Defining protein interactions with yeast actin *in vivo*

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Using the two-hybrid protein interaction reporter system, actin, profilin, Srv2p and two SH3-containing proteins are found to bind yeast actin *in vivo*. When tested for ability to interact with 35 actin mutations distributed over the monomer surface, distinct subsets of mutations characteristic for each putative ligand are found to disrupt binding. In particular, the pattern of differential interactions for the actin-actin interaction is consistent with published structures for the actin filament. Despite functional similarities, the patterns of differential interaction for Srv2p and profilin are different. In contrast, the patterns for profilin and the SH3 domain proteins suggest a shared binding site and commonality in mechanism.

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The development of genetic methods to assess protein interactions presents an opportunity to obtain biologically relevant structural information about incompletely characterized multi-protein complexes such as the actin cytoskeleton. We have used a protein interaction reporter system, known as the two-hybrid system[1], to characterize binding sites on yeast actin. This system takes advantage of the possibility of separating the *GAL4* transcriptional activator into its DNA-binding and transcriptional-activation domains. The two halves of Gal4p are each fused to other protein domains which, if they interact, can reconstitute Gal4p activity *in vivo* through their protein-protein interactions. Interactions can thus be assessed by measurements of the activity of one or more Gal4p-responsive promoters in the living cell.

The entire wild type yeast actin coding sequence and each of the previously described 'charged-to-alanine scanning mutagenesis' mutant actin sequences[2] were introduced into the two-hybrid system as fusions to the Gal4p DNA-binding domain. These fusions were then tested for their ability to interact with a number of protein actin ligand sequences that had been fused to the Gal4p activation domain. By identifying those mutations that specifically destroy binding to actin by a particular protein we have been able to characterize and in some cases 'footprint' possible binding sites for these proteins on actin. In this report we describe the application of the method to the interactions between actin and five actin-binding proteins: actin itself, profilin, Srv2p (also know as cyclase-associated protein), a new protein we call Aip1p and two SH3-domain proteins, Fus1p and Rvs167p.

Actin-interacting proteins

The single yeast actin gene (*ACT1*) was subcloned into pRB1508 in frame with the *GAL4*p DNA-binding domain to generate pRB1516. To select for potential actin-interacting proteins, strain Y190 carrying pRB1516, was transformed with a yeast cDNA library (inserts fused to the *GAL4* activation domain) in plasmid pSE1107 (ref. 3). Protein interactions were scored by the ability of the tranformed cells to express the *HIS3* gene under the control of a Gal4p-responsive promoter, conferring resistance to 3-aminotriazole Interactions were confirmed by the ability to produce β-galactosidase from a *lacZ* gene separately fused to a Gal4p-responsive promoter. Activation was not observed in the presence of lamin, p53, CDK2 or rev fusions to the Gal4p DNA-binding domain. Neither was activation observed in the absence of the actin fusion. These results provide evidence that the interactions are specific to actin.

We recovered 18 inserts from the library; these were presumed to encode actin-interacting proteins (AIPs). Analysis of the partial DNA sequences of the AIP library inserts indicated we had identified three new genes (designated AIP1, AIP2 and AIP3), seven previously sequenced genes and one likely artifact. Of the seven previously sequenced genes, four were known or suspected to bind actin (that is, *ACT1*, encoding actin itself[4,5]; *PFY1*, encoding profilin[6] (two independent isolates); *SRV2*, encoding a suppresser of activated Ras[7,8] (three independent isolates); and *RVS167*, (encoding a protein required to survive starvation[9]). The other known proteins were *OYE2*, encoding NADPH oxidoreductase[10] ('old yellow enzyme'); *GLK1*, encoding glucokinase[11] (two independent isolates); and *RPL45*, encoding a ribosomal protein[12].

Table 1 Identification of actin interacting proteins and differential interactions with actin mutants

Name	Identity	FusionJunction	Differential Interactions
pAIP70	Actin	Codon-24–375	*act1-111, 112, 113, 114, 115[1], 129, 132, 134, 136[1]*
pAIP14	Profilin	Codon 7–126	*act1-101[1], 102, 111, 112[1], 119, 123, 129, 131, 134*
pAIP10	Srv2p/CAP	Codon 287–526	*act1-105, 111, 119, 131, 132, 136*
pAIP18	Srv2p/CAP	Codon 5–526	*act1-105, 111, 113, 119, 131*
pAIP38	Rvs167p	Codon 189–549	*act1-102, 111[1], 112, 119, 123, 129, 131, 134*
pDAb143	Rvs167p	Codon 1 to 549	*act1-101[1], 102, 103[1], 105[1], 111[1], 112, 119, 123, 129, 131, 134*
pDAb161	Fus1p	Codon 97-512	*act1-101, 102, 105[1], 111, 112[1], 119, 120 123, 125, 129, 134[1], 135*
pAIP1	Aip1p (new)		nd *act1-103, 106, 109, 111, 112, 126, 134*

