*). Values are the average of two independent experiments.

probed with anti-Abp1 antibodies. Enhanced chemiluminescence signals were quantified by densitometry using ImageQuant software (Applied Biosystems), and the data were graphed as percentage of Abp1 bound versus F-actin concentration. Binding curves were generated using SigmaPlot (SPSS, Chicago, IL), and K_d values were determined from the following formula:

$$P = \frac{100 [\text{Actin}]}{K_d + [\text{Actin}]}$$

where p is the percentage of Abp1 bound, and K_d is the dissociation constant.

Immunoblotting

Yeast whole cell lysates were prepared as described previously (Kushnirov, 2000). In total, 0.15 OD₆₀₀ units of cells were loaded per lane on SDS-PAGE, fractionated, and immunoblotted. Blots were probed with a 1:8000 dilution of chicken polyclonal IgY antibody raised against yeast Abp1 (Aves, Tigard, OR). The rabbit polyclonal antibody raised against yeast tubulin (1:20,000) was a gift from Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA).

Microscopy

To assess colocalization of wild-type and mutant GFP-Abp1 proteins with actin *in vivo*, an *abp1* Δ strain (BGY021) was transformed with empty vector (pRS316) or plasmids expressing GFP-Abp1 fusion proteins (pGFPABP1, pGFP Δ ADFH, pGFPABP1-1, pGFPABP1-2, pGFPABP1-3, pGFPABP1-4, and pGFPABP1-5). Cells were grown to log phase, fixed for 10 min in 70% ethanol, and stained with rhodamine phalloidin as described previously (Dewar *et al.*, 2002). GFP-Abp1 and rhodamine-actin images were captured using a Zeiss Nikon Eclipse E600 microscope equipped with CoolSNAP_{ix} charge-coupled device camera (Roper Scientific, Duluth, GA) running OpenLab software (Universal Imaging, Downingtown, PA).

Actin Assembly Kinetics

Actin assembly reactions were performed as described previously (Goode *et al.*, 2001) but by using a mixture of 1.5 μ M yeast actin and 1.5 μ M rabbit skeletal muscle actin (1% pyrene labeled). The mixture was used because yeast Arp2/3 complex has limited capacity to be activated by Abp1 in the presence of pure rabbit muscle actin (Quintero-Monzon, unpublished data). 22.5 μ M monomeric actin in G buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 0.2 mM ATP, and 2 mM MgCl₂) was mixed with 10 nM yeast Arp2/3 complex and/or 200 nM wild-type or mutant Abp1, and added immediately to 20 $\times$ initiation salts (final reaction concentrations 100 mM KCl, 0.5 mM ATP, and 2 mM MgCl₂). Actin polymerization was monitored by excitation at 365 nm and emission at 407 nm in a spectrofluorometer (Photon Technology International, Laurenceville, NJ) at 25°C. The rate of polymerization was calculated from the slope of the curves early in the reaction where they are linear.

RESULTS

The Crystal Structure of the ADFH Domain of Abp1

To understand the molecular basis of Abp1-actin interactions, we solved the crystal structure of *S. cerevisiae* Abp1 ADFH domain (Figure 1A). The structure is built around a five-stranded mixed β -sheet, in which the first four strands (β 1, β 2, β 3, and β 4) are antiparallel, with the fifth strand (β 5) running parallel to β 4. Each face of the β -sheet contacts a pair of α -helices (α 1- α 4), with the helices running in roughly the same direction as the β -strands. The nine ADFH domains in the asymmetric unit can be superimposed with root mean square (RMS) deviations of <0.5 Å for 139 α -carbons pairs.

Table 1. Data compilation for *abp1* alleles and analogous *cof1* alleles

Abp1 mutants							Cofilin mutants ^a			
<i>abp1</i> allele	mutations	F-actin binding affinity ^b	Arp2/3 activation ^c	Overexpression effects on growth ^d	Genetic interaction with <i>sac6Δ</i> ^e	Localization to actin patches ^f	Analogous cofilin allele	Growth phenotype	F-actin binding affinity	G-actin binding affinity
<i>ABP1</i>	–	+++ (0.58 μM)	+++	Lethal	None (0)	+++	<i>COF1</i>	wt	+++	+++
<i>abp1ΔADFH</i>	Δ1–143	–	–	None	SL (100)	–	–	–	–	–
<i>abp1-1</i>	K21A, K24A	++ (2.0 μM)	+	Lethal	SS/SL (40)	+++	<i>cof1-6</i>	wt	++	N/D
<i>abp-2</i>	K80A	–/+ (>10 μM)	–	None	SS/SL (85)	+	<i>cof1-16</i>	Lethal	+	++/+
<i>abp-3</i>	K94A, R96A	+	–	Partial	SS/SL (46)	+++	<i>cof1-17</i>	Lethal	+	+
<i>abp-4</i>	D122A, D125A	+++	++/+++	Lethal	SS/SL (4)	+++	<i>cof1-20</i>	Lethal	+	+
<i>abp-5</i>	K134A	+++ (0.63 μM)	–	None	SS/SL (51)	+++	<i>cof1-22</i>	ts	++	+++

N/D, not determined; ts, temperature sensitive; wt, wild type; +++, full wt activity or function; ++/+++, almost full; ++, partial; +, weak; –/+, very weak; –, undetectable.

