Progress and variations in two-hybrid and three-hybrid technologies

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The original yeast two-hybrid system and its variants have proven to be effective tools for identification and analysis of protein–protein, protein–DNA and protein–RNA interactions. The two-hybrid assay is being applied to the entire complement of proteins of the yeast Saccharomyces cerevisiae to characterize the network of protein–protein interactions in the eukaryotic cell. The development of nontranscriptional cytosolic and membrane-associated two-hybrid methods has made it possible to detect and examine a number of protein–protein interactions in their normal cellular locations. Small-molecule hybrid systems have been developed which can be used to study protein–ligand interactions and to activate cellular processes by forcing protein associations.

Introduction

The development of the yeast two-hybrid system [1] provided a genetic means to identify proteins that physically interact in vivo. Over the past ten years, this system and its variants have been extremely useful for detecting and analyzing protein–protein interactions. Here, I will review some of the existing hybrid-based technologies, and discuss trends that include the use of small-molecule hybrid systems.

The yeast two-hybrid system was a logical extension of the popular as a tool to screen libraries in order to isolate and identify genes that encode interacting partners for a protein of interest [3]. Using a reporter gene required for growth allows easy detection of the few yeast transformants that express interacting proteins over the background of total transformants.

Variations on the original two-hybrid system

Several permutations of the original two-hybrid system have been developed over the past few years. In one class, a third protein is expressed along with the BD and AD fusions and expression of the reporter gene is used to select for interactions which occur only in the presence of a third protein (Figure 1b). Expression of a third protein may “bridge” a two-hybrid [4–5,7], by interacting with both hybrids, or may stabilize an interaction by other means, such as post-translational modification [6]. Alternatively, a third protein may prevent formation of a two-hybrid complex [4].

Reverse two-hybrid schemes have been invented that allow selection for mutations, drugs or competing proteins that disrupt two-hybrid interactions. Vidal et al. [9] used the gene URA3 as a reporter. Since expression of URA3 is toxic to cells grown on 5-fluoro-orotic acid, a two-hybrid interaction will result in cell death. Dissociation or inhibition of the interaction will lead to loss of URA3 expression, thereby allowing cell growth. The ‘split-hybrid’ system of Shih et al. [10] provides a similar selection, using the Escherichia coli tet repressor (tetR) as a reporter for two-hybrid interactions. Expression of tetR results in repression of a second reporter gene engineered to contain a tetR binding site. Disruption of the two-hybrid interaction restores expression of this reporter.

Inoyue et al. [11] developed a modification of the two-hybrid system, the differential interaction trap, that allows mutational analysis of a protein’s interactions with either of two different binding partners, X1 and X2. Each binding protein is fused to one of two different BDs that bind
to distinct sites upstream of two different reporter genes X1 and X2, each interact with a third protein Y, which is fused to the AD. Mutations in Y that affect the interaction with X1 but not X2 can be detected, and vice versa.

Another useful extension of two-hybrid technology is the addition of a hybrid RNA molecule to the system (Figure 1d). SenGupta et al. [12] developed a three-hybrid system to detect and analyze RNA–protein interactions in which the binding of a bifunctional RNA molecule links the BD and AD proteins and activates transcription of the reporter gene. Several groups have since used this RNA-based three-hybrid system to clone genes that encode specific RNA-binding proteins [13–17].

Application of the two-hybrid system at a genomic level
The two-hybrid assay has been used extensively to characterize networks of protein–protein interactions [18,19]. Bartel et al. [20] used comprehensive two-hybrid screens to create a protein linkage map of the E. coli bacteriophage T7. The current challenge is the systematic application of two-hybrid screens to every protein expressed in a eukaryotic cell. The resulting information is likely to reveal unsuspected connections between cellular processes and shed light on the roles of genes whose functions are now unknown or poorly understood. Two different strategies are being pursued to apply the two-hybrid screen to the entire complement of proteins.
is fused to a mutated version of TFIIIC that cannot bind X is fused to the Gal4 DNA binding domain and protein Y uses an artificial promoter that contains the specific transcription start site. TFIIIB interacts with Pol-III assembles the second factor, TFIIIB, to the DNA upstream of the transcription start site. TFIIIB relies on two accessory proteins. The first, transcription activators do not affect transcription by Pol III and so can be used as baits in this system. Initiation of Pol III transcription is a complex process that is regulated by numerous activator and co-activator proteins. One limitation of the Pol-II-based two-hybrid system is that it is difficult to use to find proteins that interact with Pol II transcription is a complex process that is regulated by numerous activator and co-activator proteins. One limitation of the Pol-II-based two-hybrid system is that it is difficult to use to find proteins that interact with Pol II transcriptional activators, since these proteins often constitutively activate transcription of the reporter when used as baits. Other types of proteins may also spuriously activate transcription in the two-hybrid assay. In one approach, this problem was addressed by using a system based on a Pol III promoter [25]. Pol II transcriptional activators do not affect transcription by Pol III and so can be used as baits in this system. Initiation of Pol III transcription relies on two accessory proteins. The first, transcription factor IIIIC (TFIIIC), binds to the promoter sequence and assembles the second factor, TFIIIB, to the DNA upstream of the transcription start site. TFIIIB interacts with Pol-III to initiate transcription. The Pol-III-based two-hybrid system uses an artificial promoter that contains the specific binding site for the yeast transcription factor Gal4. Protein X is fused to the Gal4 DNA binding domain and protein Y is fused to a mutated version of TFIIIC that cannot bind DNA. An interaction between X and Y will draw TFIIIC to the promoter and lead to transcription of the reporter gene. Aside from the problems of real and artificial transcriptional activators, the original transcription-based system has other limitations. For example, only interactions that can be reconstructed in the nucleus, which may not be the optimal environment for a variety of protein-protein interactions, can be detected. Full-length transmembrane proteins are often problematic in the transcription-based two-hybrid, probably because of misfolding or instability when they attempt to localize to the nucleus. Other proteins may require modification by cytoplasmic or membrane associated enzymes in order to interact with binding partners. The development of nontranscriptional cytosolic and membrane-associated two-hybrid systems has provided innovative solutions that address these limitations of the original transcriptional-based assay.