[1]Denotes a weakened interaction

Interactions with actin mutants

To characterize more precisely the binding of potential ligands to actin, we made use of a synoptic set of 35 actin mutants constructed previously by clustered charged-to-alanine scanning mutagenesis[2], a procedure designed to alter residues mainly on the surface of the molecule[13]. Each of the 35 mutations was substituted for wild-type actin in pRB1508 and transformed into strain Y190, in preparation for the two-hybrid assay. Each of the library clones carrying the putative ligands was transformed into another strain (Y187) of opposite mating type which was then mated to each of 35 strains carrying a different mutant actin fusion protein. The genetic response — growth on aminotriazole — was then measured on the diploids, each of which contains two plasmids, one with an actin mutant fused to the *GAL4* DNA-binding domain and one with a putative ligand fused to the *GAL4* activation domain.

Three types of response were noted: In the first type, mutation affected binding of all ligands. The four lethal alleles 107, 127, 128 and 130 as well as the nearly lethal allele 108 fall into this class of highly disruptive mutations. This result is taken to indicate that these actin mutations, despite their provenance, each have a profound destructive effect on actin conformation. Such mutations were ignored in the subsequent analysis. In the second type of response a mutation affected binding of some ligands, but not others. This result is taken as defining a 'differential interaction'. In the third type of reaction a mutation affected binding of none of the ligands. This class includes the lethal allele 110, the conditional lethal alleles 121, 122 and 125 and wild type alleles 104, 116 and 135. These last results provide no information save that the structure of actin is probably unchanged by the mutation with respect to its activity in the two-hybrid system. These mutations were also ignored in the subsequent analysis.

We observed that the pattern of differential interactions was not the same for different ligands (Table1, Fig. 1). Altogether we can distinguish seven classes by differential interactions among nine ligands. When we had several points of fusion for a ligand to the GAL4-activation domain (Table 1), the patterns resembled each other closely. In some cases, the patterns of interaction for different ligands resembled each other. For example, the profilin pattern resembled the pattern for Rvs167p.

The sequence of Rvs167p contains the well-documented Src-homology 3 (SH3) sequence motif. This motif has been implicated in interactions with the actin cytoskeleton[14]; for example, it has been found that the actin-binding protein Abp1p contains an SH3 domain[15]. Prodded by this discovery, we introduced the coding sequences of the SH3-containing proteins Abp1p, Fus1p[16] and Bem1p[17] into our two-hybrid assay (that is, into the

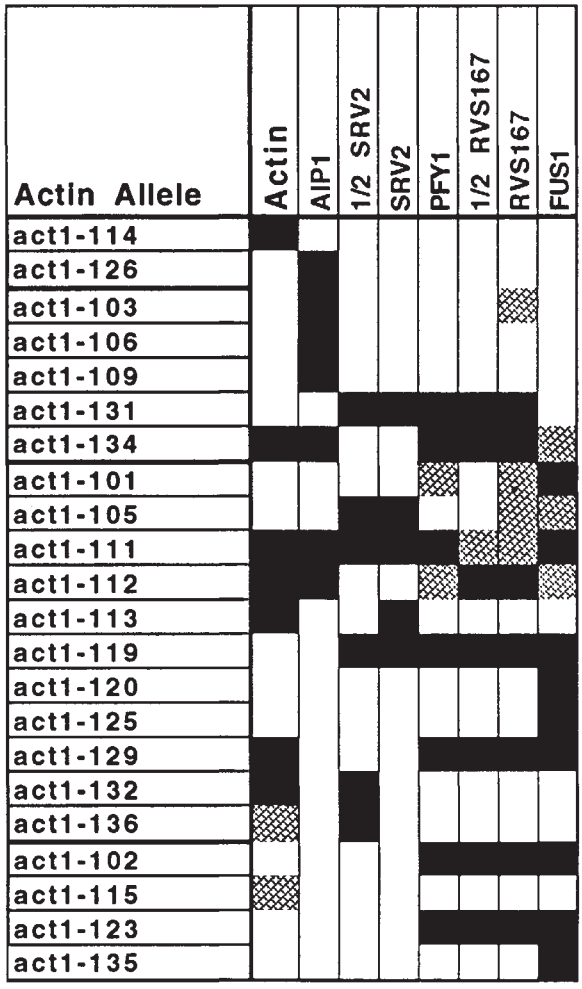

Fig. 1 Comparison of differential interactions. The solid squares indicate those mutations that disrupt binding while the stippled squares indicate mutations that weaken the interaction. Only the mutations that show differential interactions are listed; '1/2' refers to clones listed in Table 1 that carry only the C-terminal half of the relevant protein.

