Unusual Binding Properties of the SH3 Domain of the Yeast Actin-binding Protein Abp1

STRUCTURAL AND FUNCTIONAL ANALYSIS*

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The actin cytoskeleton plays a key role in many essential cellular processes, such as motility, endocytosis, secretion, and membrane recycling (1–5). As a consequence, its organization and dynamic rearrangements need to be tightly controlled spatially and temporally. A thorough understanding of the interaction network connecting all the actin-associated proteins, the scaffolds and the anchoring proteins, is likely to help to clarify the mechanisms underlying its coordinated regulation (6)

Most of the components of the yeast cell cytoskeleton have homologues in mammals where they often play similar roles (5–7). Abp1p (actin-binding protein) is a Saccharomyces cerevisiae protein homologue to the mouse mAbp1p-SH3P7, which is a Src kinase target involved in polarized cell growth and motility (8, 9). The yeast Abp1 protein is 592 amino acids long and includes an actin depolymerizing factor-homology domain at the N terminus and a SH3 domain at the C terminus of the protein (10). Abp1p is found concentrated in the actin patches that are enriched at the sites of polarized cell surface growth in the bud of budding yeast and in the mating projection of mating yeast. The overexpression of Abp1p disturbs the actin cytoskeleton and leads to an aberrant budding pattern and cortical actin assembly (11). Deletion of the ABP1 gene, on the other hand, does not cause any apparent cytoskeletal defect (12). Abp1p, however, is found to be essential when any of the genes encoding for Sac6p (the actin filament-bundling protein, fimbrin), Sla1p, Sla2p, or Prk1p is deleted (13, 51). These proteins, which functionally interact with Abp1p, are also localized in the cortical actin cytoskeleton (11, 13). The observed negative synergism in yeast cells carrying combinations of deletions in ABP1-SH3 and in the SLA1, SLA2, and SAC6 genes suggests that these gene products perform redundant essential function(s) that require the presence of an intact Abp1p-SH3 domain. Moreover, this synthetic lethality is not rescued by Abp1p devoid of its SH3 domain, suggesting a significant role for this protein interaction module (14).

To further characterize the role played by Abp1p, we have set out to identify its binding partners. More specifically, we have explored the role played by the SH3 domain in the assembly and regulation of these actin structures. The SH3 family of protein-protein interaction domains includes >1200 modules 60–70-amino acids long sharing an identity of at least 30%. More than 1000 proteins containing SH3 domains are found in many cell compartments and are involved in different functions (e.g. cell cycle control, signal transduction, or cytoskeleton organization). Typically, an SH3 domain recognizes and binds to proline-rich regions, which adopt a type II left-handed helix conformation (15, 16). The SH3 peptide recognition surface includes an hydrophobic cleft that binds to the proline-rich core and is flanked on one side by variable loops (RT and the N-Src loops) that contribute to the recognition specificity and determine ligand register and orientation (17, 18). A typical SH3 ligand contains a PXXP motif that is hosted in the hydrophobic cleft. Depending on the presence of a positive charge at the N or C terminus, the peptides bind in either of the two regulating kinase; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
Fig. 1 showed a GAL4 were found to be negative when tested with pLAM5 expressing fused to SV40 large T antigen (amino acids 87–amino acids 72 amplified in a bacterial host DH5 buffer saline containing 0.1% Tween 20. Affinity-selected clones were unbound phages were discarded by washing the resin with 1 end of M13 ability to bind to the full-length yeast Abp1 protein expressed as a GST particles displaying yeast protein fragments of which average length times of the whole genome) was a collection of recombinant phage TRP1/H9004/R/Sac (GACGTgagctcCTAGTTGCCCAAAGACAC) inserting a gaattcAAAATCCTTGGGCCACAG) inserting an Abp1-SH3-mutated in W569A. The SH3 domain (Pro-535-Asn-592) was formed after transformation into Y-187 according to the protocol by the manufacturer (CLONTECH). The positive control plasmids were from Y-190 [CLONTECH]. The β-galactosidase test in liquid was performed after transformation into Y-187 according to the protocol by the yeast strains used were HF7c (MATa ura3–52 his3–200 ade2–101 leu2–3,112 lys2–801) and Y187 (MATa leu2 trp1 ura3–52 prb1–1122 pep4–3 pre1–451 ark1–6Myc expressing 6Myc-Prk1p under its own promoter; pDD961 from pTS408, GFP-PRK1-kinase-dead K56A, Gal promoter, CEN, URA3 (13) expressing a GFP-tagged Prk1p under GAL promoter; and pDD961 from GFP-PRK1-kinase-dead K56A in which the P752PPK755 of Prk1p was mutated into alanines. The arl1 null strain DDY2115 was transformed with the following plasmids: pDD962 from pRS15, CEN, LEU2, ARK1–6Myc (13) expressing a Arl1p-6Myc under its own promoter and pDD962 from pRS315, CEN, LEU2, ARK1–6Myc kinase-dead K56A in which the sequence Lys-608-626 of Arl1p-6Myc was substituted by two alanines. To perform the pull down experiment using mutations in the SH3 targets, the arl1 null strain was transformed with pDD962 expressing the Arl1p-6Myc devoid of its polyproline tail, otherwise cells were transformed with pDD961 encoding the GFP-Prk1p with a mutated polyproline stretch. pDD961 and pDD962 were both created using a PCR-stitch technique as follows. To make pDD962, two first-round of PCR reactions were performed using pDD54 (13) as template with primer pairs 1 AF19 and JC74 and 2) JC95w and JC75. The products from these reactions were pooled and amplified in a second round of PCR using AF19 and JC95w. The PCR products were digested with XhoI and XbaI, and the removed fragment was replaced by similarly cut product from PCR round 2. A similar procedure was used to make pDD962 using pDD1160 (13) as template: SY47 and JC77 and JC51 in the first round, SY47 and SY51 in the second round, and replacing an NcoI fragment in pDD1160. All mutations were verified by sequencing.