^a Data primarily from Lappalainen *et al.* (1997). F-actin binding of Cof1–6 is from Rodal (unpublished data).

^b Data from Figure 2. K_d , where determined, in parentheses.

^c Data from Figure 6.

^d Data from Figure 5.

^e Data from Figure 4, with the combined percentage of *abp1 sac6* double mutant cells synthetic sick (SS) and synthetic lethal (SL) in parentheses.

^f Data from Figure 3.

The Abp1-ADFH domain structure exhibits the same topology as cofilin (2.1-Å RMS deviation >126 α-carbons) and twinfilin (2.3-Å RMS deviation >116 α-carbons) (Figure 1C), despite their low sequence homologies (Figure 2A). Detailed structural features of the five-stranded mixed sheet present in Abp1 are shared with cofilin and twinfilin; however, there are also small but significant differences. Cofilin possesses an additional small β-strand comprised of residues near the immediate N terminus that runs parallel to the equivalent of Abp1 β2. In Abp1 and twinfilin, the orientation of the corresponding chain segment precludes formation of this β-strand. The orientations of the four helices differ slightly in the three structures, as do the detailed conformations of some connecting loops, including the equivalents of Abp1 chain segment 24–31 between α1 and β1, segment 50–54 between β2 and α2, segment 71–80 between β3 and β4, and segment 110–113 between α3 and β5. Notably, all insertions and deletions are located within these connecting loops.

The most prominent structural difference among the three ADFH family members is in the conformation of the loop that connects β3 and β4 in Abp1 (Figure 1C). In cofilin, this loop is inclined toward the neighboring helix α4 but has no interactions with it. In Abp1, this loop is shorter than in cofilin, but it assumes approximately the same orientation and again makes no interactions with neighboring chain segments. In both Abp1 and cofilin, this loop is not well defined as judged by the quality of the electron density maps and the associated high-temperature factors. In cofilin, the bending of this loop exposes residues at the N terminus of β4 important for the F-site. Mutations at these residues (*cof1-16*) are lethal in vivo and disrupt cofilin's ability to interact with F-actin and to induce filament disassembly in vitro (Lappalainen *et al.*, 1997). In twinfilin, this loop adopts a very different orientation (toward the α3) and participates in contacts with the chain segment joining α3 and β5. Residues from this loop in twinfilin are well defined and have low-temperature factors in both independent molecules in

the crystal structure (PDB file 1M4J). In all three structures, this loop has no contacts with symmetry-related molecules.

Defining Actin Binding Surfaces on the Abp1 ADFH Domain

The structural similarity between Abp1 and cofilin suggested that they might interact with actin in a related manner. To test this possibility, we introduced point mutations in the Abp1 ADFH domain that were known to disrupt cofilin's interactions with actin (Lappalainen *et al.*, 1997). The sequences of yeast Abp1 ADFH domain and yeast cofilin are only ~20% identical (Moon *et al.*, 1993), yet many of the residues in cofilin that mediate actin binding are conserved in Abp1 (Figure 2A). We targeted those residues for mutagenesis, generating five *abp1* alleles, each with one or two charge-to-alanine substitutions. Four alleles (*abp1-2*, *abp1-3*, *abp1-4*, and *abp1-5*) are analogous to *cof1* alleles that are impaired in actin binding and cause overt phenotypes in vivo (Table 1). In addition, we generated *abp1-1*, which is analogous to *cof1-6*, an allele of cofilin with no obvious growth phenotype, but whose biochemical activities have never been tested (Lappalainen *et al.*, 1997). Our own biochemical analyses of purified Cof1-6 protein showed that it is modestly impaired in actin filament binding (Rodal, unpublished data); therefore, we investigated whether this mutation in Abp1 has similar effects (Abp1-1). Furthermore, we generated a deletion of the entire ADFH domain (*abp1ΔADFH*). Each of the six *abp1* alleles and wild-type *ABP1* was introduced into a yeast overexpression vector for purification and biochemical analyses, and into an integration vector for genetic analyses (see below).

The wild-type and mutant Abp1 proteins (without tags) were overexpressed in yeast under control of the galactose-inducible promoter and purified by multiple steps of conventional chromatography (Figure 2B). The proteins were then compared for their relative binding to a fixed concentration of F-actin in cosedimentation assays (Figure 2C).

