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TWO-HYBRID
SYSTEM**

Edited by
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& Stanley Fields

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Obtaining Structural Information about Protein Complexes with the Two-Hybrid System

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The ability of the two-hybrid system to detect biologically relevant protein-protein interactions has been well established. When this system identifies a biologically relevant protein-protein interaction, a natural assumption is that the fundamental nature of this interaction is the same as when it occurs in its normal functional context. Indeed, the successful discoveries of a large number of protein interactions through use of the two-hybrid system should be taken as evidence supporting this assumption. Once this proposition is accepted, it follows that the two-hybrid system can be used not only to detect protein-protein interactions but also to study *how* proteins interact.

The most direct approach to obtaining information on the residues of a protein that are important in binding a ligand is to solve the structure of cocrystals. Even in these favorable cases, it has often been necessary to corroborate these structural studies with genetic assays, which confirm the amino acids that form critical stabilizing contacts by the identification of mutations that cause a failure to bind. Indeed, one can often use mutations in conjunction with *in vitro* binding assays to make inferences about the mechanisms of binding without the labor of solving cocrystals (for an example, see Honts et al. 1994). These genetic and biochemical methods, although less arduous than X-ray crystallography, require that all the mutant proteins be purified and that the interaction be strong enough to be detected under possibly suboptimal *in vitro* conditions. We have shown that this kind

of information can be obtained by substituting the two-hybrid system for in vitro assay systems and obtain at least as much information about the structural requirements for binding with considerably less effort.

Our method of differential interactions requires that the interaction be amenable to two-hybrid analysis, that a three-dimensional structure exist of at least one of the protein ligands, and that an appropriate set of mutants exists in one or both of the binding partners. The advantages of this method are: the interactions are studied *in vivo*, weak interactions can be analyzed (apparent $K_d > 1\mu\text{M}$; Phizicky and Fields 1995) as well as strong ones, and data can be obtained very rapidly with much less effort than more traditional methods.

In this chapter, we explain how the two-hybrid system can be used to describe interactions between yeast actin and several yeast actin ligands. Besides covering the mechanistic details of this analysis we will show examples of the data obtained using a large set of rationally designed mutations.

Construction of Suitable Mutations

Mutations can frequently affect the structure of a protein. How generalized these effects are will vary greatly from mutation to mutation in ways that may be difficult to predict. Since the goal is to use the mutations to try to delineate binding sites, one wants to use mutations that have only localized effects on the structure. It is, therefore, critical that the mutations be carefully designed to minimize structural disturbances. If the three-dimensional structure of the protein of interest is known, then rational decisions can be made in the design of these mutations. Two general philosophies might be used: alter charged amino acids on the surface of the protein in the hope of disrupting important ionic interactions; alternatively, alter surface exposed hydrophobic residues in the hope of disrupting hydrophobic interactions. The importance of hydrophobic interactions for protein-protein interactions is well established and has been made clear by work solving the structures of dimeric proteins (Green et al. 1995; Potts et al. 1995).

When the mutations that were employed in our actin analysis were constructed, the three-dimensional structure of actin was still unknown. The actin alleles were constructed by clustered charged-to-alanine scanning mutagenesis, a rational mutagenesis procedure designed to target surface exposed residues in proteins of unknown structure. In this method, the protein sequence is scanned in a window of five amino acids; when two or more charged amino acids appear in a window, they are altered to alanine (Bass et al. 1991; Bennett et al. 1991). This method yielded 36 mutant alleles of actin (Wertman et al. 1992). When the structure for actin was published, we discovered that the mutagenesis algorithm had worked well: all but one of the alleles altered surface exposed amino acids (Kabsch et al. 1990). More recently, many of these mutant proteins have been purified and assembled into filaments *in vitro*, indicating that their structure is not

grossly damaged (Miller et al. 1995; Miller and Reisler, 1995). Clearly, these mutants were well suited for the two-hybrid analysis, or indeed any analysis of protein ligand binding.

It should be noted that charged-to-alanine scanning mutagenesis of yeast calmodulin did not have the same happy result. None of the calmodulin alleles constructed in this manner caused mutant phenotypes, indicating that in vivo the mutations had no effect on the important interactions calmodulin makes with other proteins. However, when phenylalanines (chosen on the basis of knowledge of calmodulin structure and interactions with peptide ligands; Ikura et al. 1992; Meador et al. 1992) were systematically changed to alanine, many mutants with interpretable phenotypes were obtained. These would be the most appropriate reagents for a structurally based two-hybrid analysis of calmodulin interactions (Ohya and Botstein 1994a, 1994b).