As a bait for the pull down assay, the Abp1p-SH3 domain (Pro-353–Asn-592) was expressed in PYEX(ABP1-SH3, a pGEX2T derivative in which the SH3 domain (Pro-535-Asn-592) was inserted into EcoRI-SacI, with oligonucleotides R40 and R404. The oligonucleotides R649 (TATATGagcatcATGGTTTGGAGAATTTAT) and R650 (TCTGTGagcatcTTGCTCCCAAGAGACATA) were used to insert the GAL1 gene cloned into pGEX2P-1 (EcoRI-BamHI) to determine synthesis of the yeast protein Abp1p as a GST fusion. The GST-Abp1p protein produced in the bacterial strain HB101 was immobilized on a glutathione-Sepharose resin. The bacterial strains used were DH5a (F::supE44 lacZAFav-galV69) deor endA1 gryA96 hsdR17 (r -m k-) recA1 relA1 and HB101 (supE44 hsdS20 r -b m -b) recA1 ara -14 proA2 lacY1 galR2 pvl290 xil-5 mtl-1 leuB).
The SH3 Domain of Abp1

**Table I**

Data-processing statistics of Abp1 SH3 domain

| Orientation   | No. of reflections | I/σ(I) | Rmerge (%) | Completeness (%) |
|---------------|--------------------|--------|------------|------------------|
| 1             | 56,424             | 0.842  | 50         | 90.0             |
| 2             | 56,424             | 0.842  | 50         | 90.0             |
| 3             | 56,424             | 0.842  | 50         | 90.0             |
| 4             | 56,424             | 0.842  | 50         | 90.0             |

Numbers in parentheses indicate the values in the highest resolution range (1.32–3.10 Å).

RESULTS

Identification of Ligands of the SH3 Domain of Abp1—The yeast-two-hybrid approach is the most popular selective method to search for protein partners. However, we have recently demonstrated the feasibility of achieving similar goals by panning phage-displayed libraries in which cDNA or genomic fragments are fused to phage capsid genes (34). We have observed that the two methods are partly complementary, and that a search carried out with a specific bait domain by both methods results in a better coverage of the ensemble of potential partners (35).