Independently, we determined the dissociation constants (K_d) for these interactions by varying the concentration of F-actin in cosedimentation assays (Figure 2, D and E). Abp1 Δ ADFH protein failed to cosediment with actin filaments, consistent with our previous study using GST-Abp1 Δ ADFH (Goode *et al.*, 2001). Two mutants, Abp1-2 ($K_d > 10 \mu\text{M}$) and Abp1-3 ($K_d = 8.0 \mu\text{M}$), interacted weakly with F-actin compared with wild-type Abp1 ($K_d = 0.58 \mu\text{M}$). Mutations in these two alleles are located on opposite ends of the ADFH domain crystal structure, corresponding to the G- and F-sites on ADF/cofilins, respectively, and are suggested to interact with two adjacent actin subunits in a filament (Bamburg, 1999). Abp1-1 ($K_d = 2.0 \mu\text{M}$) and Abp1-5 ($K_d = 0.63 \mu\text{M}$) exhibited much more subtle defects in F-actin binding, consistent with the modest defects in F-actin binding for analogous cofilin mutants Cof1-22 and Cof1-6. Unexpectedly, Abp1-4 showed relatively normal F-actin binding, whereas the analogous cofilin mutant Cof1-20 is severely impaired in F-actin binding and is lethal *in vivo* (Lappalainen *et al.*, 1997). These data are summarized in Table 1.

Abp1 Binding to Actin Filaments Is Required for Localization *In Vivo*

Next, we addressed the requirement of actin binding for Abp1 localization to actin patches *in vivo*. Actin patches assemble at the cell cortex where membrane and membrane proteins are internalized as vesicles, which eventually fuse with endosomal sorting compartments and shed their actin coats (Kaksonen *et al.*, 2003; Huckaba *et al.*, 2004). Actin patches are comprised of filamentous actin, as demonstrated by their staining with rhodamine phalloidin, which binds specifically to F-actin (Adams and Pringle, 1984). Although purified Abp1 binds directly to F-actin *in vitro* (Kessels *et al.*, 2000; Goode *et al.*, 2001), it is unknown whether actin binding by Abp1 is required for its localization *in vivo*. Because Abp1 makes physical associations with a number of other components of actin patches, including Srv2/CAP, Rvs167, Sla1, and Arp2/3 complex (Freeman *et al.*, 1996; Colwill *et al.*, 1999; Cope *et al.*, 1999; Goode *et al.*, 2001; Fazi *et al.*, 2002), its localization might not require direct binding to actin. To test this possibility, we compared the localization of wild-type and mutant Abp1 proteins in yeast cells.

Attempts to obtain reliable immunolocalization data using our Abp1 antibodies were unsuccessful. Therefore, we expressed N-terminally tagged GFP-Abp1 wild-type and mutant fusion proteins from low copy plasmids introduced into an *abp1* Δ strain. Immunoblotting of total extracts from these cells demonstrated that wild-type and mutant GFP-Abp1 expression levels were comparable (Figure 3A). GFP-Abp1 localized to cortical actin patches, as expected, whereas GFP-Abp1 Δ ADFH was found in the cytoplasm (Figure 3B). This provides the first demonstration in any organism that the ADFH domain is required for Abp1 localization in cells. This also demonstrates that the physical interactions of the proline-rich and SH3 domains in Abp1 with other patch components are insufficient for its localization to actin patches.

Using point mutations, we next tested whether the actin binding activity of the ADFH domain specifically was required for Abp1 localization. Abp1-2, which is strongly impaired in F-actin binding *in vitro*, was mislocalized to the cytoplasm, albeit not as severely as Abp1 Δ ADFH. Three other mutants, Abp1-1, Abp1-4, and Abp1-5, which have minimal or no defect in F-actin binding *in vitro*, localized normally to actin patches. Abp1-3, which has >10-fold reduced binding affinity for F-actin *in vitro*, showed only

Figure 3. Colocalization of GFP-Abp1 proteins with yeast actin patches. An *abp1* Δ strain (BGY021) was transformed with the indicated URA3 marked GFP-Abp1 plasmids. Cells were grown to log phase in media lacking uracil. (A) Total protein samples were extracted from these cells and immunoblotted with anti-Abp1 antibodies to detect expression of GFP-Abp1 fusion proteins. Note the absence of an endogenous Abp1 band at 85 kDa in the *abp1* Δ strain. (B) In parallel, samples of these cells were chemically fixed and processed for dual imaging of GFP-Abp1 and F-actin (rhodamine phalloidin) by fluorescence microscopy.

slightly diminished localization to patches. This is consistent with Abp1-3 having less severely impaired actin binding affinity than Abp1-2, and with data showing that *abp1*-2 is

more severely compromised for *ABP1* genetic functions (below). These data indicate that mislocalization of Abp1 in vivo arises only from severe disruptions of F-actin binding (Abp1-2 and Abp1 Δ ADFH).