In summary, the best strategy would be to make mutations covering the surface of the protein of interest using knowledge of the protein's three-dimensional structure. Lacking such information, charged-to-alanine mutagenesis has been successful for a variety of proteins, including alpha- and beta-tubulin (Reijo et al. 1994; K. Richards and D. Botstein, personal communication), tissue plasminogen activator (Bennett et al. 1991), poliovirus (Diamond and Kirkegaard 1994) and yeast signal peptidase subunit Sec11p (E. Beasley and D. Botstein, personal communication).

Once one has decided which mutations to construct, a number of methods exist for the construction of these alleles. In the case of the actin alanine scan alleles, we chose to use the Kunkel method (Kunkel et al. 1987). This and other methods have been fully described elsewhere (for example, see Sambrook et al. 1989). Movement of the mutant alleles into the two-hybrid vector can be done by standard subcloning methods; however, we have found a recombination strategy particularly convenient. When one transforms yeast with overlapping DNA fragments, one fragment carrying the bulk of the vector backbone and the other carrying the mutant allele, the recipient yeast cells will correctly reconstruct the plasmid by homologous recombination (Ma et al. 1987). In this way, passage of the new plasmid through *Escherichia coli* is entirely avoided.

Since the actin alleles were not constructed with the two-hybrid system in mind, we were forced to use a PCR strategy to move the alleles into the two-hybrid DNA-binding domain vector. To detect and avoid PCR-induced errors, each allele was amplified in three separate PCR reactions from which three independent clones were derived. All three isolates were then tested in the two-hybrid system. PCR-induced errors did occur frequently enough to present a problem: in 7 of the 36 alleles, one of the three independent isolates behaved differently from the other two. In all seven cases, the dissimilar constructions failed to interact with any actin ligands; no doubt, they contained PCR-induced mutations with a disastrous effect on the actin structure. This result allows us to re-emphasize the importance of

the proper choice of mutant alleles: most uncritically obtained alleles will be unsuitable either because they have no effect on the structure or because the effect is not localized enough.

Details concerning the construction of the “bait” construct have been covered in (chapters 2, 3, and 4). Our approach with yeast actin was to make a Gal4p fusion to the N-terminus of actin and to insert a 4-residue alanine linker between the actin and Gal4p sequences to decrease the likelihood that the Gal4p sequences would block access of actin ligands to the actin surface. We feel that our concern was justified as the only activation domain fusion with actin found to dimerize with our bait actin fusion carried 24 additional amino acid residues between the actin and Gal4p sequences. It would probably be prudent in any new endeavor to investigate a variety of linker lengths.

Identification and Construction of Ligand-Gal4p Fusions

One may already know what ligand(s) the protein of interest interacts with. In this case, ligand sequences can be moved directly into the two-hybrid activation domain vectors and tested for their ability to interact with the bait Gal4p DNA-binding domain fusion prior to undertaking an extensive mutagenesis of the bait sequences. If the Gal4p domains interfere with the interactions of interest, a considerable amount of trying various linker lengths may be required.

If one has not already determined which proteins interact, one starts by screening a library of fusions to the Gal4p activation domain (see chapters 2, 3, and 4). This will not only identify potential ligand proteins, but will also provide them as activation domain fusions that are capable of interaction. In addition, use of the library may provide additional information such as the identity of the interaction domain within a putative ligand protein.

A library approach should provide a number of constructs with different fusion junctions all capable of interacting with the bait. When these constructs are used with the mutant forms of the bait, there should be strong agreement as to which mutations disrupt the bait-ligand interaction, regardless of the fusion junction. In our actin analysis, we generally found this to be the case.

Two-Hybrid Analysis with Mutants

Analysis of many mutant alleles and many putative ligand constructs in all pairwise combinations means that a very large number of comparisons must be made. The most direct and easy way to examine and compare interactions among a large number of mutants and a large number of ligands is to introduce the DNA-binding domain and activation domain plasmids into the same cells via mating of haploid yeast cells. The DNA-binding domain fusions bearing actin mutants (in triplicate) were transformed into

haploid cells of one mating type while the constructs bearing ligand fusions to the Gal4p activation domain were transformed into yeast of the opposite mating type. The transformant strains were then mixed into small volumes (20 μ l) of sterile water and pipetted into the wells of microtiter dishes and arranged so that transformants in one mating type were arranged in the columns of the microtiter dishes while those in the opposite mating type were placed in the rows of the microtiter dishes. This can all be done with a multichannel pipettor if one takes care to avoid cross-contamination between wells. The mixed cells can then be spotted (using either a multichannel pipettor or a multipronged implement called a "frog") onto a plate on which all the strains can grow and mate efficiently (such as YEPD; see chapters 2, 3, and 4); selection for plasmids appears generally not to be required. On the next day, the mated cells are replica-plated onto selective plates in which only diploids bearing both a DNA-binding domain plasmid and activation domain plasmid will be able to grow.