By applying such a combined approach to the SH3 domain of Abp1, we have identified seven prey proteins, which were selected by either method (Table III). Our results confirm that the Abp1-SH3 binds to the adenyl cyclase-associated protein Srv2 (12). In addition, our two-hybrid screen also identifies the open reading frame product Yir003wp, the unconventional myosin Myo5p, and the kinase Prk1p as putative partners of Abp1p. Using the entire Abp1p as a bait, we have also screened a yeast genomic library displayed on filamentous phage capsids obtained by fusing random fragments of the yeast genome to the 5′ end of fl gene VIII. This approach permitted us to identify a second Ser/Thr kinase, Ark1p, as an Abp1-SH3 ligand. The 247 amino acid N-terminal catalytic domain of Ark1p is 73% similar to the catalytic domain of Prk1p. The two kinases aside from a similar C-terminal proline-rich sequence are otherwise rather divergent (13). The Ark1p peptide region selected by phage display corresponds to amino acids Arg-561-Leu-627 (Fig. 1) in which two putative SH3-binding motifs are present. Because the fragment of Ark1p was selected by panning phage-displayed libraries, we can exclude the possibility that the interaction between the Ark1p kinase and the SH3 domain is bridged by a third protein.

Another putative partner of Abp1p-SH3 discovered by phage display is Inp53p/Sip1p, which is similar to synaptotagmin, a mammalian synaptic inositol 5-phosphatase that is suggested to have a role in the regulation of membrane trafficking and actin cytoskeleton organization (36). In the short yeast synaptojanin, Ynl094wp, was repeatedly found by screening phage display genomic libraries. The shorter selected region comprises the residues from 471 to 504.

**Identification of the Abp1-SH3 Consensus Target Motif**—The Abp1p-SH3 targets selected by the two-hybrid approach contain several proline-rich regions. However, sequence alignment does not permit the definition of a clear consensus peptide.
The SH3 Domain of Abp1

The hybrid coat gene (Fig. 1) was deduced from the DNA sequence of genomic peptides (Ark1p, Prk1p, Myo5p, Yir003p) or by affinity selection of phage displayed yeast proteins. We have initially selected peptides from a repertoire of nonapeptides displayed by filamentous phage. We have characterized, and the amino acid sequence of the disulfide to the major coat protein pVIII. After three selection experiments, we had already shown that the SH3 domain of Abp1p does not bind to any of the typical SH3 ligands that we had characterized in our laboratory. To be able to identify in each protein the peptide that is bound by the Abp1p-SH3 domain, we characterized its recognition specificity by panning peptide libraries of random sequence displayed on filamentous phage. We have initially selected peptides with fixed positions and possibly a longer sequence displayed on filamentous phage. We have characterized, and the amino acid sequence of the disulfide to the major coat protein pVIII. After three selection cycles, four clones, which were found to be positive in ELISA, were characterized, and the amino acid sequence of the displayed peptide ligands was deduced from the DNA sequence of the hybrid coat gene (Fig. 1A). However, compared with other SH3 selections carried out in our laboratory, fewer clones were selected, and they were found to bind to the bait Abp1-SH3 less tightly (optical density of 0.2–0.4 in ELISA). This result indicates that the SH3 domain of Abp1p has a higher specificity, and that for tight binding, it requires a larger number of specific residues in fixed positions and possibly a longer peptide. The consensus sequence of the selected peptides, RXPPXXPKX, could be interpreted either as an extended class 2 peptide, containing an extra positively charged residue at the amino side, or as an extended class 1 peptide, containing an extra positively charged residue at the C terminus.

Because this first panning experiment did not yield a sufficient number of ligand sequences, we have also panned a second library of pentadecapeptides containing a fixed PXXPx motif displayed at a lower density by the filamentous phage pIII receptor protein (37). The three selected pentadecapeptides reveal a slightly different consensus, PXXPXRPPxW (where # represents a hydrophobic residue), that can be interpreted as an extended class 2 with a requirement for a proline after the conserved positively charged and hydrophobic residues at the C terminus.

We used the consensus sequences obtained by the two panning experiments to identify the best matches on the selected protein partners. Whenever visual inspection was not sufficient, we used a position-specific profile derived from the phage display experiments as described by Tong et al. (35). The results are illustrated in Fig. 1B. Although no protein partner contains an exact match of either consensus, the predicted peptides identify a third consensus, +XXPXXPx+PXX, that can be interpreted as a merge of the two phage-displayed consensus.