The ADFH Domain of Abp1 Is Required for Overlapping Genetic Functions with *Sac6* and *Sla2*, but Not *Sla1*

ABP1 has shared or overlapping genetic functions with three other genes, *SAC6*, *SLA2*, and *SLA1*. The SH3 domain of Abp1 is required for each of these shared functions (Lila and Drubin, 1997). However, it is unknown what other domains and activities of Abp1 are required. To address this, we used a plasmid shuffle assay to test the importance of the Abp1 ADFH and SH3 domains in rescuing lethality in double mutant backgrounds: *abp1* Δ *sac6*-102, *abp1* Δ *sla1*-7, and *abp1* Δ *sla2*-5 (Figure 4A). The SH3 domain was required for complementation of growth in all three backgrounds, consistent with the study mentioned above. In contrast, the ADFH domain was required only in the *abp1* Δ *sac6*-102 and *abp1* Δ *sla2*-5 backgrounds. Thus, actin binding is required for Abp1 functions that overlap with *SAC6* and *SLA2*, but not *SLA1*.

To test specifically whether actin binding is required for the functions shared by *ABP1* and *SAC6*, we combined our ADFH domain mutant *abp1* alleles with a *sac6* Δ mutation and examined genetic interactions. For these analyses, we integrated each of the six mutant *abp1* alleles at the *ABP1* locus. Immunoblotting of total extracts showed that wild-type and mutant Abp1 proteins were expressed at equivalent levels in these strains (Figure 4B). Each *abp1* haploid strain was crossed to a *sac6* Δ strain. The resulting diploids were sporulated, dissected, and their haploid progeny were serially diluted and compared for defects in cell growth at different temperatures (25, 30, 34, and 37°C). A large number of tetrads were analyzed (40–70 per cross) to maximize the chance of scoring intermediate phenotypes (Figure 4C). Consistent with the plasmid shuffle data described above, double mutant *abp1* Δ *sac6* Δ strains were inviable. The growth of *abp1*-2 *sac6* Δ strains also was highly compromised, consistent with Abp1-2 having strong defects in F-actin binding and localization to actin patches. The growth phenotypes of *abp1*-4 *sac6* Δ , *abp1*-1 *sac6* Δ , and *abp1*-3 *sac6* Δ strains were less severe and correlated with the degrees to which they are impaired in F-actin binding (Abp1-3 > Abp1-1 > Abp1-4). These data demonstrate that F-actin binding is required for Abp1 functions in vivo that overlap with *Sac6*. Unexpectedly, *abp1*-5 *sac6* Δ strains were severely compromised in cell growth, which did not correlate with the normal F-actin binding and localization of Abp1-5. This suggested that the surface mutated in Abp1-5 has a function other than actin binding (see below).

To test the requirement of F-actin binding by Abp1 for the *ABP1* *SLA2* shared function, we crossed *abp1* mutant strains to the *sla2* Δ 376–440 strain, which is synthetically lethal with *abp1* Δ (Wesp *et al.*, 1997; our unpublished data). Whereas a full deletion of *ABP1* was synthetic lethal with *sla2* Δ 376–440, the *abp1* Δ ADFH mutant was only synthetic temperature sensitive at 37°C (our unpublished data). Thus, the ADFH domain is only partially required in the *sla2* background, in contrast to being fully required for function in the *sac6* background. None of the other *abp1* alleles showed the same synthetic temperature sensitivity in the *sla2* background as Abp1 Δ ADFH, but it was difficult to reliably score partial synthetic defects.

Figure 4. Abp1 ADFH domain functions in *sac6*, *sla1*, and *sla2* backgrounds. (A) Yeast strains DDY995 (*abp1* Δ , *sla1*-7), DD996 (*abp1* Δ , *sla2*-5), DD997 (*abp1* Δ , *sac6*-102), each carrying a *URA3*-marked wild-type *ABP1* plasmid to maintain cell viability, were transformed with the following *HIS3*-marked plasmids: empty vector (pRS313), pABP1 (pDD187), pABP1 Δ SH3 (pDD188) and pABP1 Δ ADFH (pBG497). Cells were grown to saturation in His⁻ media, plated as serial dilutions on Ura⁻ or 5-fluoroorotic acid⁺ media, and grown at 25°C for 3 d. (B) Abp1 expression levels in strains with integrated *ABP1* alleles. Cells were grown to log phase, and total protein samples were immunoblotted and probed with Abp1 antibodies and tubulin antibodies (loading control). (C) Genetic interactions between *abp1* alleles and a *sac6* Δ mutation. Haploid strains carrying integrated *ABP1* alleles (Table S1) were mated with a haploid *sac6* Δ strain (DDY217). The resulting diploid strains were sporulated, tetrads were dissected, and the growth of haploid progeny was measured. The percentage of double mutant haploid progeny found to be lethal or temperature sensitive was determined for each directed cross. n, number of double mutant haploids analyzed.