Hybrid systems based on other types of proteins

The original two-hybrid system activates transcription via RNA polymerase II (Pol II), one of three types of RNA polymerases found in eukaryotic organisms such as yeast. Each RNA polymerase transcribes different sets of genes. Pol II transcribes genes whose RNAs will be translated into proteins, while Pol I and Pol III transcribe RNAs that have structural or catalytic roles in the cell. Initiation of Pol II transcription is a complex process that is regulated by numerous activator and co-activator proteins. One limitation of the Pol-II-based two-hybrid system is that it is difficult to use to find proteins that interact with Pol II transcriptional activators, since these proteins often constitutively activate transcription of the reporter when used as baits. Other types of proteins may also spuriously activate transcription in the two-hybrid assay. In one approach, this problem was addressed by using a system based on a Pol III promoter [25]. Pol II transcriptional activators do not affect transcription by Pol III and so can be used as baits in this system. Initiation of Pol III transcription relies on two accessory proteins. The first, transcription factor IIIIC (TFIIIC), binds to the promoter sequence and assembles the second factor, TFIIIB, to the DNA upstream of the transcription start site. TFIIIB interacts with Pol-III to initiate transcription. The Pol-III-based two-hybrid system uses an artificial promoter that contains the specific binding site for the yeast transcription factor Gal4. Protein X is fused to the Gal4 DNA binding domain and protein Y is fused to a mutated version of TFIIIC that cannot bind DNA. An interaction between X and Y will draw TFIIIC to the promoter and lead to transcription of the reporter gene. Side from the problems of real and artificial transcriptional activators, the original transcription-based system has other limitations. For example, only interactions that can be reconstructed in the nucleus, which may not be the optimal environment for a variety of protein-protein interactions, can be detected. Full-length transmembrane proteins are often problematic in the transcription-based two-hybrid, probably because of misfolding or instability when they attempt to localize to the nucleus. Other proteins may require modification by cytoplasmic or membrane associated enzymes in order to interact with binding partners. The development of nontranscriptional cytosolic and membrane-associated two-hybrid systems has provided innovative solutions that address these limitations of the original transcriptional-based assay.

Fields and co-workers [24] have taken advantage of the availability of sequences for the approximately 6,000 open reading frames (ORFs) in the yeast genome. Each ORF can be expressed as a BD fusion in haploid yeast of one mating type and these yeast can be mated to an ordered array of ~6,000 test strains of the opposite mating type that express the complete set of ORF AD fusions. Advantages of the array approach are that the process is easily automated, requires no large-scale sequencing, and has built in controls for specificity of interactions. It is unknown, however, how many interactions may be missed by using full-length ORF fusions because of misfolding, toxicity or steric inhibition.

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Johnson and Varshavsky [26] have developed a cytoplasmic two-hybrid assay based on split-ubiquitin (Figure 2a), which has recently also been used for membrane proteins [27]. Ubiquitin is a small, 76 amino acid protein. Attachment of a ubiquitin molecule to a cellular protein marks that protein for proteolytic cleavage. Proteins fused to ubiquitin are rapidly cleaved in vivo by ubiquitin-specific proteases. If the carboxy-terminal fragment of ubiquitin (Cub) is fused to a reporter protein and co-expressed with the amino-terminal fragment (Nub), the two halves will reconstitute native ubiquitin, resulting in cleavage of the reporter-ubiquitin fusion and activation of the reporter protein. To adapt the system for the detection of protein-protein interactions, a mutant Nub, unable to interact with Cub on its own, is fused to one protein and a Cub-reporter hybrid is fused to its prospective interacting partner. Interaction between the two proteins allows ubiquitin to be reconstituted, leading to cleavage and release of the reporter protein. Aside from its ability to detect protein-protein interactions in the cytoplasm, the split-ubiquitin system also has the advantage that the signal for an interaction can be changed by changing the nature of the reporter protein.

