9 Molecular Matchmaking: Techniques for Biomolecular Interactions

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9.1 Introduction

Protein interactions play pivotal roles in virtually all the cellular processes. They are intrinsic to every cellular process, ranging from DNA replication, transcription, splicing, and translation, to secretion, cell cycle control, signal transduction, metabolism, formation of cellular macrostructures, and enzymatic complexes. Thus the identification of protein–protein interactions remains fascinating and very helpful in understanding biological phenomena.

9.2 Tools for the Study of Protein–Protein Interactions

In recent years, the convergence of biochemistry, cellular, and molecular biology has made available a number of powerful techniques for studying such interactions. Together, these constitute an impressive collection of tools for studying interactions among proteins. These techniques vary in their sensitivity, efficiency, and rapidity, but judicial deployment of a combination of them has proved to be effective and reliable.

Two broad approaches are generally applied to the study of protein–protein interactions: experimental and computational. Computational methods (Valencia and Pazos 2002) are used to infer protein interaction networks and predict the function of proteins. When the molecular structure of two proteins is known, the molecular prediction (or docking problem) of protein interactions

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can be analyzed. Therefore, as more genomic, structural and protein interaction data become available, the ability to predict protein interactions in silico is strengthened. The experimental approaches include physical/biochemical, genetic and biophysical methods to select and detect proteins that bind another protein. Traditionally, the tools available to analyze protein–protein interactions in multicellular organisms have been restricted to biochemical (also referred to as physical methods) approaches. However, despite obvious advantages, biochemical approaches can be time-consuming. Biochemical methods that detect proteins that bind to other proteins generally result in the appearance of a band on a polyacrylamide gel. Under this category, protein affinity chromatography, affinity blotting, co-immunoprecipitation, far-westerns, cross-linking are popular techniques to detect proteins that interact with a known protein (Phizicky and Fields 1995). Certain spectroscopic techniques, including fluorescence polarization spectroscopy (FPS), surface plasmon resonance, and mass spectroscopy, are used for several cases of protein interactions. Biacore's surface plasmon resonance technology has become widely popular. This is a label-free technology for monitoring biomolecular interactions as they occur. It also uses spectroscopy to measure changes in molecular size. The instrument monitors changes in refractive index that occur at a liquid/metal interface when biomolecules interact. Several new fluorescent imaging-based biophysical techniques are also available for studying protein–protein interactions, such as fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), fluorescence correlation spectroscopy, and biomolecular fluorescence complementation (Boute et al. 2002). Other widely applicable methods are library-based methods. A variety of methods have been developed to screen large libraries for genes or fragments of genes whose products may interact with a protein of interest. As these methods are by their nature highly qualitative, the interactions identified must be subsequently confirmed by biochemical approaches. Library screens are generally performed in bacteria or yeasts, organisms with rapid doubling times. Thus, these procedures can be completed rapidly. Protein probing and phage display are common library screening techniques. Protein probing uses a labeled protein as a probe to screen an expression library in order to identify genes encoding interacting proteins. Since all combinations of protein–protein interactions are assayed, including those that might never occur in vivo, the possibility of identifying artifactual partners exists and is a typical disadvantage of most exhaustive screening procedures. A second drawback derives from the use of a bacterial host, where not all post-translational modifications needed for the interaction might occur. Despite obvious advantages, biochemical approaches can be tedious and time-consuming. Also coming along the pike is the application of microarrays and protein chips to protein–protein interactions (MacBeath and Schreiber 2000). All in vitro methods suffer from one common drawback, i.e., the genes encoding the interacting proteins are not readily available. An answer to this problem was the introduction of the yeast two-hybrid system by Fields and Song in 1989.