The lethal Abp1 Overexpression Phenotype Requires Actin Binding and Arp2/3 Complex Interactions

Expression of wild-type Abp1 on a high copy plasmid causes about fivefold overexpression compared with endog-

Figure 5. ADFH domain contributions to the phenotype caused by Abp1 overexpression. (A) An *abp1Δ* strain (BGY021) was transformed with galactose-inducible *URA3*-marked Abp1 expression plasmids (pRB2139 and pBG498-pBG503; Table S1). Cells were grown to saturation in *Ura*[−] media, plated in 10-fold serial dilutions on *Ura*[−] media containing either glucose or galactose, and grown for 4 d at 25°C. (B) Abp1 expression levels in cells after galactose induction. Cells were grown to OD₆₀₀ = 0.1 in *Ura*[−] media containing glucose, transferred to *Ura*[−] media containing galactose, and grown for 24 h. Total protein samples were extracted from these and control cells (wild type, BGY012; *abp1Δ*, BGY021) and then immunoblotted and probed with Abp1 and tubulin antibodies (loading control).

enous Abp1, leading to impaired cell growth at higher temperatures, loss of actin polarization, and enlargement of mother cells at the expense of bud growth (Drubin *et al.*, 1988). We tested the *abp1* alleles in this overexpression assay to determine whether F-actin binding is required for the overexpression phenotype. To further sensitize the assay, we overexpressed Abp1 under control of the *GAL1/10* galactose-inducible promoter, which resulted in higher levels of overexpression, and lethality at all temperatures (Figure 5A). All constructs were overexpressed to comparably high levels relative to endogenous Abp1 (Figure 5B).

Like wild-type Abp1, Abp1-1 and Abp1-4, which have normal or only modestly impaired F-actin binding *in vitro* and normal localization to actin patches *in vivo*, were lethal when overexpressed (Figure 5A). In contrast, Abp1ΔADFH and Abp1-2 showed no obvious growth defects when overexpressed. This indicates that direct binding to F-actin is required for the Abp1 overexpression phenotype. Consistent with the observed intermediate defects in F-actin binding for Abp1-3, overexpression of this mutant caused an intermediate growth phenotype. Abp1-5, which binds normally to F-actin and localizes normally *in vivo*, failed to cause a growth phenotype when overexpressed. Thus, the Abp1 overexpression phenotype depends not only on F-actin binding but also on another function of the ADFH domain. These data are consistent with the genetic interactions between *abp1-5* and *sac6Δ* (Figure 4), which suggest that the surface mutated by Abp1-5 performs a nonactin-binding function of physiological importance.

Figure 6. ADFH domain contributions to the Arp2/3 nucleation-promoting activity of Abp1. (A) Actin (3 μM) (1% pyrene labeled) was polymerized in the presence and absence of 10 nM yeast Arp2/3 complex with and without 200 nM Abp1 proteins (wild type and mutant) as indicated. The curves are averaged from four independent reactions. (B) Bar graph showing the quantitative effects of each Abp1 protein on Arp2/3 complex. Rates of actin filament assembly in reactions from A were determined from the slopes of the curves and normalized to the effects of wild-type Abp1 on Arp2/3 complex (set at 100%). Error bars show the SD of the four reactions.

ADFH Domain Requirements for Abp1 Stimulation of Arp2/3 Complex *In Vitro*

To better understand the unexpected genetic results for Abp1-5 (Figures 4 and 5), we considered what biochemical activities might be performed by the ADFH domain other than actin binding. The only other activity of Abp1 that is known to depend on its ADFH domain is activation of the Arp2/3 complex (Goode *et al.*, 2001). Therefore, we compared the ability of purified wild-type and mutant Abp1 proteins for their ability to activate the Arp2/3 complex (Figure 6). Wild-type Abp1 and Abp1-4 activated the Arp2/3 complex with similar efficiencies. Abp1-1 partially activated the Arp2/3 complex. Abp1-2, Abp1-3, and Abp1ΔADFH had no significant ability to activate Arp2/3 complex. Thus, Abp1 activation of Arp2/3 complex is highly sensitive to partial defects in F-actin binding. For these mutants, the ability to stimulate the Arp2/3 complex correlated closely with ability to bind F-actin (Abp1 ~ Abp1-4 > Abp1-1 > Abp1-3 > Abp1-2 > Abp1ΔADFH). From these data, we conclude that F-actin binding is required for Abp1 activation of Arp2/3 complex, which supports the proposed filament recruitment mechanism.

