Review

Redefinition of the Yeast Two-Hybrid System in Dialogue with Changing Priorities in Biological Research

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Ilya G. Serebriiskii, Vladimir Khazak¹, and Erica A. Golemis

Fox Chase Cancer Center, Philadelphia, PA and ¹Small Molecule Therapeutics/Morphochem, Monmouth Junction, NJ, USA

ABSTRACT

Examination of the pattern of reagent creation and application in the two-hybrid system since 1989 reveals the expansion of a simple core technology to address increasingly sophisticated problems in protein interaction. As the technology has matured, its clear suitability for large-scale proteomic projects has made a major focus of its application the generation of global organismal protein interaction networks. In an inversion of emphasis, the increasing availability of such information now provides a master plan with the potential to specify the most promising directions for biological investigations (i.e., by directing the physiological validation of predicted critical protein-protein interactions). Recent derivatives of the two-hybrid system enable the targeting of such key interactions by facilitating the identification of essential amino acids conferring protein interaction specificity and of small molecules that selectively disrupt defined interaction pairs. Finally, the creation of mammalian expression systems based on two-hybrid principles became a new tool to create and probe novel biological systems. Taken in sum, this trajectory emphasizes the point that the creation of tools and the evolution of the idea of what is an interesting biological problem are in intimate dialogue.

INTRODUCTION

Research efforts in the biological sciences proceed along two separable tracks. The first track is oriented toward biological discovery and utilizes hypothesis-driven research to characterize the functioning of cells and organisms. The second track is oriented toward technology development and implementation and, as such, is frequently viewed simply as an engineering support system for the first track. However, ample evidence documents the fact that the invention of new technologies acts as a major force in creating novel targets for hypothesis-driven research by expanding the horizons available for study. For example, the development of facile DNA sequencing capability in 1977 (54,74) was arguably the foundation of the entire field of molecular biology, providing unprecedented mechanistic insights into the function of not only DNA but also RNA and protein. This development also reshaped the concept of what constituted an interesting course of experimentation around which to develop a hypothesis. While not all technologies have such a profound impact, most exist in a state of dialogue with the current topics of high biological interest, such that continued exercise of the technique gradually revises the research focus it was originally developed to support.

The yeast two-hybrid system (Figure 1) (28) was originally devised as a simple means to probe protein-protein interactions by engineering the readout of such interactions to be the transcriptional activation of easily scored reporter genes. In the most basic schema-

tic of the system, a first protein is expressed as a translational fusion to a DNA-binding domain (DBD) of known binding site specificity, while its interactive partner is expressed as a translational fusion to a transcriptional activation domain (AD). These two elements are co-introduced into yeast with one or more reporter genes that are made to be transcriptionally dependent on activation through a cognate-binding site for the DBD. Interaction of the DBD fusion (or "bait") with the AD fusion (or "prey") positions the activation domain in proximity to the reporter unit, activating its transcription. Over the 11 years since its introduction, this system has been modified and re-modified, while the biological and technological applications of the system have greatly expanded. In this review, we document and discuss the development of the two-hybrid system, first as an agent of biological discovery, second as a tool in proteomics, and finally as a means towards engineering novel pharmaceutical agents and biological systems, against the changing background of interesting biological questions.

TWO-HYBRID FIRST PHASE: A CANDLE IN THE DARKNESS

When the two-hybrid system was first defined as a potential tool to probe protein interactions in 1989 (28), it was in the context of a very large black box, or at least dark gray box, representing general understanding of the details of intracellular signaling machinery. For some organisms, notably bacteria and the simple eukaryote *Saccharomyces*

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cerevisiae, the power of genetic manipulation combined with biochemistry allowed reasonably thorough identification of the protein constituents of essential metabolic pathways. It also allowed definition of an initial framework of functionally interacting proteins for a number of pathways in signal transduction and cell cycle (e.g., compare References 11 and 86). For higher eukaryotes, comprehension of signaling networks was much more limited. Many discrete proteins had been nominated as interesting by varied criteria, including defined activity or intracellular localization. However, absent from lengthy processes of co-purification, the key partners of these molecules were unclear. Finally, for all proteins, given the relatively limited status of knowledge about the modular nature of protein interaction domains, it was of considerable interest to have a convenient means to define regions governing protein association.

Based on these priorities, the major emphases in the use of the two-hybrid system were as a tool to dissect defined pairs of known interacting proteins, characterizing residues critical for the association, and as paramount goal, as a tool to define a set of interactive partners for known proteins of critical function. Domain definition was a relatively straightforward undertaking involving targeted study of a small number of defined clones. In contrast, two-hybrid system use in identifying interactive partners required screening of complex libraries. This immediately emphasized the importance of having a second reporter gene transcribed following bait-prey interaction. This was done to limit the occurrence of artifactual or “false positive” results due to mutations or epigenetic events impacting a single two-hybrid reporter and to provide an active means of selection of positive interactors. From 1991 to 1993, a number of groups introduced two-hybrid systems in which the interaction of bait and prey activated two parallel reporters, one being the *lacZ* gene originally used by Fields and colleagues and the second being an auxotrophic gene (either *HIS3* or *LEU2*) whose activation would enable positive colony outgrowth on selective medium (Table 1) (14,23,37,96). These techniques were rapidly

and widely adopted, with the number of papers citing two-hybrid system applications growing exponentially through the 1990s (Figure 2).