Currently, the yeast two-hybrid system is the most widely used genetic assay for the detection of protein–protein interactions (Fields and Sternglanz 1994; Fashena et al. 2000; Bartel and Fields, 1995). The yeast two-hybrid system has become popular because it requires little individual optimization and because, compared with conventional biochemical methods, the identification and characterization of protein–protein interactions can be completed in a relatively short time-span and is inexpensive. Most importantly, novel protein–protein interactions can be easily selected from a pool of potential interaction partners (e.g., a cDNA expression library; Gyuris et al. 1991; Chevray and Nathans 1992) and genetic systems not only yield information on the interaction itself but also directly provide the cDNA encoding the novel interaction partner. Furthermore, no previous knowledge about the interacting proteins is necessary for a screen to be performed. Since its conception, the two-hybrid system has become one of the most widely used experimental methods. The basic method is constantly being improved and widely used with a range of improvements and modifications to overcome drawbacks and limitations. It is no longer applicable to study only protein–protein interactions but has been extended to allow screening for DNA and RNA interactions, assaying interactions in the cytosol rather than being limited to the nucleus, and screening in bacterial or mammalian hosts.

9.2.1 The Two-Hybrid System

The classic two-hybrid assay exploits the modular nature of the yeast *Saccharomyces cerevisiae* transcriptional activator, GAL4, required for the expression of genes encoding enzymes for galactose utilization (Johnson 1987). GAL4 consists of two separable and functionally distinct essential domains: (a) the DNA binding domain (DBD; Keegan et al. 1986) which binds to specific DNA sequences [upstream activation sequences (UAS; Giniger et al. 1985)] in GAL4 responsive promoters, and (b) a transcription activation domain (TAD; Ma and Ptashne 1987) required for the transcriptional activation of the GAL4 responsive genes. Theoretically the two-hybrid principle is very straightforward. To study interaction between two proteins X and Y, protein X (the bait) is fused in-frame to DBD and protein Y (the prey) is fused to the TAD, where either hybrid protein alone fails to activate the transcription. The bait and prey fusions are co-expressed in yeast, where the interaction of proteins X and Y reconstitutes the proximity of GAL4 domains, reconstituting a functional transcription factor, and transcription of downstream reporter occurs. Commonly, auxotrophic markers that can be selected for are used in combination with the *lacZ* gene encoding the bacterial β-galactosidase. The common auxotrophic markers HIS3 and LEU2 allow the selection of interactions by monitoring growth on selective plates lacking histidine or leucine, respectively, whereas lacZ can be easily measured using a colorimetric assay.

9.2.2 The Split-Ubiquitin System

This is a genetic technique, based on the split-ubiquitin system (Johnsson and Varshavsky 1994a, b; Stagljar et al. 1998), which offers the advantage that it can be used to detect interactions between virtually any type of protein in the cell – that is, between two integral membrane proteins, between a membrane protein and a cytoplasmic protein, or between two cytoplasmic proteins, provided that one of them is artificially anchored to the membrane. To date, this system is the most widely used of the alternative yeast-based two-hybrid systems.

The split-ubiquitin system is an alternative assay for the in vivo analysis of protein interactions. The system pioneered/proposed by Johnsson and Varshavsky (1994a) was originally developed to detect interactions between soluble proteins and later modified to work with membrane proteins.

9.2.3 Reverse Two-Hybrid System

In this system, the conventional yeast two-hybrid system has been modified to allow genetic selection of events responsible for the dissociation of particular interactions, e.g., mutations, drugs, or competing proteins. For the reverse two-hybrid system, yeast strains are generated such that the expression of interacting hybrid proteins increases the expression of a counter-selectable marker that is toxic under particular conditions (negative selection; Vidal et al. 1996a). Under these conditions, dissociation of the interaction provides a selective advantage (as the counter-selectable marker is no longer expressed), thereby facilitating detection: a few growing yeast colonies in which hybrid proteins fail to interact can be identified among millions of non-growing colonies expressing interacting hybrid proteins. This system has a variety of uses. For example, mutations that prevent an interaction can be selected from large libraries of randomly generated alleles (Vidal et al. 1996b). Similarly, molecules that dissociate or prevent an interaction can be selected from large libraries of peptides or compounds.