The data in Figure 6 reveal an additional role for the ADFH domain in Arp2/3 complex regulation. Although Abp1-5 binds normally to F-actin, it shows no ability to stimulate Arp2/3 complex activity. This means that Abp1-5 uncouples the F-actin binding function of the ADFH domain from a distinct role in Arp2/3 complex activation. We tested

purified ADFH domain for direct physical interactions with Arp2/3 complex, but could detect none (Quintero-Monzon, unpublished data). Although we cannot rule out the possibility of direct interactions, we suspect a different mechanism (see *Discussion*). Regardless of the precise mechanism, these data define a new and unexpected functional site on the ADFH domain that is important for Arp2/3 complex activation and for *ABP1* function in vivo, as demonstrated by the genetic interactions between *abp1-5* and *sac6* (Figure 4) and the nonlethal overexpression phenotype of *abp1-5* (Figure 5).

DISCUSSION

Abp1 is a conserved multidomain actin binding protein that regulates actin dynamics and endocytosis. However, its precise cellular functions have been difficult to pinpoint, because Abp1 is a complex protein with numerous ligands, and its cellular functions(s) in yeast overlap genetically with at least three other genes. Here, we make two important contributions to understanding Abp1 function. First, we report the structure of the *S. cerevisiae* Abp1 ADFH domain and characterize its interactions with actin by using point mutations. Structures have been reported for the two other actin-binding proteins that contain ADFH domains (ADF/cofilins and twinfilin), so this completes the cross-family gallery and enables close comparisons of structure and function. We find similarities in how Abp1 and ADF/cofilins interact with actin, but also key differences, offering insights into how these two proteins differentially affect actin dynamics. Second, we use point mutants to demonstrate the

importance of actin binding for Abp1 localization and function in vivo. Abp1 localization to actin patches and the lethal growth phenotype caused by Abp1 overexpression both depend on F-actin binding. Furthermore, we uncouple the genetic functions of *ABP1* shared with *SAC6*, *SLA1*, and *SLA2* and show that each function requires a distinct set of Abp1 domains and/or activities (F-actin binding, Arp2/3 complex stimulation, and SH3 domain function). This indicates that Abp1 performs distinct adapter functions in vivo that depend on linking different subsets of its ligands.

Differential Actin-binding Interactions by ADFH Family Members

The ADFH domain is a versatile actin-binding module found in three conserved families of actin binding proteins, ADF/cofilins, twinfilin, and Abp1 (Lappalainen *et al.*, 1998). A side-by-side comparison of their structures (Figure 1C) shows that Abp1 most closely resembles ADF/cofilin. This is consistent with these two proteins binding to F-actin, whereas twinfilin does not. The ability of ADF/cofilin to interact with F-actin requires its G- and F-sites (Figure 7A). The G-site is predicted to interact with subdomains 1 and 3 of one actin subunit, whereas the F-site interacts with subdomains 1 and 2 of the next actin subunit in a filament (Ono, 2003). Together, these two interactions can weaken the lateral and longitudinal contacts between subunits and induce filament severing and rapid dissociation of subunits from filament ends (Bamburg *et al.*, 1999; Galkin *et al.*, 2001, 2003). Now with crystal structures and mutagenesis analyses reported for each of the three major ADFH family members, a

Figure 7. Functional models for Abp1 and ADF/cofilin. (A) The crystal structures of *S. cerevisiae* Abp1 (left) and *S. cerevisiae* cofilin (right) are shown in the same view. Regions on these molecules important for F-actin binding are shaded and labeled (sites 1 and 2 for Abp1; G- and F-sites for ADF/cofilin). Residues mutated in *abp1* and *cof1* alleles are colored and marked by allele numbers (Table 1 and Figure 2). Abp1 Site 2 (disrupted by *abp1-1* and *abp1-2*) is similar to ADF/cofilin F-site (disrupted by *cof1-6* and *cof1-16*); both make important contributions to F-actin binding. In contrast, ADF/cofilin G-site is far more extensive than Abp1 site 1. The G-site in ADF/cofilin includes two acidic residues (mutated by *cof1-20*) that are crucial for both G- and F-actin binding (Lappalainen *et al.*, 1998); however, the same two residues in Abp1 (mutated by *abp1-4*) make no detectable contribution to actin binding. A third site required for efficient F-actin binding and disassembly by ADF/cofilin is located in helix 4 (mutated by *cof1-22*). In Abp1, this helix does not contribute significantly to F-actin binding, but instead is essential for Arp2/3 complex activation. Thus, helix 4 plays a key role in specializing ADFH domain functions in different family members. (B) Rendered structures of yeast Abp1 and cofilin, highlighting charged residues mutated by selected alleles (numbered) and neighboring exposed hydrophobic residues (purple). Note the belt of exposed hydrophobic residues near helix 4 in cofilin is absent in Abp1.

close examination of their structures and functions can be made to identify determinants of their specific activities.