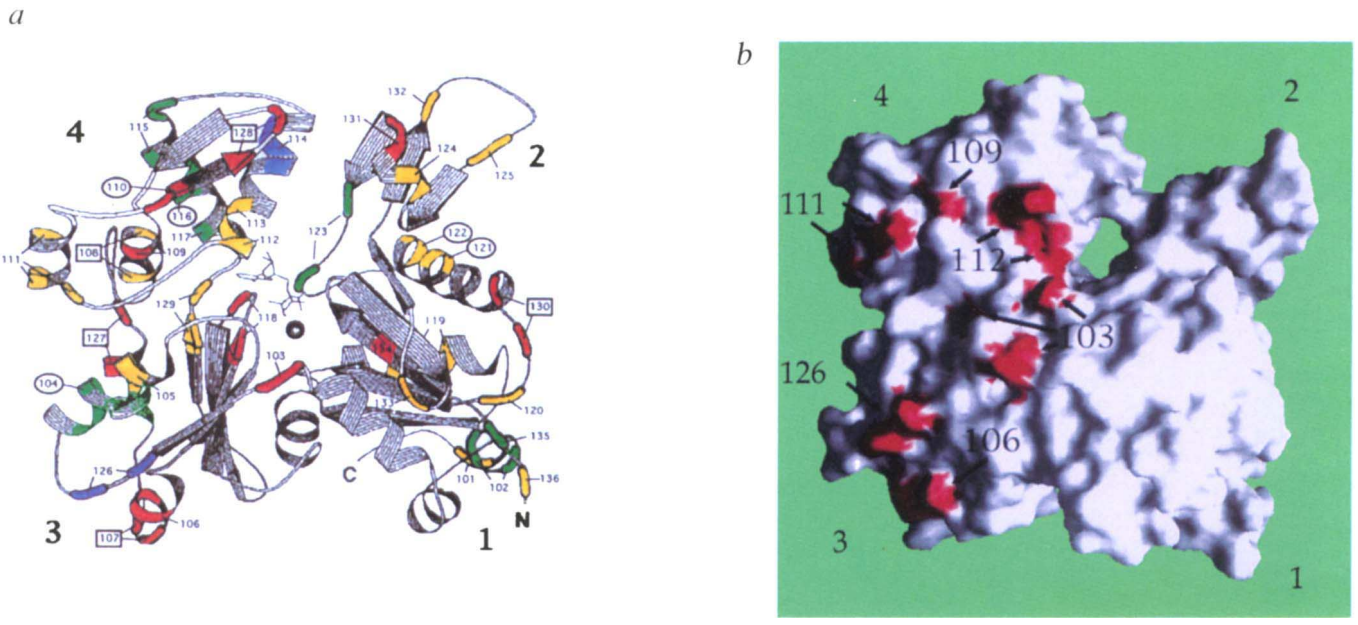

Fig. 2 Modelling of the actin-Aip1p differential interactions. *a*, The clustered charged-to-alanine scan alleles are shown on the ribbon structure of rabbit muscle actin[2]. Subdomain numbers are indicated at the corners. This is designated as the 'front view'. Allele numbers of mutations that disrupt all interactions in the two-hybrid system are boxed and mutations that fail to disrupt any of the tested interactions are circled. Blue, dominant lethal phenotype; red, recessive lethal; green, 'wild type'; and yellow, temperature-sensitive. *b*, The solvent exposed surface of a monomer of rabbit muscle actin is shown. That portion of the surface formed by the side chains of mutations that disrupt the actin-Aip1p interaction is shown in red. The view displayed corresponds to the same front view as in *a*. Modelling was performed on a Silicon Graphics Iris computer running GRASP software[36].

ligand plasmid pRB1506) and tested the differential interactions as above. In the case of Fus1p we observed a pattern strikingly similar to that found for profilin and Rvs167p (Fig. 1, Table 1). With Abp1p and Bem1p all interactions were weak, even with wild-type actin, so a definitive test was not possible. We subsequently found that disruption of the *RVS167* reading frame, immediately upstream from the C-terminal SH3 domain, destroys the ability of the fusion protein to interact with actin in the two-hybrid system (data not shown). This result argues strongly for direct involvement of the SH3 domain in the actin interaction.

Molecular modelling

We extended our analysis to the examination of the differential interaction data on the three-dimensional structure of actin[18]. A ribbon diagram of actin, on which the 'Ala scan' alleles have been displayed is shown in Fig. 2*a*. In many cases those mutations that disrupt particular interactions map to restricted regions on the surface of the actin monomer. One such result is displayed in Fig. 2*b*. Here the solvent-exposed surface of actin is calculated, as well as those regions of the surface (shown in red) altered by mutations that destroy binding of actin to a new protein we call Aip1p. The only disruptive allele not visible in this display is *act1-134*, which also happens to be the only Ala scan allele that alters residues that are entirely buried in the structure. The data displayed in this manner are highly suggestive of a discrete binding site for Aip1p on subdomains three and four of actin.