As a result of these undertakings, from 1995 and for several years following, typical mammalian proteins were constituents of small interactive sets, with a protein of defined activity at the center of a starburst of connections to proteins of known or unknown function. In many cases, the validity of identified partner molecules was bolstered by clearly reasonable biological connectiveness with the starting bait. In other cases, surprising identifications led to the question of how reliable the two-hybrid technique was in practice, causing some researchers to perform tests of system reliability (5,25,78,79). This

work indicated that, while some specific protein combinations yielded artifactual results when scored in a two-hybrid milieu, such false positives could frequently be eliminated through either simple secondary characterizations or by comparison against a database of common false positives. Particular classes of proteins, including proteins intrinsically associated with membranes, proteins that activated transcription strongly, and proteins that interfered with yeast metabolism when overexpressed (78,79,89) were identified as generally unreliable in yeast two-hybrid experiments. Of these proteins, some can be studied in alternative genetically based protein interaction technologies that are more distantly related to the yeast two-hybrid system, as reviewed in Reference 27.

Figure 1. The yeast two-hybrid system. Two chimeric proteins are expressed in yeast. The DBD-fusion can bind consensus sites in a promoter upstream of a reporter gene but cannot activate transcription. The AD-fusion can activate transcription of the reporter gene only when interaction with the DBD-fused protein draws it to the promoter.

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Table 1. Diverse Yeast Two-Hybrid Systems

System	DNA Binding Domain	Activation Domain	Reporters	Inducible Library Expression
Two-Hybrid; "Improved Two-Hybrid"; Matchmaker 3	GAL4	GAL4	HIS3, LacZ HIS3, ADE2, LacZ	no
ProQuest: Reverse Two- Hybrid			HIS3, URA3, LacZ	
InteractionTrap	LexA	B42	LEU2, LacZ	yes
Modified Two-Hybrid	LexA	VP16	HIS3, LacZ	no
Dual Bait	LexA and cl	B42	LEU2, LacZ; LYS2, GusA	yes
Two Bait	LexA and TetR	B42	LacZ; URA3	yes

A number of different groups have contributed to the development of reagents that utilize two-hybrid principles, while varying the specific selection of DBD, AD, and reporters. Some commonly utilized variants are listed here and further described in the main text. Primary references for the cited examples are as follows: for two-hybrid, advanced two-hybrid, and Matchmaker 3 (4,6,14,23,28), and the Clontech Laboratories Web site (www.clontech.com); for Proquest, the Gibco-BRL information at the Life Technologies Web site (www.lifetech.com); for the Interaction Trap (37); for the modified two-hybrid system (95,96); for the dual-bait system (80); for the two-bait system (102). More extensive descriptions of reagents, system-compatible libraries, and links to commercial suppliers are available at <http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>.

Finally, a limited number of proteins exist in a gray zone, as "red herrings". For example, Daxx was originally identified as novel cDNA that interacted with a Fas cell surface death receptor bait (104) but was subsequently found to possess a naturally broad interaction capacity (56) and a biology that confounded the original assumptions made about its function. This example emphasized the need for caution in following up initial interaction assignments. Nevertheless, for the majority of intracellular proteins, the two-hybrid system appeared to be capable of detecting protein-protein interactions over a physiological range.

As two-hybrid technology became established, a number of investigators seeking to gather information about the natural functioning of biological systems began tinkering with the core reagents to determine whether they could be modified to identify interactions between proteins and an enlarged class of ligands. The engineering approaches adapted to this endeavor were in many cases ingenious and resulted in some system derivatives of remarkable elegance. A preliminary relatively simple undertaking was the effort to exam-

ine protein interactions between baits and preys in which the association of the pair was known or thought to be dependent on the presence or action of a third component. For example, proteins that normally function as constituents of large complexes may bind with low affinity to other single protein components of the complex, prohibiting detection, whereas, if two separate partner molecules are present, the cumulative effect of combining interactions among all three proteins could contribute to stabilization and subsequent detection of interactions. To this end, a third plasmid, expressing an additional "stabilizing" partner, is co-introduced with bait into yeast before performance of a targeted assay or library screen, in a tri-hybrid system. This approach has been used with numerous classes of proteins, including: p57Kip2 and other components of the cell cycle machinery (70); the *Schizosaccharomyces pombe* proteins scd1 (Cdc24p), scd2 (Bem1p), cdc42sp, and ras1, involved in morphogenesis (13); the signaling complex of EGF-receptor, Grb2, and Sos (112); and others (69,87,88,91). An alternative example of third-party action is the requirement of an additional protein to confer posttrans-

lational modifications that would not occur in yeast. For instance, many biologically important signaling events in higher eukaryotes depend on protein recognition of tyrosine-phosphorylated substrates. Yeast possess few, if any, endogenous tyrosine kinases, making it impossible to detect such interactions in a simple two-hybrid system. In 1995, Osborne et al. (64,65) first demonstrated that a "tribrid" system, with the Lck tyrosine kinase overexpressed separately in parallel with an IgE-receptor bait, was suitable to confer appropriate phosphorylation on the bait to allow detection of interaction with a prey containing the SHIP SH2 domain. Various classes of posttranslational modification have been described, and those that are specific to higher eukaryotes can theoretically be reconstituted in yeast to enable tribrid screening. The only caveat to this approach is that the third party must not possess an activity that is deleterious to the viability of the yeast, a point that must be determined empirically.