What determines whether an ADFH domain binds to G-actin, F-actin, or both? Actin monomer binding by ADF/cofilins and twinfilin requires key charged surfaces on both the G-site and helix 3 (Figure 7A) (Lappalainen *et al.*, 1997; Ojala *et al.*, 2001; Guan *et al.*, 2002). Twinfilin and ADF/cofilin are functionally conserved at both of these surfaces, whereas Abp1 sequence diverges at helix 3 (the two charged residues critical for G-actin binding in ADF/cofilin and twinfilin are not conserved in the Abp1 sequence; Figure 2). This likely explains why Abp1 does not bind G-actin. On the other hand, F-actin binding by ADF/cofilins requires its G- and F-sites, but not helix 3. Twinfilin diverges structurally from ADF/cofilins at the F-site (Paavilainen *et al.*, 2002), explaining why it does not bind to F-actin. The connecting loop between $\beta 3$ and $\beta 4$ in twinfilin adopts a conformation that positions it away from $\alpha 4$, disrupting the F-site. The $\beta 3$ - $\beta 4$ connecting loop in Abp1 is similar to ADF/cofilins, which stresses the importance of this structure in the F-site in predicting F-actin binding activity among ADFH family members. Consistent with this prediction, the recently solved structure of another ADFH family member, coactosin, resembles Abp1 and ADF/cofilins at the F-site (Hellman *et al.*, 2004; Li *et al.*, 2004; Liu *et al.*, 2004), and this protein binds specifically to F-actin, although its activities have not yet been dissected by mutagenesis (de Hostos *et al.*, 1993).

Why do ADF/cofilins promote actin filament severing and disassembly, whereas Abp1 does not? Two key differences in their F-actin binding surfaces suggest a possible explanation. First, ADF/cofilins have a more extensive G-site (site 1 in Abp1) (Figure 7A). The ADF/cofilin G-site includes two conserved charged residues, D123 and E126 (yeast cofilin nomenclature), that are critical for actin filament binding and depolymerization in vitro and in vivo (Lappalainen *et al.*, 1997). The analogous residues in Abp1 (D122 and D125, mutated in Abp1-4) make no contribution to F-actin binding or Abp1 localization and function in vivo. This result was unexpected because the two residues are well conserved, unlike the charged residues on helix 3. However, adjacent to this site in ADF/cofilins is a patch of exposed hydrophobic residues, which is lacking in Abp1 (Figure 7B). Although charged residues at this site clearly are required for F-actin binding and disassembly in ADF/cofilins (as shown by the severe defects of Cof1-20), they may not be sufficient and may require the neighboring hydrophobic surfaces for this activity. Consistent with this possibility, the predicted target of the ADF/cofilin G-site on actin (subdomains I and III) is a hydrophobic-rich surface.

The second place where the F-actin binding surfaces of Abp1 and ADF/cofilins diverge is in helix 4 (Figure 1). Three charged residues mutated in *cof1-22* contribute to F-actin disassembly activity in vitro and in vivo (Lappalainen *et al.*, 1997; Lappalainen and Drubin, 1997), but Abp1 diverges at this site. Only one of the three charged residues is conserved in Abp1, and it makes no apparent contribution to F-actin binding affinity (Abp1-5). As described above for the *cof1-20* site, we note that adjacent to the *cof1-22* site in ADF/cofilins is a patch of exposed hydrophobic residues, lacking in Abp1 (Figure 7B). Thus, combined charged hydrophobic surface interactions at these sites may be important for specifying filament severing and depolymerization by ADF/cofilins. We also propose that helix 4 acts as a functional determinant in Abp1, because Abp1-5 abolishes Arp2/3 complex activation (Table 1), as discussed below.

Mechanism of Arp2/3 Complex Activation by Abp1

Yeast Abp1 directly associates with the Arp2/3 complex and stimulates its actin nucleation activity (Goode *et al.*, 2001). Abp1 and cortactin have been categorized as class II NPFs, because they bind to actin filaments but not monomers (Welch and Mullins, 2002). It is hypothesized that class II NPFs stimulate Arp2/3 complex nucleation activity by strengthening its association with the sides of preexisting actin filaments (Goode *et al.*, 2001; Uruno *et al.*, 2001; Weaver *et al.*, 2001). Here, we used point mutants in the actin-binding domain of Abp1 to demonstrate that F-actin binding is required for its nucleation-promoting activity, which strongly supports the filament recruitment hypothesis. In addition, we uncovered an unexpected second function of the ADFH domain in activating Arp2/3 complex that is independent of F-actin binding affinity. This function maps to the C-terminal portion of helix 4 and is disrupted by a single point mutation (K134A) in *abp1-5*. Abp1-5 has no apparent defect in F-actin binding affinity, yet abolishes Arp2/3 complex activation and the lethality caused by *ABP1* overexpression in vivo. Thus, this mutation uncouples the F-actin binding and Arp2/3 activation functions of the Abp1 ADFH domain. Furthermore, it suggests that the lethality in cells caused by Abp1 overexpression involves misregulation of Arp2/3 complex (Figure 5) and that normal functional interactions with the Arp2/3 complex are required for the Abp1 cellular function that is shared with Sac6 (Figure 4). This latter observation is consistent with previous results showing that the Arp2/3-activating acidic motifs of Abp1 are required in the *sac6Δ* background (Goode *et al.*, 2001).