The advantage of defining binding sites in this manner is that the information is obtained from fully solvated proteins interacting in living cells. In order to ask whether our results agree with structural information obtained from more 'classical' methods, we compared our data with a current model for actin-actin interactions in F-actin (Fig. 3). The differential interactions observed for the actin-actin interaction were modelled on the solvent exposed surface of an actin monomer excised from the refined Holmes model for the actin filament[19] (Fig. 3). Nearly all of the disruptive alleles (shown in red) map to the 'back side' of the monomer, suggesting that we are detecting a back-to-back dimer of the actin fusion proteins. Those mutations that merely weaken the interaction have been omitted from this analysis. These data are consistent with the interactions across subfilaments observed in the F-actin model (shown in blue in Fig. 3). Regions of overlap between the mutants and regions of actin-actin proximity are shown in lavender in the figure.

The Holmes filament model predicts that the major stabilizing interaction across subfilaments is formed by the insertion of a hydrophobic loop (amino acids 262–274) into a hydrophobic hole formed in part by the cleft between subdomains two and four[20,19] (Fig. 3). The other portion of the hydrophobic hole is formed by the cleft between subdomain one and three at the bottom of the monomer. Of the mutations that show differential interactions, alleles 112, 113 and 114 map to an α-helix of subdomain four which forms or supports one half of

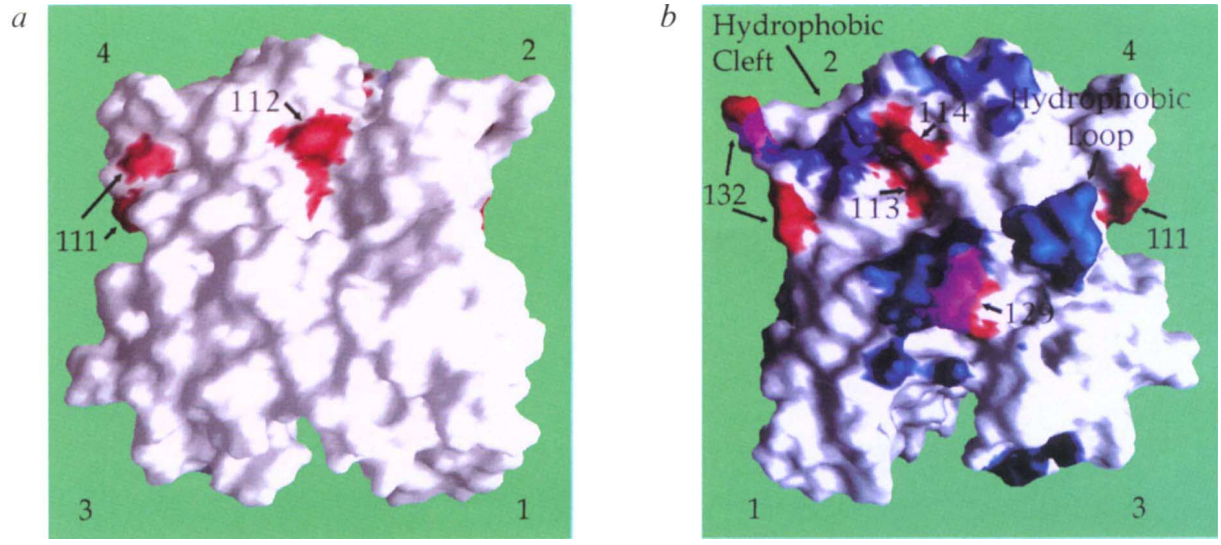

Fig. 3 Modelling of the actin-actin differential interactions. The solvent-exposed surface of a single monomer from the proposed actin filament structure[19] is shown. The amino acid side chains of residues altered by mutations that disrupt the actin-actin interaction are displayed in red. Surface that would be 3Å or closer to monomers in the opposing sub-filament are shaded blue. Overlap between disruptive mutations and regions of actin-actin proximity are shaded in lavender.*a*, The front view of the subunit is shown. *b*, The back view of a subunit is shown. Actin filament coordinates were provided by K. Holmes.

the hydrophobic cleft. In addition, allele 132 maps to the other side of the cleft. Allele 111 has three mutations in the α-helix which forms part of the pocket in which the hydrophobic loop resides in monomeric actin[18]. These mutations may result in localized structural disturbances that affect how the loop swings out from the monomer; alternatively they may significantly increase the local hydrophobicity, thereby increasing the amount of energy required to swing the loop out from the body of the molecule. Alleles 115 and 129 map to the backside of the monomer and appear to correspond to other regions of close contact, distinct from the hydrophobic loop. Allele 134 is once again not visible because it alters residues buried in the structure.