Once the diploids have grown into colonies, they can be assessed for activation of the two-hybrid reporters and, most importantly, the degree of activation can be compared among the whole set. We found that the β -galactosidase reporter was too variable for this type of analysis, although it is a useful and comforting confirmatory test. We favor using the His3p enzyme reporter. Enzyme levels are assessed by the ability of yeast to grow on minimal plates containing an inhibitor of the His3p enzyme, 3-amino-1,2,4-triazole (3-AT). We found that plate media containing 25, 50, or 100 mM 3-AT were adequate to assess the relative levels of activation resulting from interaction between the ligand-Gal4p activation domain fusion and the actin bait variants. The 3-AT selection is tightest when minimal media containing low adenine concentrations are used, presumably because adenine is a potential source of imidazole. In our experiments, we used strains Y187 and Y190 (Durfee et al. 1993) allowing the 3-AT selections to be done on SD medium containing 10 μ g/ml adenine.

In our two-hybrid analysis, we examined combinations of 36 actin alleles (including wild type, an absolutely essential control on every plate) and 10 ligands for interaction, for a grand total of 360 assessments of protein interaction. We performed 36 (plus wild type) assays per plate and thus could examine all 360 combinations at three different 3-AT concentrations on a total of 30 plates.

Analysis of Differential Interaction Data

One can view a data set of differential interactions with a given ligand and a large number of mutant alleles from a variety of perspectives. One of these concerns the behavior of each mutant allele; another concerns which mutations affect the interaction with a given ligand; and the third concerns the spatial relationships among mutations that have common effects on interaction with a given ligand.

In our experiments with actin, mutant alleles could be placed into three groups based on how they behaved with the 10 ligands. The first class of alleles included those that encoded actin proteins that bound all the ligands as well as wild-type actin did. The second class comprised those that failed to bind any of the ligands. The third class contained those that bound some of the ligands as well as wild-type and other ligands poorly or not at all. It is this last class that is the most informative about protein-protein interactions.

Surprisingly, there was a correlation between the two-hybrid phenotype of the mutants and the severity of the growth defects of strains bearing the mutant alleles. Those of the first class, whose products bound all ligands well, were, with only one exception, alleles with no growth defect relative to yeast expressing wild-type actin. The surface characteristics and structure are presumably unperturbed in these mutant proteins. Alleles of the second class were, with one exception, recessive lethal alleles that presumably encode either extremely unstable proteins and/or proteins that are folded very differently than the wild-type protein.

Alleles of the third class that encode proteins that bind some ligands but not others, are less generalized in their effects on the surface and structure of actin than those mutations of the second class. It is from these mutations that we expected to extract structural information about the actin-ligand complexes. A more conservative view might be that only those mutations that affect a few interactions should be considered. In any case, one is tempted to weight more heavily the behavior of these alleles in developing a hypothesis about a ligand's binding site.

Figure 6-1 shows a bar graph of the mutations, in order of decreasing severity of their growth phenotypes, relative to how many interactions they disrupt in the two-hybrid system. There is a clear correlation between the severity of the two-hybrid defects and the severity of their growth defects. Interestingly, presumed dominant mutants affect few interactions, and, therefore, may be sequestering or otherwise inhibiting essential components of the actin cytoskeleton. Since actin self-assembles into filamentous actin (F-actin), it is possible that the dominant mutants might poison actin assembly.

We found that the data for a given ligand are generally internally consistent when modeled on representations of the known structure of actin. In some cases where information already exists about interactions between actin and a protein ligand, the data obtained by two-hybrid analysis are in agreement. Figure 6-2 shows a surface representation of the back of monomeric actin (G-actin). In dark gray (and outlined in black) are the mutations that affect the two-hybrid interaction between actin and Oye2p and in light gray are those mutations that have no effect. The data are internally consistent in that all the mutations are located very near each other in the structure of actin. The data are clearly informative: the region of actin