We do not yet understand how Abp1 helix 4 participates in Arp2/3 activation. We considered that this helix might directly interact with Arp2/3 complex. This seemed reasonable given the similarity of Arp2 and Arp3 subunits to actin and previous reports that ADF/cofilin binds to Arp2/3 complex with low affinity (Blanchoin *et al.*, 2000). However, we could find no evidence of a direct interaction in binding assays by using purified Abp1 ADFH domain and Arp2/3 complex (Quintero-Monzon, unpublished data). Thus, although we cannot rule out this model, our data do not support it. Many other mechanisms are possible. For instance, this surface of the ADFH domain could make productive intramolecular interactions with a different part of Abp1 involved in Arp2/3 activation, such as the acidic motifs (Figure 1B). Alternatively, this surface could interact with and change the conformation of F-actin to promote Arp2/3 complex activation without changing Abp1 affinity for F-actin. Further investigation will be required to determine precisely how helix 4 contributes to Arp2/3 complex activation.

Abp1 Functions as a Modular Adapter In Vivo

Over a decade ago, it was demonstrated in yeast that an *abp1Δ* mutation is synthetic lethal with loss of function mutants in three other genes: *sac6*, *sla1*, and *sla2* (Holtzman *et al.*, 1993). However, until now it has been unclear whether these genetic interactions resulted from loss of the same or distinct cellular functions of Abp1. Here, we have uncoupled *ABP1* functions, demonstrating that Abp1 cellular functions rely on different combinations of domains and activities. We focused primarily on the Abp1-actin interaction and showed that it is differentially required in vivo, being strictly required for cell viability in the *sac6* background, only partially required in the *sla2* background, and not required at all in the *sla1* background. The precise functions of Abp1 that overlap with Sac6, Sla1, and Sla2 remain to be defined. Sac6

(fimbrin) bundles and stabilizes actin filaments (Adams *et al.*, 1991; Goodman *et al.*, 2003), whereas Abp1 alone has no direct effects on actin filament stability or organization *in vitro* (Kessels *et al.*, 2000; Goode *et al.*, 2001). However, it has been hypothesized that Abp1 may be similar to cortactin and stabilize Arp2/3-nucleated branched actin filaments (Olazabal and Machesky, 2001; Welch and Mullins, 2002). Thus, in the capacity of stabilizing branched actin filaments Abp1 function may be required in the absence of Sac6. This model also is consistent with the observed synthetic genetic interactions between *abp1-5* and *sac6Δ* mutations (Figure 4C).

Although actin binding is differentially required for Abp1 functions *in vivo*, the SH3 domain of Abp1 is required for all of its genetic functions (Lila and Drubin, 1997; Figure 4). This suggests that Abp1 functions as a modular adapter *in vivo*. For functions shared with Sac6, it links F-actin, Arp2/3 complex, and SH3 domain ligands. For functions shared with Sla1, it bridges a different set of ligands that does not include F-actin. Defining more precisely each biological role of Abp1 will require identifying the ligands required for each of its functions. Many Abp1 ligands are known. It is strongly established that Abp1 binds through its proline-rich and SH3 domains to Rvs167/amphiphysin (Colwill *et al.*, 1999), Sla1 (Warren *et al.*, 2002), Srv2/cyclase-associated protein (Freeman *et al.*, 1996; Lila and Drubin, 1997), and the actin-regulating kinases Ark1 and Prk1 (Cope *et al.*, 1999). In addition, many other SH3 domain- and proline-rich domain-containing proteins associate with Abp1 in genome-wide screens and proteomic studies, including App1, Cla4, Hua2, Myo5, Scp1, Sjl2, Yor284, and Ysc84 (Uetz *et al.*, 2000; Ito *et al.*, 2001; Fazi *et al.*, 2002; Ho *et al.*, 2002; Tong *et al.*, 2002). The complexity and overlapping nature of this web of interactions lends support to our central conclusion that Abp1 is a versatile adapter molecule and that different combinations of ligands interact with Abp1 at different times in the cell and/or with different subpopulations of Abp1.

ACKNOWLEDGMENTS

We are grateful to A. Manning for technical assistance, M. Welte for generously microscope use, and F. Solomon for providing yeast tubulin antibody. We thank H. Balcer, J. D'Agostino, K. Daugherty, and M. Gandhi for critical reading of the manuscript. S.C.A. was supported by National Institutes of Health Grant GM-53807. B.L.G. was supported by National Institutes of Health Grant GM-63691 and a Pew Scholars award.

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