Interactions formed between actin and profilin, a known regulator of actin assembly, have also been well characterized structurally. The recently published structure of the actin-profilin co-crystal structure[21], predicts two regions of contact for profilin on actin and two novel sites of actin-actin contact. One of the profilin contacts on actin was previously identified in cross linking studies[22]. We compare our data with contacts predicted from the profilin-actin co-crystal structure in Fig. 4. A monomer of β-actin is shown with regions of profilin proximity displayed in yellow and the two regions of actin proximity displayed in two shades of blue. The differential interactions observed with alleles 101, 102 and 119 are in agreement with the strong profilin contact described on the bottom of actin. Furthermore, allele 101 includes mutation E364A; Glu 364 is the amino acid that has been cross-linked to profilin in solution[22]. Note that allele 101 only weakens the actin-profilin interaction. It may be that the residues altered in allele 101 are in close proximity to profilin but do not play critical roles in the stabilization of the complex. In any case, this region corresponds to the best-defined site of profilin-actin interaction. It is also noteworthy that allele 111 disrupts the actin-profilin complex. This mutation lies immediately under the second actin-profilin contact predicted by the co-crystal structure[21]. The disruptive effect of this mutation provides genetic support for the existence of this second contact *in vivo*.

Despite these limited correlations, the bulk of the actin-profilin differential interaction data are difficult to interpret in the context of the actin-profilin crystal structure. In addition, the disruptive mutations do not map to a single region of the monomer. This observation indicates that multiple regions of the monomer are important for the profilin interaction, which in turn suggests that the formation of a complex between actin and profilin is more involved than simple one-to-one heterodimer. In addition, we cannot rule out that some of the actin mutations are having disruptive effects far from their location in the structure.

Structural implications

The combination of systematic mutagenesis of yeast actin with the two-hybrid protein interaction reporter system provides an additional tool for understanding the many dynamic interactions actin forms in the execution of its many functions. Possibly the simplest example of the power of this approach comes from the modelling of differential interactions for the newly discovered putative actin-binding protein Aip1p (Fig. 2*b*). These mutations all map to a small and contiguous region on the surface of actin's subdomains three and four.

The usefulness of this kind of analysis is illustrated by our examination of the data for the actin-actin interaction in the context of the proposed interactions formed within filamentous actin[19] (Fig. 3). These results pro-

vide further genetic evidence in support of the hydrophobic loop to hydrophobic plug interaction. This interaction is predicted by the Holmes model to be the major stabilizing contact formed between subfilaments in the filament[19]. It has been shown previously that a mutation in this loop causes actin filament assembly to be cold sensitive[23]. It is interesting to note that the hydrophobic plug of β-actin, in the profilin-β-actin structure, is held quite strongly against the main body of the molecule[21]. This is despite the existence of two inter-actin contacts in the actin-profilin crystal structure. These actin-actin contacts are quite different from those predicted by the Holmes filament model and their existence has resulted in a spirited debate over the possibility of an alternative model for the actin filament. The actin-actin differential interaction data is not consistent with the actin contacts formed in the actin-profilin crystal structure and therefore our data would not be consistent with filament models based on these actin contacts.

Actin dimers have been previously described in *in vitro* filament assembly reactions[24]. Two kinds of actin dimers have been identified. The so called 'lower dimer' forms during the lag phase of the reaction and is unable to nucleate filament assembly. The second dimer — the 'upper dimer' formed during the polymerization phase — is capable of nucleating filament assembly and is believed to be structurally similar to F-actin. The crosslink formed in the upper dimer is found to be between Cys 374 on one subunit and Lys 191 of the other subunit[25]. We believe these residues could be in close proximity in a back-to-back dimer such as the one we have invoked to explain our actin-actin differential interaction data. If there is correspondence between our structure and the upper dimer, then our system provides an opportunity to further explore the structure of this filament assembly intermediate.

In modelling our data, we have assumed that mutations which cause differential interactions have only localized effects on the structure. We recognize that in many cases the effects of the mutations may have more global and/or distant effects on the structure of actin. This does not appear to be the case for the Aip1p differential interaction data which maps to a strikingly distinct region on actin, or for the actin-actin differential interaction data which maps to the backside of the actin monomer. However, the situation for the profilin data is much more complex. Besides unpredictable effects on the structure, we should also consider that some of the mutations may effect ATP binding which in turn may result in differential interactions with some or many of the actin ligands tested in our system. This may be the case for some of the mutations that disturb the actin-profilin interaction, thereby resulting in the confusing pattern observed in the modelling of this data (Fig. 4).