Ark1p, Prk1p, Srv2p, Imp32p, and Yir003wp contain a peptide that matches most of the extended consensus. Myo5p and Ynl094p contain a peptide in which the conserved KP motif is preceded by a long stretch of prolines.

Mapping of the SH3 Target Peptides on Prk1p and Ark1p—To verify by an independent method the physical interaction between the SH3 domain of Abp1p and the Prk1p kinase, we have used Abp1p-SH3 fused to glutathione S-transferase to pull down Prk1p fused to an N-terminal 6Mye-tag expressed in prk1Δ yeast cell. As shown in Fig. 2A, ~5% 6Myc-tagged Prk1 protein is pulled down by the Abp1p-SH3 domain. By contrast, most of the binding is abolished when the conserved tryptophan in the SH3 domain (Trp-569) is changed to an alanine. Several bands can be identified with an antibody against the Myc-tag antibody in yeast extracts expressing the Myc-tagged Prk1 protein, suggesting that this protein is degraded either in vivo or during the preparation of the protein extracts. However, only the full-length protein is pulled down by the SH3 domain of Abp1p. This is consistent with our identification of the SH3-binding site with the proline-rich region at the C terminus of the Prk1 protein.

Similarly, we confirmed by a pull down experiment the in
The SH3 Domain of Abp1

Proline-rich Peptide of Prk1p and Ark1p Is Essential for Localization of the Kinases to Actin Cortical Patches—Cope et al. (13) have shown that the localization to the actin cortical patches of Prk1p and Ark1p is dependent on Abp1p. To verify whether the in vivo co-localization of Abp1p with both Prk1p and Ark1p is mediated by the direct physical interaction that we have identified in vitro, we looked at the localization of GFP-tagged kinases in abp1 null yeast cells expressing different derivatives of the Abp1 protein. In this experiment, we used both kinases carrying an inactivating mutation in their enzymatic domain (kinase-dead), because elevated levels of either Ark1p or Prk1p result in abnormal cell morphology and ultimately in cell death (13). Each of these two strains was further transfected with one of three different plasmids expressing (i) a wild type Abp1p, (ii) an Abp1p-deleted of the SH3 domain, or (iii) an Abp1p with a mutated (W569A) SH3 domain. Normal cortical spots of kinase localization are visible only in cells that are transfected with a plasmid expressing the wild type Abp1p, demonstrating that the SH3 domain is essential for proper localization of the two GFP-tagged kinases (Fig. 3, upper panel). However, we did notice a residual cortical localization of Prk1p even in the absence of Abp1p, whereas Ark1p was completely delocalized.

To further characterize the SH3 interaction in vivo, we examined the localization of a GFP-tagged Prk1p kinase that was altered in its Abp1-SH3-binding motif in the C-terminal region. The cells were transformed with a plasmid encoding a GFP-PRK1 gene fusion in which the four residues (750<sup>PPPKe</sup>755) had been changed into alanines. As a result, the Prk1p mutated at the polyproline site is no longer localized to the cortical patches (Fig. 3, lower panel). These results identify the SH3 domain of Abp1p and the polyproline motifs of Prk1p and Ark1p as essential regions for the proper cellular localization of both kinases to the cortical actin patches.

**FIG. 2.** Affinity purification. The prk1 null strain (A) and the ark1 null strain (B) were transformed with pDD558 expressing 6Myc-Prk1p and pDD852 expressing Ark1p-6Myc, respectively. Both kinases were expressed under the control of their native promoters. The bait used for affinity selections are GST alone, GST fused to the Abp1-SH3 domain, GST fused to the Abp1-SH3 mutated in a conserved tryptophan (W569A), and GST fused to the Abp1-SH3 full-length as indicated above each lane. The faster migration of the band corresponding to Ark1p in the GST-Abp1-SH3 lane in B is caused by the co-migration of the bait protein as verified by Ponceau staining. C, extracts from prk1 (a and b) or ark1 (c and d) null strains transformed with plasmids expressing GFP-Prk1p (Extract a), GFP-Prk1p-polyproline-mutated (Extract b), Ark1p-6Myc (Extract c), and Ark1p-polyproline-deleted-6Myc (Extract d). These extracts are affinity-selected over immobilized GST or GST fused to Abp1p-SH3 mutated in the C-terminal region. The input extract is from 10<sup>8</sup> cells, and the pulled down extract is from 10<sup>9</sup> cells incubated with 20 µg of bait proteins.