While a great many biologically significant protein interactions involve protein association with other proteins, proteins clearly also associate with other classes of macromolecule, including

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DNA, RNA, and lipids. To date, no report has been made of a successful modification of the two-hybrid approach to study protein-lipid interactions, although some conceptually related systems enable the study of lipid-bound proteins. However, DNA and RNA have proven to be fruitful targets. The one-hybrid and one-and-a-half hybrid systems have been used to identify transcriptional regulatory proteins or replication factors that either bind DNA independently or bind DNA as obligate partners of defined proteins (8,16,21,22,42,100). In a one-hybrid system, a DNA motif of interest is used to replace the binding site for Gal4p or LexA located in standard reporter constructs (*lacZ*, *HIS3*, *LEU2*), while either a defined DBD for the site or a cDNA library is expressed as fusion to an AD (i.e., as a standard two-hybrid prey). The binding of the AD fusion to its cognate motif directly activates the reporter. The one-hybrid system was first outlined by Wilson et al. (101) to define binding site mutants for the nuclear receptor NGF-1B, was subsequently used in library screening by Li and Herskowitz (51) to identify the replication factor ORC6 and has been used by numerous other groups (8,16, 21,22,42,100). The one-and-a-half hybrid system is to the one-hybrid system what the three-hybrid system is to the two-hybrid, in that assembly of a reporter-bound activation complex relies on the presence of a third component expressed from an additional plasmid that heterodimerizes with the prey. This approach has been less heavily mined but has been effective in a number of instances, including the probe of transcription factors SAP-1 and SRF binding to serum response elements, factors BETA2 and E47 binding the insulin promoter E-box, and the function of other proteins involved in activation of *ftz*-dependent promoters (20,58) and in a related approach (109).

The work of Aronheim and co-workers (1,2,12,53) and of Stagljar et al. (85) has begun to adapt two-hybrid principles to the analysis of proteins that must be associated with membranous structures to possess physiological activity. This class of proteins has tended to pose problems in attempts to utilize them in classic two-hybrid ap-

proaches, requiring translocation of a bait or prey fusion to the nucleus to activate transcriptional reporters. In the systems of Aronheim and Stagljar, clever adaptations of interaction readout allow the assessment of interaction in situ at membranes. In the SOS-recruitment system (2), one interacting component is localized to the plasma membrane via fusion to a myristylation motif, while the second is expressed as a fusion to the human SOS protein, homologous to the *S. cerevisiae* Cdc25p guanine nucleotide factor for Ras2p. In a temperature-sensitive *cdc25-2* mutant strain, only yeast expressing interacting partners can recruit the SOS-fusion component to the membrane, allowing it to grow with Ras2p at the restrictive temperature. This system has been successfully used with a number of different interactive pairs (43,108). The separate system developed by Stagljar and

colleagues adapts the split-ubiquitin system of Johnsson et al. (47) to create a readout for protein-protein interactions. In this system, one transmembrane protein (TM-A) is expressed as a fusion to the N-terminus of ubiquitin, while a second transmembrane protein (TM-B) is expressed as a fusion to the C-terminus of ubiquitin, and to a LexA-VP16 fusion protein. Interaction between TM-A and TM-B reconstitutes ubiquitin, which is then recognized by the ubiquitin-specific protease UBP and cleaved, resulting in the release of LexA-VP16 from sequestration at the plasma membrane. The released LexA-VP16 transits to the cell nucleus, where it activates *lacZ* and *HIS3* reporters as in a conventional two-hybrid system. The application of these systems promises to enlarge the category of proteins to which two-hybrid screening can be productively applied.

Figure 2. Increase in two-hybrid system citations. Number of citations in Medline that describe use of the yeast two-hybrid system for targeted applications or for library screening, 1989–1999, by year. Numbers were arrived at by searching Medline by permutations of the keywords two-hybrid, yeast, and protein interaction, followed by scanning of content. This figure thus represents an approximation of publication numbers but may not be completely accurate. Total number of citations to date approaches 3000.

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One of the more baroque manipulations of the two-hybrid reagents has been directed at the investigation of protein-RNA interactions, initially described by two groups (71,77). In these systems, the DNA-binding fusion (bait) itself is no longer used as a probe for interaction partners. Instead, it is used as a "hook" to display an RNA molecule that will function as bait for an RNA-binding protein (Figure 3). This is accomplished by fusing the DBD to a protein with a defined RNA-binding specificity. Hence, a DBD-MS2 phage coat protein fusion will bind RNA molecules that contain an MS2 target stem-loop sequence (77), or a DBD-Hiv Rev protein fusion will bind RNAs containing a Rev response element (RRE) (71). The actual bait in these systems is a separately synthesized RNA molecule that combines sequences of defined specificity for the bait (the MS2 stem loop or an RRE) and bait RNA sequences of interest (Figure 3). This hook-and-bait combination can then be used to identify AD-fused proteins that bind to the bait RNA sequence. Alternatively, if an RNA-binding protein has been defined and it is desirable to identify novel RNA sequences to which it can bind, it is possible to keep bait and AD-fused protein invariant, while screening a library of RNAs that vary to keep the hook-binding component (e.g., the MS2 stem loop) while providing a battery of potential binding sites for the AD-fused protein of interest. This latter approach has been used successfully to identify novel physiological binding sites for the Snp1p (*S. cerevisiae* homolog of U1-70K) protein (76). While such three-hybrid applications of RNA binding are reputed to be more prone to detection of low-affinity or false-positive interactions than simpler two-hybrid systems (67), the utility of this approach in targeted situations and screening is clear (10,68,83,110,111), and their reliability in library screening is likely to continue to improve.