It is also possible to make inferences about the consequences of particular mutations on protein structure *in vivo* using our methods. By assessing the ability of mutant actins to bind several different actin-binding proteins, we have been able to determine how generalized the mutations are in their effects. It is not surprising that many of the lethal alleles destroy all interactions tested while conversely many of the 'wild type' alleles fail to disrupt any of the interactions tested. This information is likely to be useful for deciding which mutant alleles should be used for further genetic analysis of local effects on the actin monomer surface. For instance, muta-

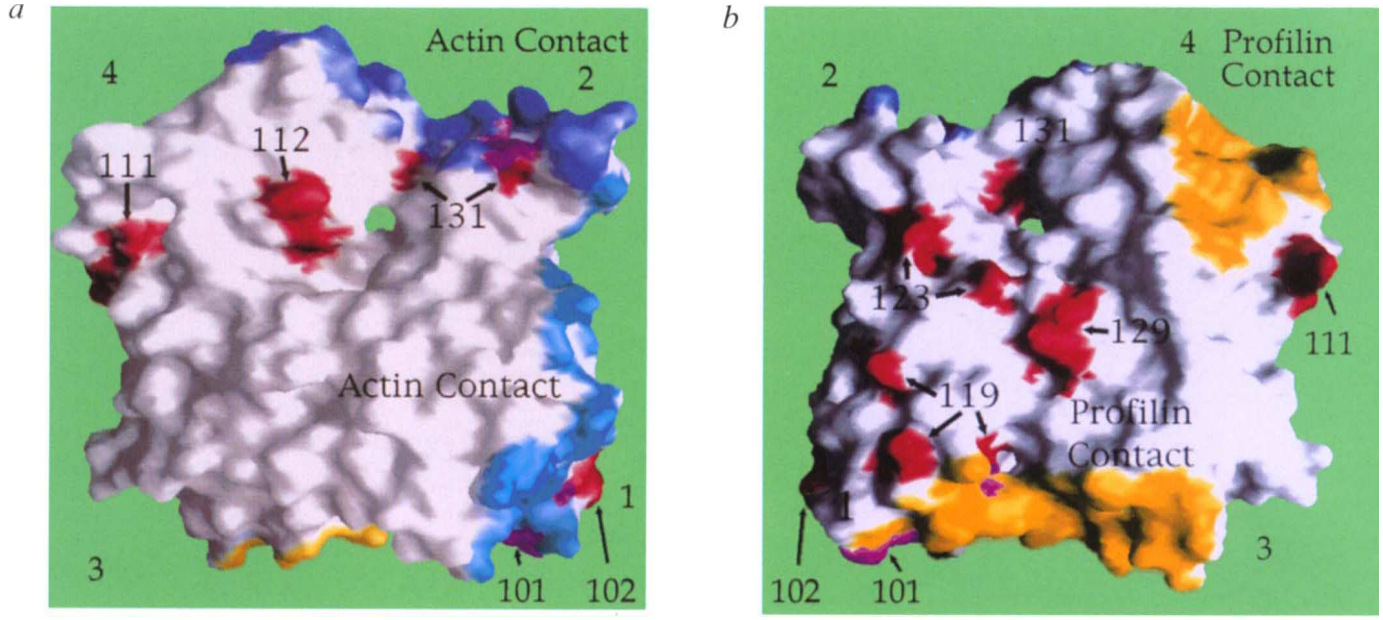

Fig. 4 Modelling of the actin-profilin differential interactions. The solvent-exposed surface of a single monomer of b-actin from the actin-profilin ribbon structure[21] is displayed. The two regions of actin that would be 3Å or closer to profilin in the ribbon structure are in yellow. The two regions of actin that would be 3Å or closer to other actin molecules in the riboon are shown in two shades of blue. Mutations that disrupt the actin-profilin interaction are shaded red and regions of overlap are lavender. *a*, The front view of the subunit is shown. *b*, The back view of a subunit is shown. Actin-profilin ribbon coordinates were provided by C. Schutt.

tions that affect only a restricted set of protein ligands are more likely to have phenotypes restricted to the functions of those ligands.

Biological implications

Most of the actin interacting proteins display distinct patterns of differential interactions. One of the most interesting findings we have made is that profilin, Rvs167p and Fus1p are exceptions to this rule, having very similar patterns. Profilin is an important regulator of actin assembly. It has been shown to sequester actin monomers, activate nucleotide exchange on actin, inhibit the actin ATPase, participate in assembly at the barbed end of actin filaments and play a role in the regulation of phospholipase C-γ1 (for reviews see refs 26, 27). On the other hand, Rvs167p and Fus1p are both SH3 domain containing proteins with no other apparent sequence similarities. SH3 domains - have been identified in a large number of proteins but the function of these domains has not yet been elucidated. They have been observed in proteins involved in tyrosine kinase pathways, signaling pathways involving small RAS-like GTPases and in proteins involved in the organization of the cortical actin cytoskeleton[14]. It has been postulated that these domains mediate the interaction of signal transduction pathways with the cortical actin cytoskeleton.