**FIG. 3.** Localization of Prk1p to actin cortical patches depends on an intact polyproline motif at its C terminus. Yeast cells were transfected with plasmids directing the synthesis of GFP-Prk1p (upper panel, PRK1) or GFP-Prk1p carrying a mutation in the polyproline stretch (lower panel, PRK1 mut poly P). In the panels on the left, the clear spots are attributed to fluorescence of the GFP-Prk1p hybrid protein. Actin was visualized in the two panels on the right by staining with rhodamine-conjugated phallolidin (Rh). To avoid disturbing cell physiology by overexpressing the Prk1p kinase, a mutationally inactivated kinase was used in this experiment (GFP-Prk1p K56A).

Interaction of the SH3 Domain of Abp1p with the C-terminal interaction between Abp1p and the Ark1p tagged with 6Myc at the C terminus (Fig. 2B). The Ark1p-6Myc was efficiently pulled down by GST-Abp1-SH3. By contrast, Abp1-SH3 (W569A) mutated in the conserved tryptophan does not bind to Ark1p. Furthermore, the SH3 domain seems to be as efficient as the full-length Abp1p protein in the pull down assay. These results further stress the primary role of the SH3 domain of the scaffold protein Abp1p in binding to the two regulatory kinases.

Moreover, we have used the pull down assay to confirm that the peptides identified by matching the phage display consensus to the Ark1p and Prk1p protein sequences indeed mediate the interaction with the SH3 domain. To this end, we have modified the two kinase genes by altering the sequences encoding the putative SH3-interacting motifs. As a result, four residues in the polyproline stretch (750<sup>PPPKe</sup>755) at the C-terminus of GFP-Prk1p were changed into alanines, and the 19 residues (808<sup>KPTPPKPSHLKPKPPP</sup>826) in the C-terminal region of Ark1p-6Myc were deleted and substituted with two alanines. The two kinases, altered in their putative SH3-binding motifs, no longer bind to the Abp1-SH3 as judged by the pull down experiments (Fig. 2C).

**Fig. 3. Localization of Prk1p to actin cortical patches depends on an intact polyproline motif at its C terminus.** Yeast cells were transfected with plasmids directing the synthesis of GFP-Prk1p (upper panel, PRK1) or GFP-Prk1p carrying a mutation in the polyproline stretch (lower panel, PRK1 mut poly P). In the panels on the left, the clear spots are attributed to fluorescence of the GFP-Prk1p hybrid protein. Actin was visualized in the two panels on the right by staining with rhodamine-conjugated phallolidin (Rh). To avoid disturbing cell physiology by overexpressing the Prk1p kinase, a mutationally inactivated kinase was used in this experiment (GFP-Prk1p K56A).
Three-dimensional Structure of the Abp1-SH3 Domain—The interaction of a standard SH3 ligand and its receptor domain can be schematically split into two contributions. The first is rather nonspecific and derives from PXXP contacting the two hydrophobic pockets, while the second derives from further often electrostatic interactions in the so-called specificity pocket. As outlined above, the SH3 domain of Abp1p displays a peculiar recognition specificity, and differently from most SH3 domains described so far, it does not bind tightly to any short class 1 or class 2 peptide that we have tested. Abp1-SH3 ligands are characterized by two positive side chains separated by seven of eight residues containing a P motif. To understand the structural basis of this novel specificity, we have determined the three-dimensional structure of the Abp1-SH3 domain at 1.3-Å resolution. The high resolution provides a detailed model in which double conformations and numerous solvent molecules could be determined accurately (Fig. 4A). The overall structure is similar to other SH3 domains consisting of a compact \( \beta \)-barrel of five anti-parallel \( \beta \)-strands forming two orthogonal \( \beta \)-sheets (Fig. 4B). At first sight, the SH3-binding pockets, which normally host the PXXP-binding motif, do not reveal any peculiar characteristics that would readily explain the unusual binding properties of Abp1p. The residues that normally flank the hydrophobic cavities can be superimposed to the equivalent residues of the Abl and Src SH3 domains for instance (Fig. 5).