TWO-HYBRID SECOND PHASE: IMPOSING ORDER ON CHAOS

By the mid-1990s, two-hybrid systems were shown to be generally reliable and highly accessible. In the back-

ground, the Human Genome Project was gathering steam, raising the specter of an exhaustively described DNA content within the decade. Inevitably, the paradigm of two-hybrid system usage would expand to consider the reagents as primary tools for proteomics and "functional genomics", in which the goal was not to use two-hybrid screening on a small scale to support specific biological questions, but rather to create large profiles of protein-protein interactions, which would help in the process of nominating new interesting questions for investigation. The basic physiology of yeasts makes them readily adaptable to culture in high-throughput formats such as microplate arrays. In the analysis of cell cycle kinases, Finley et al. (29) used directed crosses of arrayed MAT α and MAT α haploid yeast, in which yeast of one mating type contained a series of baits and reporters,

while yeast of the opposite mating type contained a series of preys, allowing easy scoring of a large set of interactions in diploids obtained through mating. This work was a pilot demonstration of the utility of this approach as an alternative or supplement to straightforward library screening. Over the last several years, the mechanics of analysis of two-hybrid reagents via robotics and the means of integrating the information into other classes of proteomic analysis have been increasingly well established (17,24,66,82,90,99). Genomic investigations generally use a combination of approaches, including interaction mating (29) on mass cultures or arrayed whole genome bait sets, direct transformation, and comparison of results obtained with baits derived from full-length cDNAs or cDNAs truncated either arbitrarily or by design.

Since 1996, the number of two-hy-

Figure 3. A yeast tribrid system to characterize protein-RNA interactions. Top, a DBD-fusion hook provides a protein of known RNA-binding specificity tethered to the reporter gene promoter. The bait is a composite RNA molecule, with one region containing sequences allowing binding to the hook, and the second region allowing binding to a second known or novel RNA-binding protein. As in a simple two-hybrid system, the prey is an activation-fused protein. Shown below, the system of Sengupta et al., as applied by Bacharach and Goff (3), with MS2 phage protein used as hook and an MS2-binding element-HIVPsi sequence composite RNA bait used to recruit AD-fused HIV gag protein.

brid, proteome-oriented investigations reported has been rising each year. Some of this work focuses on the fine analysis of proteins associated with specific complexes organized around a discrete biological function. The 1997 study of pre-mRNA splicing-associated factors by Fromont-Racine et al. (33) not only tied a number of open reading frames (ORFs) of unknown function to the spliceosomal machinery but also defined parameters for assessing the quality of predicted interactions. Flores et al. (31) have exhaustively probed the interactions between subunits of *S. cerevisiae* RNA polymerase III using a two-hybrid approach. However, the main thrust of these studies has been the ambitious undertaking of analyzing the complete interaction profiles of whole genomes. Simpler explorations have dealt with the interactions of proteins encoded by viral genomes, including those of *E. coli* bacteriophage T7 (7), vaccinia (55), and hepatitis C (30). Of free-living organisms, efforts underway address bacterial genomes such as *Helicobacter pylori* (<http://pim.hybrigenics.com/pimriderlobby/PiRiderLobby.htm>) and the yeast *S. cerevisiae* (44,89) (http://depts.washington.edu/sfields/projects/yplm_home_page.html), in collaboration with CuraGen (<http://CuraGen.com>); *Caenorhabditis elegans* (98) and *Drosophila* (in the laboratory of R. Finley; <http://cmmg.biosci.wayne.edu/finlab/finlabhomeb.html#Projects>), in collaboration with G. Rubin and CuraGen; and humans (39), in collaboration with Myriad Genetics (<http://www.myriad-pronet.com>).

These combined efforts have produced a flood of information, both in terms of specific facts about individual interactions and in terms of general information about the nature of interaction networks. Gleaning through the several studies, you can find specific trends emerging. First, not surprisingly, the interactions detected depend significantly on the nature of the bait; frequently, interactions can be detected with individual domains of proteins that are masked when the full-length protein is used. Second, the two-hybrid approach is not saturating in identifying interactions. As discussed in Reference 90, for the test case of Skp1, when an attempt at globally identifying interactors for this molecule were made by two-hy-

brid versus mass spectrometry (work of R. Aebersold, University of Washington, Seattle, WA, USA), 11 interactors were identified by two-hybrid and eight by mass spectrometry; however, only two interacting proteins were isolated by both approaches. The two-hybrid approach likely biases for interactions that are of moderate to significant strength between two individual proteins, while

the mass spectrometry approach identifies molecules that associate tightly with a complex but may only interact weakly with individual members of that complex. Hence, such a discrepancy is not completely surprising. However, it suggests that two-hybrid interaction networks will provide a general guide for interaction profiles but will need to be merged with results from many other