9.2.4 Sos Recruitment System (Cyto Trap Yeast Two-Hybrid System)

This system was developed by Aronheim et al. (1994, 1997). It is another modification of the yeast two-hybrid system to bypass the reconstitution of transcription factor and takes advantage of a cell proliferation signaling pathway. In this

system, the protein–protein interactions are artificially tethered to yeast cell membranes. Interaction is detected by activation of the Ras signal transduction cascade by localizing a signal pathway component, human Sos (h-Sos), to its site of activation in the yeast plasma membrane.

9.2.5 Yeast One-Hybrid System

The one-hybrid system is an extension, by simplification, of the two-hybrid concept. The yeast one-hybrid or single hybrid system is a genetic system to identify DNA binding proteins. It provides a genetic screen to identify cDNAs encoding polypeptides that bind short sequences (motifs) of DNA, usually *cis*-acting regulatory elements of expressed genes (Li and Herskowitz 1993; Inouye et al. 1994). In this method also, the bipartite structure of the yeast transcription factor GAL4 is exploited. Each cDNA in the library being explored is expressed as a fusion protein with the activation domain of the GAL4 protein. This fusion protein interacts directly with a DNA binding site/target element and transactivates reporter genes (*HIS3*, *lacZ*). The usual upstream activating sequences (within the promoters of these reporter genes) in the yeast two-hybrid systems are replaced by the target DNA motif. This motif is introduced in multiple copies to provide increased sensitivity to the screen.

9.2.6 Double Interaction Screen

Yu et al. (1999) developed the double interaction screen (DIS) to identify partners of DNA binding transcription factors. DIS is a modification that combines yeast two-hybrid and one-hybrid screens, used to identify partners of DNA binding transcription factors. As in the one-hybrid screen, a *cis*-acting regulatory element is cloned upstream of reporter genes *lacZ* and *HIS3*. This DNA motif is known to be a direct target of the transcription factor (TF) in question, i.e., protein X, and also contains binding sites for other transcription factors whose activities are independent of protein X. Thus, two baits are available in the screen, the *cis*-regulatory element itself, [which is used in the first screen to "anchor" a native full length TF (protein X) to DNA upstream of reporter gene] and X anchored to the regulatory element via native binding sites. Next, screening of the cDNA library allows identification of three types of proteins: (a) DNA binding proteins that interact directly with the regulatory element, (b) protein bait partners that also bind to specific DNA sequences, and (c) protein bait partners that interact only at the protein level.

9.2.7
Yeast Three-Hybrid or Tri-Hybrid System

Different cellular mechanisms often involve interactions between more than two proteins. The three-hybrid system is based on the reconstitution of a transcriptional activator complex either to search for or to study a protein that interacts with two others, providing information about ternary complexes. The technique detects either direct or mediated interactions between two fusion proteins. As in the yeast two-hybrid system, one protein is a fusion with DBD (that is DBD-X) and the other with the AD (that is AD-Y) of the GAL4 proteins. Different variations that involve third partners as native proteins, in the absence of any fused domains, are referred to as "tribrid" systems. The third protein can act either as a bridging factor (it interacts with both X and Y, which alone do not interact with each other), a stabilizing factor (it promotes/induces/strengthens the weakly interacting proteins X and Y), or as a regulating factor (it post-translationally modifies X and/or Y in order for them to interact, and in this case it may not necessarily be part of the reconstituted transcriptional activator). In either case, the third partner allows transcriptional activator formation and stimulates reporter gene transcription by the reconstituted transcription factor. Hence, the interaction between X and Y is mediated by the third protein. Another utility of the three-hybrid system is that, if X and Y interact and reconstitute the transcription factor, the system can be used to search for inhibitors. The three-hybrid system actually encompasses a range of different systems to study RNA–protein, small organic ligand–receptor or protein–protein interactions, which all have in common the basic principle of the two-hybrid systems but are mediated by a third partner. These third partners are quite diverse, from proteins to small molecules and nucleic acids.