FIG. 5. Superposition of the Abp1p-SH3 domain (red) with Sem5-SH3 (green) and c-Abl-SH3 (blue). The residues in the Abp1-SH3-PXXP-binding site are labeled. They overlap almost perfectly to those in Sem5-SH3 and c-Abl-SH3. The figure was produced using MOLSCRIPT (49). Y53, Tyr-53; Y9, Tyr-9; W35, Trp-35; N52, Asn-52; Y7, Tyr-7; E6, Glu-6.

By considering the multiple alignment of the SH3 domains of known specificity and by concentrating on the residues that in the three-dimensional structure flank the ligand-binding pocket, the most striking peculiarity observed in Abp1-SH3 is Glu-6 that precedes the conserved tyrosine at position 7. The vast majority of SH3 domains have a hydrophobic residue at the Glu-6 position. Although this side chain is not in direct contact with the ligand in the SH3-peptide complexes of known structure, it is possible that the presence of a negative charge in an otherwise hydrophobic surface results in a decrease of the affinity for a “typical” PXXP peptide. Possibly because of the suboptimal interaction with the PXXP motif, the Abp1-SH3 physiological partners extend their contacts beyond the first hydrophobic pocket that is normally boxed by the aromatic ring of Tyr-7. Notably, just beyond the Tyr-7 side chain, the surface of Abp1-SH3 displays a second glutamate residue (Glu-21) in a position that in most SH3 domains is occupied by a positively charged residue. Because until now we have been not successful in our attempts to co-crystallize the Abp1-SH3 domain with its target peptides, we have designed site-directed mutants to test this model. In one mutant, Glu-6 was changed into Leu as found in most SH3 domains, and in a second one, Glu-21 was substituted by Lys.

When these mutant domains were assayed in pull down experiments, they proved to be at least as efficient as wild type in binding to Prk1p (data not shown). This result proves that neither of the residues tested plays a major role in ligand binding. However, consistent with the model, the mutant G6L has acquired the ability to bind to a classical (class 1) peptide, LSSRPLPTLPS, thus pointing to the important role of the side chain at the position corresponding to Glu-6 in shaping the typical SH3-binding pocket (Fig. 6C).

DISCUSSION

The large scale two-hybrid screening experiments have failed to confirm the characterized interactions of Abp1p with Rvs167p and Srv2p (11, 12, 38) and have revealed a new putative partner of Abp1p, Yor284wp (39, 40). However Yor284wp does not contain proline-rich regions that are typical signatures for SH3 domain targets.

In this study, by combining yeast two-hybrid screenings and selections of protein fragments displayed on filamentous phage capsids, we have identified six new ligands of the Abp1-SH3
domain that are potentially physiologically relevant. These include the two Ser/Thr kinases, Ark1p and Prk1p. By mutating either a conserved tryptophan on the Abp1-SH3-binding surface or the polyproline target peptides near the C termini of the two kinases, we have shown that this SH3-mediated interaction is responsible for the proper localization of both kinases to actin cortical patches.

Prk1p was suggested to be a negative regulator of endocytosis by several lines of evidence. In vivo, a loss of Prk1p activity suppresses the effects of a loss of function mutation in Pan1p, the yeast homologue of Eps15. By phosphorylating Pan1p on threonine residues, Prk1p hinders the interaction of Pan1p with the endocytosis protein, End3p (41). The formation of the heterotrimeric complex Sla1p-End3p-Pan1p is required for proper cortical actin organization and endocytosis (2, 42).