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approaches to yield a complete picture of protein association. Third, the number of interactors identified by individual proteins varies over a considerable range. Up to approximately 50% of proteins assessed do not yield reproducible interactors, with reproducible defined as the same interacting pair identified in two separate rounds of mating a prey against an arrayed bait library (89). Of the baits that do not yield interactors, many are metabolic enzymes or proteins whose biology suggests they may primarily function as monomers in the absence of associated complexes (for examples, see http://depts.washington.edu/sfields/projects/ypm_home_page.html). Of proteins that do yield interactors, depending on the study (44,55,89,98), between three and nine interactors per bait are obtained [with recorded highs, including lower-confidence interactors, ranging from 29 to 40 for single baits (32,97,98)], supporting the growing perception that many proteins function in complexes organized around shared function. Finally, as interaction assignment moves towards the comprehensive in the most advanced project, for *S. cerevisiae* (75), over 2300 interactions have been identified that involve over 1500 proteins in a single web. Continuing the trend in smaller complexes, groups of proteins that associate to accomplish linked functions tend to connect to other groups of proteins with similar function [e.g., with proteins defined as affecting cell polarity, providing connections to proteins clustered as involved in vesicular transport, cell structure, cytokinesis, and cell cycle (75)]. While these results are not completely surprising, they mark a milestone: for the first time, proteomics provides an omniscient view of the interlocking design elements of an entire eukaryote, yielding a pattern that will inevitably be strongly echoed as databases are assembled for higher organisms. One pauses for a moment in wonder.

TWO-HYBRID THIRD PHASE: INTELLIGENTLY ENGINEERING THE FUTURE

Returning to earth, it is evident, based on the preceding recitation of genomic projects, that this work is largely

moving from the academic domain to a new form as a searchable information resource, generally sponsored by an affiliated bio-information technology partner. It is likely that the field of two-hybrid proteomics will be saturated in the next few years. In reaction to the burgeoning databases, four courses of future research emerge. One approach is to attempt to justify the predicted interaction networks with reality, through exploration and validation of the physi-

ological context within which the predicted interactions occur. A second approach is to attempt to refine knowledge of physiological interactions, mapping domains and key residues required for protein association through the use of two-hybrid and related technologies. A third approach is to respond creatively to the interaction databases using the contained information as a basis for engineering targeted disruptions of specific protein-protein interactions

Figure 4. The reverse two-hybrid system. In the reverse two-hybrid system developed by Vidal et al. (92), reporter 1 (*HIS3*) is used for positive selection, while reporter 2 (*URA3*) is used for counterselection. Top, interaction between DBD- and AD-fused proteins results in growth on medium lacking histidine, but lethality on medium containing 5FOA, a toxic metabolite of the *URA* pathway. Below, following mutagenesis of DBD- or AD-fusion protein, missense mutations that weaken the interaction of DBD- and AD-fusions can be separated from nonsense mutants that result in loss of either fusion by comparing profile on histidine- and 5FOA medium. Mutations that weaken the interaction will display slow growth on both media, while mutations or truncations that completely abrogate the interaction will result in moderate to strong growth on 5FOA medium, but no growth on histidine medium.

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that alter physiology in a desirable way, either through mutation or through the use of pharmacological agents. The global and necessary research encompassed in the first course of action is beyond the scope of this review. The second and third approaches are discussed together in this and the following section. A fourth approach is to declare victory and move on: that is, to concede that the main work of global interaction determination will soon conclude and that a productive strategy is to take the reagents created for two-hybrid studies and apply them to new problems, as discussed in the final section.

Essential sequence motifs or domains required for protein-protein interaction can be simply determined by the two-hybrid system, by examining the interaction properties of DBD- and AD-fused targeted truncations or mutations of full-length interacting proteins; indeed, this was one of the first productive uses of the system (14). A higher-throughput means of identifying interacting domains within a larger sequence was soon described by Stagljar et al. (84), based on the fragmentation of a cDNA encoding one interacting partner, shotgun cloning of fragments into an AD-fusion vector, and screening against a predefined partner fused to a DBD. These are both examples of positive selection for a minimal interaction domain. Other problems that bear on the identification of mutations that disrupt interactions and on the determination of interaction specificity are significantly more complex and require re-engineering system components.

One of the most attractive features of two-hybrid screening is the ability to identify positive-interacting colonies out of a lawn of non-interacting proteins. Using conventional reagents, mutations that lead to loss of interaction would not be detected; however, by inverting the selection strategy, in the “reverse two-hybrid system”, this problem was circumvented by Vidal and co-workers (93). In this strategy (Figure 4), interaction of DBD- and AD-fused partners induces the expression of the *URA3* gene, completing the yeast pathway for uracil synthesis. Growth of yeast expressing the *URA3* gene on medium containing 5-fluorotic acid (5-FOA), a toxic metabolite of the

Ura3p protein, is counterselected; hence, only yeast containing forms of DBD- and AD-fusion that have no power to interact are able to grow. Similarly, counterselectable reagents have been developed based on acquisition of sensitivity to cycloheximide (107). Such strategies can be efficiently combined with a mutagenic approach to identify small changes in amino acid sequence that disrupt the association of proteins that normally interact with high affinity (92,105) and can be adapted into probes of small molecule interaction disruptants, as described below.