9.3
Procedure

1. Take 50 µl of freshly grown appropriate yeast reporter strain. Inoculate into a 250-ml baffled flask containing 100 ml of YPD. Place on shaker at 30 °C with shaking (150 rpm) overnight.
2. Check cell density of $1–4\times10^7$ using a spectrophotometer ($OD_{600} = 1.00$).
3. Transfer cells into two 50-ml sterile falcon tubes and centrifuge at 3000 rpm for 2 min at room temperature.
4. Resuspend the cell pellet with 10 ml of Lithium acetate (LiAc) solution, centrifuge at 3000 rpm for 5 min, and discard the supernatant.
5. Resuspend cells in 500 µl of LiAc solution with gentle shaking and store tubes in ice until further use.

6. Take 100 µl of cells in a sterile micro centrifuge tube, add 10 µl of plasmid DNA, mix well, and incubate at room temperature for 5 min.
7. Add 280 µl of PEG 3350 solution and mix by inverting the tube 4–6 times.
8. Incubate at 30 °C for 45 min.
9. Add 43 µl of DMSO and mix by inverting the tube 4–6 times.
10. Heat shock at 42 °C for 5 min, chill on ice for 1–2 min.
11. Centrifuge at 4000 rpm for 1 min at room temperature and resuspend cells in 0.1 ml of sterile H_2O.
12. Spread plate transformation mix on selective media plates and incubate at 30 °C for 3 nights.
13. Pick the largest colonies and restreak them on the same selection medium for master plates. Plates sealed with parafilm may be stored at 4 °C for 3–4 weeks.

9.3.1 Reagents, Materials, and Equipment

Regular molecular biology laboratory equipment, like microcentrifuge, incubator, water bath, and a laminar hood.

9.3.1.1 Reagents and Materials

YPD or the appropriate SD liquid medium, sterile 1xTE/LiAc (prepare immediately prior to use from 10× stocks), sterile 1.5-ml micro centrifuge tubes for the transformation, appropriate SD agar plates (100-mm plates), appropriate plasmid DNA in solution, appropriate yeast reporter strain for making competent cells, Herring Testes carrier DNA (10 mg/ml; denature the carrier DNA by placing it in boiling water for 20 min and immediately cool it on ice), sterile 40–50% PEG-LiAc solution (make PEG solution in 1× 0.1 M LiAc), 10× TE buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5, autoclaved), 0.1 M LiAc, 100% DMSO, glass spreader to spread cells on plates.

9.3.1.2 Composition of Reagents

1. YPD medium: yeast extract (1 g/100 ml), peptone (2 g/100 ml), dextrose (2 g/100 ml).

2. YPD plates: yeast extract (1 g/100 ml), peptone (2 g/100 ml), dextrose (2 g/100 ml), agar (2 g/100 ml).
3. LiAc solution: 0.1 M LiAc (0.1 g/10 ml), 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (50 µl/10 ml).
4. 50% PEG 3350 solution: 50% PEG 3350 in LiAc solution.
5. 10× dropout (SD) LT^-: YNB (1.87 g/250 ml), dextrose (5.0 g/250 ml), agar 5.0 g/250 ml, amino acid mixture* (25 ml/250 ml), H_2O (225 ml), histidine (500 µl).
6. 10× dropout (SD) LTH^-: YNB (1.87 g/250 ml), dextrose (5.0 g/250 ml), agar (5.0 g/250 ml), amino acid mixture* (25 ml/250 ml), H_2O (225 ml).
7. 10× TE pH 8.0: 10 mM Tris-HCl (6.0578 g), 1 mM EDTA (1.8612 g).

* Amino acid mixture: L-isoleucine (300 mg/l), L-valine (1500 mg/l), L-adenine hemisulfate (200 mg/l), L-arginine HCl (200 mg/l), L-lysine HCl (300 mg/l), L-methionine (200 mg/l), L-phenylalanine (500 mg/l), L-threonine (2000 mg/l), L-tyrosine (300 mg/l), L-uracil (200 mg/l).

9.3.2 Notes and Points to Watch

- For the highest transformation efficiency, use the competent cells within 1 h of their preparation.
- Prepare the media plates in advance and allow them to dry at room temperature for 2–3 days.
- To obtain even growth on plates, continue to spread the transformation mix over the agar surface until all liquid has been absorbed.
- Calf thymus DNA is not recommended as carrier DNA.

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