Our results however, suggest a more active role for these protein sequence motifs. The similarity of the differential interaction data sets between profilin and two SH3 domain-containing proteins indicates that SH3 domains may be actin-binding motifs that involve, directly or indirectly, interactions with actin like those found for profilin. This suggests that the binding of SH3 domains to actin may have similar consequences to profilin-actin binding and suggest a role in regulation of actin assembly. This result is not as surprising as one might think, as profilin and SH3 domains have other features in common. Both SH3 domains[28] and profilin[29] bind proline rich sequences. Even more compelling is the observation that the crystal structures of SH3 domains and profilin are similar[21, 30].

We have not ruled out an equally interesting explanation for similarity in differential interactions: namely, that the SH3-containing proteins are interacting with actin indirectly through profilin itself. We consider this explanation to be somewhat less likely than that of direct interaction for a reason related to the two-hybrid assay. A role for profilin in the assay would require profilin to exist in the nucleus, a compartment of the cell in which profilin is not normally found. Discrimination between these possibilities awaits direct measurements of actin binding by Rvs167p and Fus1p.

In contrast to our results with the SH3 domain proteins, we found that the pattern of differential interactions of Srv2p was very different from that of profilin. Only three of eleven differentially acting alleles are shared between the two data sets, making it unlikely that these proteins execute their functions by interacting with actin in the same manner. This is particularly interesting because the apparent function(s) of profilin and Srv2p are known to be inter-related, and in some ways quite analogous.

Our data provide insight into the relationship of Srv2p and actin. The phenotypes reported for the loss of the carboxy-terminus of Srv2p are typical of those observed in mutants of actin and actin-binding proteins[31, 32]. Therefore, it was not surprising that Srv2p binds to actin, although our results are the first indication of a direct interaction. Furthermore, the comparison between intact and truncated fusion proteins shows that it is indeed the C terminus of Srv2p that is involved in actin binding, as suggested from the mutant studies.

Only two of ten differentially-acting alleles are shared between the data sets for actin and the intact Srv2p fusion protein. This finding indicates that Srv2p binding does not require actin to dimerize, suggesting in turn that Srv2p may be a monomer-binding protein that regulates actin cytoskeleton assembly, in the same way that profilin does. Such a model is supported by the observation that overexpression of profilin suppresses the phenotypes associated with C-terminal truncations in Srv2p[33]. Combining these suggestions with the association of Srv2p with adenylate cyclase and its role in the display of activated RAS phenotypes[31, 32], one may envision that Srv2p directly couples the RAS pathway to regulation of actin cytoskeleton assembly.

Finally, we believe that defining differential interactions in the two-hybrid system, which is quite simple to do operationally, will become a generally useful approach for testing predictions made by X-ray crystallography. Since our methods assess protein interactions occurring within living cells, agreement between these genetic and physical techniques should be seen as providing strong mutual support for the particular interaction under investigation.

Methods

Plasmids and strains. pAS1-CYH2 (S. Elledge, personal communication) is a derivative of pAS1 (ref. 3) with the *CYH2* gene inserted upstream of the *ADH* promoter. pRB1508 is a CEN version of pAS1-CYH2. It was constructed by cutting pAS1-CYH2 with *Sac*I, blunting this site and then excising the 2622 bp, *GAL4-CYH2* cassette with a *Sal*I digest. This fragment was then cloned into the SalI and SmaI sites of the MCS of YCplac22 (S. Elledge, personal communication).

pRB1516 was constructed using the polymerase chain reaction (PCR). Use of the 5' primer (DAaO1; GGCCATGGCTG CCGCTATGGATTCTGGTATGTTCTAGCGC) results in the insertion of 3 alanine codons upstream from the initiator ATG. The 3' primer, DAaO2, has the sequence CTCGTCGACGATACACGG TCCAATGGATAAACA. Vent polymerase (New England Biolabs) and these primers were used to amplify yeast actin from plasmid pRB155, a pUC19-based plasmid which contains the 3.8 kb *Eco*RI genomic fragment of the yeast *ACT1* gene (unpublished). The resulting product was digested with *Nco*I and *Sal*I and inserted into the same sites of pRB1508. The correct sequence of the *ACT1* insert was confirmed by dideoxy sequencing.

The entire *RVS167* reading frame was inserted in triplicate by PCR into vector pRB1506 to create pDAb143, 144, and 145. 5' primer sequence is CTCAGATCTTGATGAGTTTTAAAGGGTTTA and 3' primer is GGGAATTCGCCCGTAATAGATAGACT. The *RVS167* reading frame was amplified off genomic DNA from strain FY23x86 (F. Winston, personal communication), in three separate reactions, using these primers and Vent polymerase. The fragment was digested with *Bgl*II and *Eco*RI and cloned into the same sites of pRB1506.

pRB1506 (a.k.a. pACTII); (S. Elledge, personal communication) is a derivative of pSE1107 (a.k.a. pACT)[3] which has an expanded

multi-cloning site. *FUS1* was inserted in triplicate into pRB1506 to create pDAb160, 161 and 162. 5' sequence is CGCCCATGGTGTCCATTTCAAATCCACCCATG and 3' primer sequence is CCGCTCGAGGGGTTGCCTTGGAGGCAATCGGCC.