A complementary strategy to the use of mutations to specifically restrict protein function is to identify, or develop de novo, small peptides that are capable of achieving similar ends by blocking essential protein-protein interactions. Such peptides have the advantage that they would be easy to use in many desirable experimental manipulations in cases when it is impossible to replace an endogenous form of a gene with a specific mutation (e.g., by synthesis of the peptide inside cells from expression plasmids or by introduction exogenously by uptake of peptides from medium or by microinjection). The yeast two-hybrid system has been shown to be an

effective tool at identifying peptides with desirable properties. Initial studies identified peptides capable of binding specifically to targets such as Rb (103) or Cdk2 (19), with some highly selective peptides as little as seven amino acids in length (103). Subsequent experimental analysis has demonstrated that peptides thus identified can be valuable tools in elucidating and/or selectively disrupting fundamental cellular processes, including cell cycle control in embryonal development (49) or cultured cells (26), and in signal transduction (34,63). By modeling on the structure of such bioactive peptides as models, it is additionally conceivable that it will be possible to design related small molecular compounds (18), whose use in modulating interactions is discussed further below.

Signaling networks are intrinsically complex, with key molecules interacting with multiple partners. Use of the two-hybrid system to identify mutations that disrupt protein-protein interactions is useful; it is particularly useful if it can be applied to identify mutations that allow the dissection of pathways, such that for signaling protein A that normally associates with B and C, mutant forms of A can be derived that as-

Figure 5. The dual-bait system. As described in text, AD-fused “A” normally interacts with DBD1-B and DBD2-C. Using the dual-bait system in conjunction with cassette mutagenesis, variant forms of AD-A can be selected that display restricted interaction profile, binding only DBD1-B (shown) or DBD2-C (not shown).

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sociate with only B or only C. To address such issues, two-bait or dual-bait two-hybrid systems have been developed (36,41,46,80,102). In these systems (Figure 5; also see Table 1), the dually interacting "A" is expressed as an AD-fusion, while B and C are expressed as fusions to two separate DBDs [e.g., DBD1 is Gal4p and DBD2 is LexA (46), or DBD1 is LexA and DBD2 is the cI lambda repressor (80)], each directing the expression of one or more reporter genes. Cassette mutagenesis of the AD-A component, followed by the screening for mutations that produce selective loss of activation of DBD1-B or DBD2-C responsive reporters, has been used to efficiently identify mutants that restrict signaling output [e.g., Reference 72 identifies mutants of Pak kinase (AD-A) that preferentially associate with Cdc42 (DBD1-B), instead of associating with similar affinity with Cdc42 and Rac GTPase (DBD2-C)]. It is to be anticipated that eventual combination of dual-bait screening strategies with convenient reverse selection will further enhance the power of this approach. Finally, an additional application of these reagents of interest is their potential in selective library screens to identify AD-fused preys that interact selectively with baits DBD1-X or DBD2-Y. This can be used in cases where X is a wild-type form of a protein of interest and Y is a mutant [e.g., Cdc42 vs. Cdc42 Δ 8, altered in its insert region/Pak-binding domain (81) or Ras vs. constitutively active Ras^{V12}], a polymorphic form of the same protein, or a closely related family member [e.g., Ras vs. Rap1a/Krev-1 (80) or Cdc42 vs. Rac (72)]. In sum, these approaches should contribute greatly toward refining knowledge of sequences needed to confer interaction, while allowing the derivation of mutants with novel restricted function that can be used as probes in biological experimentation.

TWO-HYBRID, ALTERNATIVE THIRD PHASE: BETTER LIVING THROUGH CHEMISTRY

A much-avowed goal of the field of pharmacogenomics and the 21st-century pharmaceutical industry has been to

devise perfectly targeted small molecules that specifically modulate disease-specific signaling pathways while inducing minimal side effects. At present, the signaling pathways perturbed in a number of clinically significant diseases are beginning to be well understood, raising the reasonable hypothesis that the use of drugs or other small molecule agents that disrupt the associations of protein constituents of these pathways might be ideal candidates to use in targeted therapies. A significant body of work suggests that the two-hybrid system can be used to identify drugs or to identify drug-protein interactions. The first reported analysis of small molecule function in a two-hybrid application was the work of Chiu et al. in 1994 (15). In this study, rapamycin was used as a co-factor in conjunction with a DBD-FKBP12 bait to identify preys such as RAP1 that bound only to the FKBP12-rapamycin complex. Similarly, Lee et al. (50) used the combination of a thyroid hormone plus DBD-thyroid hormone receptor to find TRIP-1, a protein whose binding required the ligand-receptor combination. In principle, both of these initial studies are conceptually similar to the three-hybrid work described previously, the sole difference being that the third component is not a protein.

These first screens identified protein preys that interacted with small molecule-protein-associated complex targets; a greater challenge was to refit the system to specifically detect interactions between protein and drugs. In 1996, Licitra and Liu (52) described a three-hybrid system for the detection of protein-drug interactions that utilized a display principle similar to that used in the protein-RNA three-hybrid noted above (77). In this case, a DBD fused to the hormone-binding domain of the glucocorticoid receptor was used as a hook, while the actual bait was a chemically synthesized molecule that fused dexamethasone to FK506. The binding of the dexamethasone moiety to the DBD-glucocorticoid receptor allowed display of FK506, which enabled the isolation of the FK506-binding partner FKBP12 from an AD library. A significant feature of this strategy was the fact that it was possible to titrate the interaction of dexamethasone-FK506 with

AD-FKBP12 by adding native FK506 to the culture medium, potentially allowing the convenient assessment of relative binding affinities of synthetically derived FK506 analogues. Separately using the binding properties of FK506 and FKBP12 as a model, Huang and Schreiber (40) established a reverse two-hybrid system in which the interaction of LexA-activin R1 receptor and AD-FKBP12 is dependent on galactose-mediated induction of the LexA fusion and results in the activation of a *lexAop-URA3* reporter, thus causing death on medium containing 5-FOA. In this system, titration of FK506 results in disruption of the interaction between DBD-activin receptor and AD-FKBP12 and, hence, is positively scored; strikingly, the robustness of the system allows interaction blockage to be scored in microcosm via assay in "nanodroplets" (106), a modality amenable to high-throughput analysis (9).