The reporter may be a transcription factor, which is able to enter the nucleus and activate transcription when released from a membrane-bound fusion protein [27]. Another possibility is to use an enzyme that is activated by ubiquitin cleavage, whose activity may be detected by its effect on cell phenotype. The use of small ubiquitin fragments in the hybrid proteins may also be advantageous because it minimizes the possibility of sterical hindrance.

Rossi et al. [28] described an alternative cytoplasmic two-hybrid system to detect protein-protein interaction by intracistronic complementation of β-galactosidase mutants. Proteins of interest are fused to two nonfunctional weakly complementing β-galactosidase deletion mutants. β-galactosidase activity results from the forced interaction of the β-galactosidase peptides if the two hybrid proteins interact.
One attractive feature of this particular system is that the assay is direct and does not depend on other cell functions. In addition, the level of β-galactosidase activity should correlate with the strength of the interaction.

A membrane-associated two-hybrid system has been devised that makes use of the Ras signaling pathway. Ras is a membrane-associated signaling protein that binds and hydrolyzes GTP. Ras alternates between an active GTP-bound form that sets off a signal transduction cascade leading to cell growth, and an inactive GDP-bound form. Ras activity is regulated by two classes of proteins: GTPase activating proteins (GAPs) that increase Ras’ rate of GTP hydrolysis, and guanine exchange factors (GEFs) that stimulate release of GDP and uptake of GTP, thus reactivating its signaling activity. This signaling pathway is conserved amongst eukaryotes. The two-hybrid system takes advantage of the ability of the human GEF hSos to substitute for the yeast Ras GEF, Cdc25, in a cell with a mutant cdc25 gene. If hSos is recruited to the cell membrane, its interaction with Ras will stimulate guanyl nucleotide exchange and activate the signal transduction cascade (Figure 2b) [31]. Interaction between a hSos–bait hybrid protein (hSos–X) and a protein partner (Y) localized to the cell membrane will draw hSos to the membrane where it can interact with and activate Ras, allowing cell growth and survival. An interesting extension of
this system has been used to identify proteins that bind 3-phosphoinositide second messengers \textit{in vivo} [32••]. Membrane-bound phosphoinositide 3-kinases (PI3Ks) mediate the cellular effects of a variety of extracellular stimuli by phosphorylating the D3 position of the inositol ring of phosphoinositides. The 3-phosphoinositides generated bind proteins containing pleckstrin homology domains (PHs) which then activate downstream signaling processes [33]. In TOPIS (targets of PI3K identification system) assay (Figure 2e), PI3K is co-expressed with a hybrid protein consisting of a PH domain fused to a constitutively activated (i.e. GTP-bound) form of Ras in a Ras mutant cell. The PH binds the 3-phosphoinositides generated by PI3K, drawing the hybrid protein to the membrane where the activated Ras is able to substitute for the nonfunctional mutant, allowing cell growth. This specialized assay demonstrates that membrane-localized hybrid systems, in particular, should be a powerful tool as they are adapted for the study of a number of specific processes that only occur at the cell membrane.

A similar membrane-associated two-hybrid assay has been developed and is based on the interaction of the yeast G protein \( \alpha \) subunit with the \( \epsilon \) factor receptor Ste2. Recruitment of a chimeric G protein \( \alpha \) subunit (Gpa1) hybrid to the membrane via interaction with a Ste2 hybrid protein leads to a signal transduction cascade and rescues the viability of \textit{gpa1} mutant cells [34].

Small-molecule hybrid systems

The development of small-molecule hybrid systems began with the use of cell-permeable chemical inducers of dimerization (DIDs) to force oligomerization of chimeric receptors in mammalian cells [35]. The intracellular domain of the T lymphocyte antigen receptor was expressed as a fusion with the immunophilin FK506-binding protein (FKBP12). Addition of an FK506–FK506 dimer, FK1012, induced aggregation of the T lymphocyte antigen receptor, activating a signaling pathway. Small-molecule ligands have since provided a general method of dimerization (CIDs) to force oligomerization of proteins, including programmed cell death (apoptosis) [36–40]. Signal transduction can be manipulated to lead to a variety of outcomes for cells expressing the hybrid proteins, including programmed cell death (apoptosis) [41] and reversible cell proliferation [42]. Effects are dose-dependent at nanomolar concentrations, and the method can be effective in multicellular organisms [43,44]. These features make CIDs attractive subjects for pharmacological research as candidates for drugs that control the fate or function of genetically modified cells in potential gene therapy treatments.