All 36 actin alleles[2] were moved into pRB1508 in the same manner as used in the construction of pRB1516 except that the templates for the Vent amplifications were the derivatives of pKFW46 (ref. 2), carrying these alleles. Each construct was made in triplicate from three separate amplifications. Allele 118 was omitted from the analysis as it was found to contain additional mutations.

Strains Y190 and Y187 are *cyh2* marked derivatives of Y150 and 153 (ref. 3).

Two-hybrid screen. The λYES cDNA library (S. Elledge, personal communication) was titered and amplified in bacterial strain LE392. Plasmid inserts were excised from the λYES cDNA library by infection of strain BNN132 (ref. 34). The screening of the library was carried out in two separate experiments. The conditions for the first experiment were as follows. Strain Y190 was co-transformed with pRB1516 plus library DNA by a lithium acetate based protocol (S. Elledge, personal communication) and plated on SD + 10μgml^{-1} adenine plus 50 mM 3-aminotriazole. The plates were incubated at 25 °C for nine days at which time cells with activated *HIS3* expression were clearly evident above background. In the second library screening strain Y190, already carrying pRB1516, was transformed in the same manner with library DNA but was plated on 100 mM 3-aminotriazole-containing media. The plates were incubated at 25 °C for 14 days. In total, approximately 5 x 10^6 transformants were screened in this manner.

His3p selections were done in SD medium containing only 10 μg ml^{-1} adenine plus 25, 50, or 100 mM 3-amino-1,2,4-triazole.

To assess β-galactosidase activity, yeast strains were patched on SC-Trp-Leu-Ura or SC-Trp-Ura. These patches were lifted onto Schleicher and Schuell, BA85 nitrocellulose filters (cat# 20440) and the filters were immersed in liquid nitrogen for 10 s. The filters were then placed on Whatman paper soaked with 1 mg ml^{-1} 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (Clontech) in 0.1M $NaHPO_4$/0.01M KCl/0.001M $MgSO_4$/0.04M 2-mercaptoethanol pH 7. The filters were then incubated at 30 °C.

Specificity was tested by mating strain Y190 carrying pRB1516 with strain Y187 carrying pAS1-CDK2, pAS1-lamin, pAS1-p53 and pAS1-rev (a gift of S. Elledge). Diploids were selected and His3p or β-galactosidase expression was examined. Solo activation was tested by streaking strain Y190 carrying the library isolate plus pRB1516 on SC-LEU + 5 μg ml^{-1} cycloheximide plates. The resulting strains were confirmed as having lost the *TRP1/CYH2* marked plasmid, pRB1516 by a failure to grow on SC-TRP media and were similarly tested for activation of His3p or β-galactosidase expression.

Double stranded, dideoxy sequencing was performed with the Sequenase reagent kit (U.S. Biochemical). Primer 2-H1 (TGATGAAGATACCCCACC) was used to sequence into the 5' ends of the AIP library inserts. Primer 2-H4 (GCGACCTCATGCTATACC) was used to sequence into the 3' ends of the AIP library inserts.

Differential interaction screen. Fusions to the activation domain of *GAL4* in vector pSE1107 were transformed into strain Y187 by electroporation. Similarly, pRB1516, pAS1-lamin and all the actin alleles fused to the DNA-binding domain of pRB1508 (triplicate constructs) were transformed into strain Y190. Multiple colonies of the primary transformants were picked and mixed in 25% glycerol. These cells suspensions were then gridded into 96 well microtiter plates and mixed in all possible combinations. 1.5μl of these mixed cell suspensions were spotted on YPD plates and incubated overnight at 30 °C to allow mating. The next day the mated cells were replica plated to SD + Ade and plates containing 25, 50 or 100 mM 3-aminotriazole and incubated at 25° and/or 30 °C. This experiment was repeated a second time, except only single isolates of the actin allele fusions were used. Added to the analysis were triplicate constructs carrying a fusion of *FUS1* to the *GAL4* activation domain of plasmid pRB1506 and triplicate constructs of the entire *RVS167* reading frame fused to the activation domain of pRB1506. We retested a third time by replica plating selected diploids from the SD + Ade plates of the second experiment, directly onto the aminotriazole containing media and incubating these plates at 30 °C. The results of this third analysis cleared up any discrepancies from the first two experiments. Since the truncated Srv2p fusion of pAIP10 appeared to form a very strong interaction with actin, differential interactions were scored by incubating plates containing 100 mM 3-aminotriazole at 35 °C and by scoring on media containing 200 mM 3-aminotriazole at 30 °C.

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