In our two-hybrid screen using Abp1-SH3 as a bait, we also selected the unconventional (type I) myosin Myo5p and the product of an open reading frame of uncharacterized function, Yir003wp. The interaction with Myo5p has the potential to occur in vivo, because this motor protein co-localizes with Abp1p in cortical patches. Another protein that is implicated in endocytosis, the yeast synaptojanin Inp52p/Sjl2p, was also found to bind to Abp1p-SH3 in our screenings. The interaction we have characterized between Abp1p and Inp52p provides a possible mechanism for the localization of Inp52p to actin patches following hyperosmotic stress (43). Finally the screening of phage-displayed genomic libraries has identified the product of the reading frame Ynl094wp as a partner of Abp1p. Collectively, the newly discovered Abp1p ligands together with the information already available on their function suggest a large web of SH3-mediated interactions that regulate the dynamic assembly of actin and link it to the endocytic process.

Abp1p contains three acidic repeats containing the conserved DDW motif that was shown to mediate the association of cortactin, WASP, and Myo5p to the Arp2/Arp3 complex (44, 45). One of these motifs is solvent-exposed in the N-Src loop of the SH3 domain. However, our selection experiments have not revealed any Abp1-SH3 ligand that is a component of the Arp2/Arp3 complex. Furthermore the full-length protein Arc40p, a component of the Arp2/Arp3 complex that binds the acidic motif at the tail of the Myo3p (44), could not bind to Abp1-SH3 in a two-hybrid assay or in an in vitro assay with purified proteins (data not shown). Recently, the two other acidic regions of Abp1p have been proven to be essential for Arp2/Arp3-binding and actin polymerization (46).

These data emphasize the role of the yeast Abp1p as an F-actin-associated link between protein complexes involved in actin polymerization and a number of proteins implicated during endocytosis. Moreover, Abp1p can recruit to the actin patches of regulatory kinases, which have an inhibiting role on endocytic proteins. By phosphorylating their targets, Abp1p-associated Prk1p and Ark1p may cause a disruption of the endocytic machinery and allow vesicles to pinch off from the membrane and be transported away by actin polymerization. Abp1p in this manner could be coordinating the membrane fission and actin-promoted movement. Fig. 7 illustrates the protein network centered on the Abp1 protein.

Several proteins involved in endocytosis and cytoskeleton dynamics contain an SH3 domain (47). Recently, a number of reports have shown that the binding potential of the SH3
The SH3 Domain of Abp1

when they are queried with domains that do not bind to typical class 1 or class 2 domains. For instance, we have not been able to predict the recognition specificity of the Abp1-SH3 domain by using the SPOT algorithm (48) or even recognize that its preferred ligand has an atypical sequence. This implies that the peculiarity of the Abp1-SH3 domain cannot be revealed by simple alignment of the domain sequences and comparison of the residues that were seen in contact with the target peptides in the complexes of the known structure. Indeed, by superimposing the high resolution three-dimensional structure of Abp1-SH3 with that of other SH3 domains, like Abl or Scr, it was not immediately apparent why the Abp1 domain should fail to recognize the typical class 1 or class 2 consensus sequences. The binding surface in the two hydrophobic pockets that normally host the PXXP motif are highly comparable (Fig. 5). When the different SH3 domains are represented as surfaces, colored according to charge (Fig. 6), the only difference that stands out is the negative charge of Glu-6 in a position that is occupied by a hydrophobic residue in most SH3 domains. We have shown that this residue, although it does not make contact with the ligand peptides in the SH3 complexes of known structure, contributes to the characteristics of the peptide-binding pockets. In fact, by changing Glu-6 into a Leu, we were able to modify the recognition specificity of the Abp1-SH3 domain and promote the recognition of a classical class 1 peptide (Fig. 6C). This observation provides an important clue to pinpoint those in a multiple alignment of SH3 domains that are not likely to be good receptors of typical PXXP peptides.

According to our model, the hydrophobic pockets in Abp1-SH3 do not represent an optimized receptor for classical PXXP peptides. As a consequence, the interaction energy with a peptide of typical length is below a threshold of physiological relevance (10–100 μM). To make up for the extra energy, the natural ligands of Abp1-SH3 would extend their contacts beyond the typical SH3-binding surface. Our side-directed mutagenesis experiments exclude that the "anomalous" glutamate at position 21 of the Abp1-SH3 domain may be involved in binding to this extended peptide target.

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