Two groups have begun to report results suggesting that two-hybrid methodologies can be successfully applied to the isolation of drugs with desirable properties from combinatorial libraries. At a recent CHI conference (Exploiting Yeast Molecular Biology for Therapeutics, 2000), Khazak and co-workers from SMT/Morphochem (Monmouth Junction, NJ, USA) reported the identification of novel compounds that specifically disrupted the interaction of Ras and Raf through use of a two-hybrid assay. A cI-H-Ras^{C186G} [mutated to eliminate CAAX sequences conferring membrane localization (38)] bait and an AD-cRaf prey were used for screening. Co-expression of this bait and prey induces transcription of a colorimetric reporter (*lacZ*) and the *LYS2* gene, in a standard "positive selection" for interaction. Compounds (77 000) from a large chemical library were screened against the test strain and in parallel, against a negative control strain containing a nonspecific pair of interacting proteins. For this screening, an arrayed agar diffusion plate format was used, in which plates have been overlaid with soft agar seeded with test yeast. Concentrated solution containing compounds to be assayed is applied to a small spot on a plate and allowed to diffuse out in a halo. A particular advantage to this approach is

that it allows simultaneous assay of a gradient of concentrations of drug. Compounds that were able to block expression of β -galactosidase in the test strain but not in the control strain were selected for secondary tests in the tissue culture cells. Ras activity in carcinogenesis requires the ability to associate with and phosphorylate Raf (96,113), thereby constitutively activating MAPK signaling. Strikingly, three structurally related compounds independently selected in the screen were able to inhibit luciferase activity of the MAPK-responsive cFos-luciferase reporter gene in NIH3T3 and CHO cells in a dose-dependent manner. These three compounds additionally inhibited signaling through the MAPK pathway in serum-induced CHO cells with a 50% maximal inhibitory concentration (IC_{50}) of 6–17 μ M, which is comparable to concentrations for other effective chemotherapeutic agents. In the subsequent testing of two of the most

promising compounds, neither exhibited activity towards p38 kinase even at 30 μ M concentration, indicating specificity of action. These compounds are currently being evaluated for effects on transforming properties of Ras.

At present, the most promising description for the use of the two-hybrid system in drug screening comes from the work of Young and colleagues at Wyeth-Ayerst (Princeton, NJ, USA) [Reference 107 and presentation at the CHI conference, Exploiting Yeast Molecular Biology for Therapeutics, 2000]. Young's team has developed a novel two-hybrid counterselectable system, in which the interaction of DBD- and AD-fusions causes the synthesis of the CYH2 gene, making cells sensitive to cycloheximide; abrogation of interaction via the addition of a small molecule is necessary to allow viability on medium containing cycloheximide. The work of this group has focused on identifying compounds that modulate

the function of N-type calcium and potassium channels. The N-type calcium channel is a multi-subunit protein that has been implicated in neurotransmitter release in central and peripheral nervous systems. Selective blockers of the N-type channels provided a neuroprotective effect in animal models of head trauma and stroke. The counterselectable yeast two-hybrid screen was designed for the identification of compounds that would inhibit the interaction between the DBD- β 3 subunit and the AD- α 1B subunit intracellular juxtamembrane loop and potentially work as calcium channel antagonists. Compounds (156 000) from a complex random chemical library were screened against the test strain and a negative control strain (expressing an interacting but unrelated protein pair) using an arrayed agar diffusion plate format. Notably, 10 distinct compounds rescued yeast growth in the test strain but not in the negative control strain. The

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most potent compound, WY141520, reversibly blocked N-type calcium current with an IC_{50} of 95 μ M in superior cervical ganglion (SCG) neurons. Comparative analysis of WY141520 in SCG neurons proved that compound does not simply block the channel pore, as does known channel inhibitor and toxin SNX-111, but rather inhibits the interaction of the α and β subunits of channel. Further, this compound did not affect function either sodium or potassium channels, supporting specificity action. More recent work from the Wyeth-Ayerst group has focused on the targeting of potassium channels. The K^+ channel is a tetrameric multi-subunit complex that consists of 4 α and 4 β subunits encoded by *Kv1.1/Kv β 1* genes. In the hippocampus, heterotrimeric association of the *Kv1.1*, *Kv1.4*, and *Kv β 1* subunits is likely to form rapidly inactivating channels that modulate glutamate release from intrinsic and afferent pathways. Modulation of activity of the K^+ channel by preventing the association of *Kv1.1/Kv β 1* proteins might have a therapeutic utility in epilepsy and ischemic stroke (73). Using a DBD-*Kv β 1* bait and AD-fusion to the *Kv1.1* intracellular receptor S4-S5 loop as interactor in a screen of 176 000 compounds, "positives" were obtained with a "hit rate" of 0.0094%. One lead compound, WAY-941, eliminated N-type inactivation of the K^+ channel with IC_{50} 7.65 μ M in CHO cells with overexpressed *Kv1.1* and *Kv β 1*. The compound eliminated N-type inactivation of the *Kv1.4* channel but not the *Kv4.2* channel when the proteins were transiently expressed in CHO cells. Secondary tests in *Xenopus* oocytes with overexpressed *Kv1.1/Kv β 1* and *Kv1.4* demonstrated selectivity for some hit compounds toward *Kv1.1/Kv β 1* channels. Thus, yeast two-hybrid screen for inhibitors of the K^+ channel has yielded the first small molecule compounds that are able to disactivate heteromeric *Kv1.1/Kv β 1* complexes in a potent and dose-dependent manner.