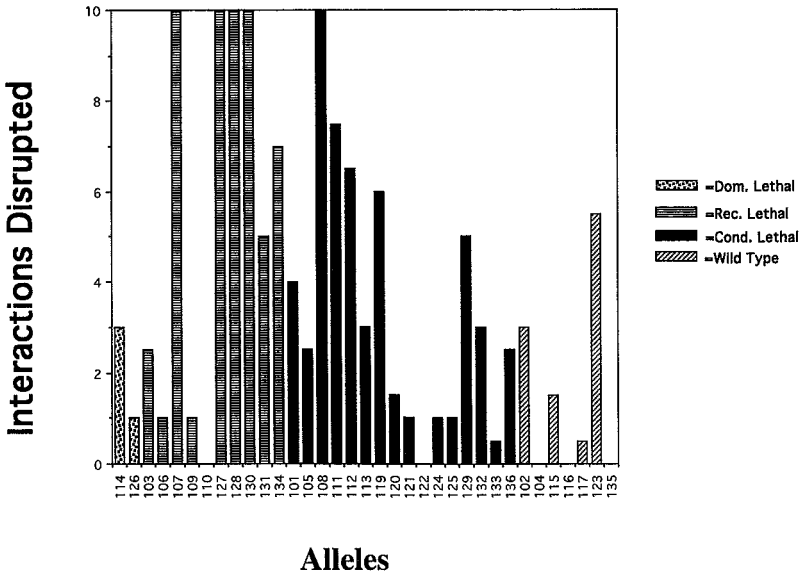


Fig. 6-1. Number of ligand interactions disrupted by the actin mutants. This bar graph displays the number of ligand interactions disrupted in the two-hybrid system by the actin alanine scan alleles. The growth phenotypes of the alleles are indicated and coded according to the accompanying key at right and the alleles have been grouped by decreasing severity of growth phenotypes moving from left to right.

identified is completely buried in F-actin and therefore Oye2p is expected to be an obligate G-actin binding protein.

The equipment used for modeling of our two-hybrid data was a Silicon Graphics Iris running GRASP (Nicholls et al. 1991) software. We have also used the Insight II (Biosym Technologies) software but we found GRASP to be more adept at rendering molecular surfaces. Complete information about all these programs are beyond the scope of this review and can be found elsewhere.

Prospects

We have found additional benefits to the construction of our two-hybrid reagents. Clearly, the analysis can be extended simply with any additional actin ligands we or others identify, because of our use of the mating of yeast to bring the interacting proteins together. Although we have yet to test this hypothesis, we expect that our yeast actin fusion will be capable of interacting with actin-binding proteins from organisms other than yeast. Many protein-protein interactions have been evolutionarily conserved and

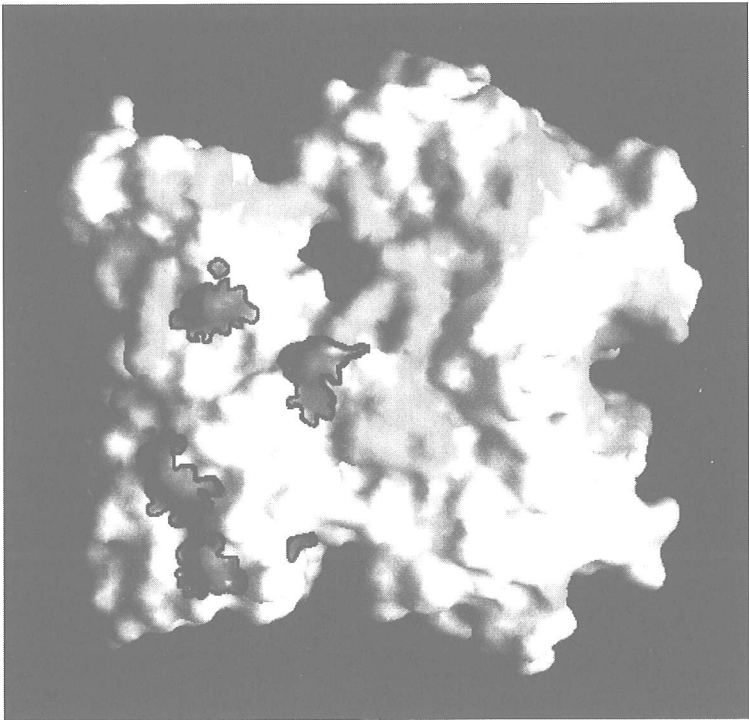


Fig. 6-2. Modeling of actin mutants that disrupt the actin-Oye2p interaction. Displayed is the calculated, solvent exposed surface of the back of G-actin. Mutants that disrupt the interaction between actin and Oye2p in the two-hybrid system are outlined in black and indicated in dark gray. Mutants that have no effect on the interaction are shown in light gray. This rendering was executed with GRASP software (Nicholls et al. 1991) on a Silicon Graphics Iris Computer.

we expect that in some cases the mechanics of these interactions are conserved as well.

We anticipate using the two-hybrid constructs to identify new mutations in either actin or the ligands that precisely affect particular interactions. Reintroduction of these mutants into yeast will then allow us to determine the functional significance of certain actin-ligand interactions. If the three-dimensional structure of the ligand is known, then the analysis affords the possibility of identifying the actin-binding site on the ligand. Such information will be very useful for modeling entire complexes and should lead to testable hypotheses concerning their structures.

Clearly, the two-hybrid system can be used for much more than merely detecting relevant protein-protein interactions and can be adapted to study the nature of protein-protein interactions as well.

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