Yeast transcription-based three-hybrid systems [40,45] have been developed in which a cell-permeable heterodimeric ligand activates expression of a reporter gene if the two ligand-binding domains are present as BI and AD fusions. This method can identify small-molecule ligands that interact with a specific ligand. It has been particularly useful as a genetic tool for ‘redesigning’ protein–ligand interfaces. For example, the molecules used as CIDs often bind to native cell proteins with toxic effects. The three-hybrid system was employed to address this problem. Libeerle \textit{et al.} [46••] selected for FKBP12 mutants that bind specifically to a nontoxic derivative of rapamycin. The reverse two-hybrid system has been combined with a miniaturized cell-based ligand screening technique to create a method for selecting small-molecule inhibitors of protein-protein interactions [47•]. A similar system based on the small-molecule three-hybrid system could easily be used to screen for new CIDs. Both applications should be useful devices for pharmacological research.

Conclusions

The original yeast two-hybrid system, along with its variants, will continue to be useful for investigation of protein–protein, protein–nucleic acid and protein–small-molecule interactions. In particular, application of the two-hybrid assay on a genomic scale should provide a starting point for more detailed analysis of the many genes whose molecular functions are currently unknown. Nontranscriptional cytosolic and membrane-associated two-hybrid systems are also apt to be powerful tools for the study of protein interactions, especially those that occur only at specific cellular locations. Finally, hybrid systems using small-molecule ligands should provide interesting leads for pharmacological research, especially when combined with large-scale screening.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as: • of special interest
** of outstanding interest


This paper presents an adaptation of the original two-hybrid system for the study of proteins that interact simultaneously with two partners. Two different DNA-binding domain fusions are present which bind to distinct sites controlling the transcription of two different reporter genes. Interaction of an activation domain fusion protein with each of the DNA-binding domain fusions is detected by selecting for expression of the associated reporter.


Legendre's group describes their strategy for mapping all protein-protein interactions in S. cerevisiae. A genomic library was screened with DNA-binding domain fusions of all predicted protein-coding genes in S. cerevisiae. Positive clones from the first screen that encode probable in vivo interactions were sequenced and selected for the second screen.


The authors describe a membrane-associated version of the split-ubiquitin two-hybrid system. Cleavage of the Cub (carboxy-terminal fragment of ubiquitin) reporter fusion releases a transcription factor, which is then able to enter the nucleus and activate transcription of a reporter gene.


This paper describes a membrane-associated two-hybrid system in yeast. Interacting protein fusions recruit Sox to the membrane where it stimulates guanylate nucleotide exchange on yeast Ras, rescuing a temperature-sensitive (Ras) mutant. The system was advanced as an alternative for studying protein interactions with transcriptional activators, but should be particularly useful for membrane-associated proteins, which are often problematic for the original two-hybrid system.


Phosphatidylinositol 3-kinase (PI3K) controls a variety of cellular responses by generating 3-phosphoinositide second messengers that activate downstream signaling pathways by binding pleckstrin homology (PH) domain proteins. In this, the authors describe a membrane-associated hybrid system to identify PH domain proteins that bind PI3K-generated phosphoinositides. Co-expression of PI3K and a hybrid protein activated Ras fused to a PH domain that binds the 3-phosphoinositide recruits the temperature-sensitive Ras mutant. The specialized assay demonstrates that membrane-localized hybrid systems can be a powerful tool as they are adopted for the study of a number of specific processes that occur at the cell membrane.


Rapamycin is useful for controlling protein dimerization in vivo because it can interact with two proteins (FK506 binding protein 12 [FKBP12] and FKBP12–rapamycin-associated protein [FRAP]) simultaneously. One limitation on its use is that its binding to native FRAP has an inhibitory effect on cell growth. Liberles et al. addressed this problem by synthesizing an altered form of rapamycin and used a small-molecule three-hybrid system to identify mutations in the ligand-binding domain of FRAP that stabilized binding to the modified rapamycin.

47. Huang J, Schreiber SL: A yeast genetic system for selecting small molecule inhibitors of protein-protein interactions in nanodroplets. Proc Natl Acad Sci USA 1998, 95:13396-13401. Huang and Schreiber combined a miniaturized high-throughput screening method with a reverse two-hybrid assay to create a system for selection of small-molecule inhibitors of protein-protein interactions. High local concentrations of ligands were obtained for testing by photochemically controlled release from small beads. Their method used inducible activation domain and DNA-binding domain fusions and antibiotic resistance reporter genes.