At present, with the field in its infancy, it is not clear how useful protein-interaction directed compounds will prove to be in clinical applications. However, several points suggest that this approach will turn out to be useful.

First, it is extremely easy to do high-throughput screening; Young et al. (107) note that 572 compounds can be assayed per plate, or 30 000/day, in a three-day assay cycle with work performed by a single technician. In contrast to earlier worries that anticipated the "impermeability" of yeast to specific compounds would limit applications, this does not pose a problem in practice (personal communications, J. Huang, K. Young, and unpublished results, V.K.). Approximately 10% of small molecule compounds assayed induce cytotoxicity in yeast, indicating a considerably higher percentage of compounds are probably entering yeast efficiently, which in retrospect is not too surprising, given the average molecular weight of assayed molecules is less than 500 Da. Several experts in the field note that compounds that present permeability issues in yeast are likely to be similarly troublesome in humans, so such compounds are disfavored in any case. The hit rate for molecules with desired blocking effect remains at approximately 0.01% of screened compounds and a variety of classes of interacting pair targets; of these hits, between 50% and 75% display desired biological effects upon physiological validation. Finally, the existence of parallel work studying the direct interaction of drugs with yeast physiology (35) suggests that screening for clinically significant drugs can be integrated into the larger framework of *S. cerevisiae* genomics and proteomics, accelerating discovery (48,57,94). This will be a fascinating field to watch over the next few years.

TWO-HYBRID SYSTEM IN BIOLOGICAL CONTEXT: PROSPECTS

In the last decade, the remarkable rate of progress in the field of protein interactions means the landscape of scientific inquiry has been transformed. Although this review has focused on documenting achievements obtained with the two-hybrid system, this technology is only one of many different techniques that have been created to identify, refine, localize, and quantitate protein interactions. Based on the combined application of these techniques, a

map of how proteins interact in networks is likely to be completed for many organisms, at least in rough outline, within the next few years. How best to react to the interaction webs? First, for some years, react with skepticism. Present efforts have defined proteins that can conceivably interact in artificial DBD-/AD-fused test tubes. Taking the predictions as a beginning point, one begins the process of testing predictions through biological assays, including the design of genetic crosses between knockouts: cell biological examinations of the effect of perturbing partner "A" on partner "B": scrutiny for mutations in partner "X" when partner "Y", known to be frequently mutated in a human pathological condition, fails to show mutations in some affected families. Pursuit of such experimentation for some years, performed in response to the directions suggested by non-hypothesis driven application of the technology, will likely validate a significant fraction of the predictions. Further, integration of protein interaction map data with the output of other technologies, such as the use of gene arrays, will tend to augment the power of these data sets. For example, if the application of the pharmacological agent "YYY" to yeast induces a change in transcriptional profile comparable to that obtained by mutation of yeast gene "XXX1", and if the yeast protein encoded by *XXX1* possesses a known series of interacting proteins (t, u, and v), then disruption of interactions between *XXX1* and its partners becomes rapidly nominated as candidate for target of drug action.

In parallel, benefits derived from the efforts of researchers adding tools to the box by creating novel derivatives of the two-hybrid system will begin to open new research areas. The ability to finely dissect complex signaling networks through the use of two-hybrid predicted mutations, peptides, and drugs to block specific molecular couplings has the potential to provide an unprecedented level of control in biological manipulation. The insights gathered through the use of such reagents will undoubtedly contribute to the nascent field of intelligent organism redesign, in the creation of genetically modified organisms to support agriculture and therapeutic applications. Final-

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ly, an inversion of perspective concerning what is technology and what is biology may provide insights into some of the more creative future uses of two-hybrid technology. In a series of recent studies (45,59–62), the laboratory of R. Muller has described the adaptation of two-hybrid reagents to create novel transcriptional regulatory systems. In this strategy, although protein interaction still bridges a DBD- and AD-fusion, the interactor moieties are invariant, and these fusion components only serve as elements in a circuit. By expressing DBD- and AD-fusion under the control of discrete mammalian promoters of overlapping specificity patterns (45,62), or by constructing feed-back-amplification loops using the DBD/AD-fused combinations (59), Muller et al. are able to begin to construct targeted specificity of reporters to meet desirable needs for gene therapeutic and other applications. In the end, a technology that was built by modeling on biological paradigms can

now become a building block to generate new biological systems.

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Address correspondence to:

Dr. Erica A. Golemis
Fox Chase Cancer Centre
7701 Burholme Avenue
Philadelphia, PA 19111, USA
e-mail: ea_golemis@fccc.edu