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Chemical Dimerizers and Three-Hybrid Systems: Scanning the Proteome for Targets of Organic Small Molecules

Review

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The integration of technological advances in areas as diverse as chemical biology, proteomics, genomics, automation, and bioinformatics has led to the emergence of novel screening paradigms for analyzing the molecular basis of drug action. This review summarizes recent advances in three-hybrid technologies and their application to the characterization of small molecule-protein interactions and proteome-wide identification of drug receptors.

Introduction

A critical and difficult challenge in many areas of biomedical research has been the identification and characterization of protein targets of organic small molecules. For instance, research pursuant to the "chemical genetics" paradigm utilizes small molecules as molecular probes for a quantitative and temporal perturbation of protein function [1, 2]. This approach emulates all the principles of genetics, but rather than relying on genetic mutations to dissect protein function, it uses small molecules. Although this approach holds great promise for deciphering functions encoded by the human genome, its current utility is limited by the difficult task of identifying the intracellular target spectrum for any given small molecule probe. The identification of protein targets of small molecules is of course also crucial in pharmaceutical research, where a lack of a thorough understanding of mechanism of action may significantly limit the lead optimization process and the development of therapeutics with optimal efficacy and safety profiles. Identification of small molecule targets could also lead to novel therapeutic applications for a drug or drug candidate.

In the wake of the sequencing of the human genome, as well as various other genomes, the past few years have seen a significant increase in the development of systematic approaches designed to elucidate the role of small molecules in perturbing cellular processes. These approaches have variously been labeled with the terms chemical biology, chemical genomics, and chemical proteomics, and integrate various technological advances in areas of assay design, chemistry, functional genomics, proteomics, automation engineering, and bioinformatics with access to an increasing number of cloned genes and their associated sequences. For instance, genome-wide gene expression profiling, and, to an increasing extent, protein expression profiling are being used to profile the effect of a small molecule on signaling pathways [3–8], and a variety of chemically reactive probes are being used to profile and identify enzymes or other protein targets in complex mixtures based on their catalytic or ligand binding activities [9–16]. Clearly, the sequencing of the human genome and the many recent technological advances have increased the versatility, sophistication, and scale with which complex data sets can be generated, analyzed, and interpreted—notwithstanding the many challenges still associated with managing the volume and quality of large data sets.

An integrative technology-driven approach, such as that seen in functional genomics and proteomics research, should also facilitate the development of systems for more rapid and large-scale identification of small molecule ligand binding proteins. Traditionally, the identification of receptors for small molecules has relied on in vitro biochemical methods, such as photocrosslinking, radiolabeled ligand binding, and affinity chromatography. Affinity chromatography-based approaches have improved significantly in recent years, aided by technological advances in protein separation and analysis. However, these methods are still laborious and time consuming, interactions identified by such methods may not be direct (proteins may be purified as components of protein complexes), and cloning of cDNAs encoding candidate targets is uncoupled from the target identification process. To circumvent these problems, several functional cloning methods have been developed in recent years. These include yeast three-hybrid (Y3H) [17], drug-western [18], phage display cloning [19], and mRNA display cloning [20]. In all of these methods, the identification of ligand binding proteins is inherently linked to the selection of cDNA sequences encoding such interactors. The Y3H system (as well as other three-hybrid systems) distinguishes itself from these other methods in that interactions between small molecules and proteins occur in living cells rather than in vitro.

The development of three-hybrid systems, such as Y3H, has drawn from recent advances in (1) the design and application of synthetic small molecules that can interact with two proteins simultaneously, also known as chemical inducers of dimerization (CIDs), or chemical "dimerizers", and (2) two-hybrid protein-protein interaction screening technologies (such as the yeast two-hybrid system, or Y2H). This review summarizes the evolution of and recent advances in three-hybrid technologies for the analysis of small molecule-protein interactions as well as current and foreseeable applications in chemical biology and drug discovery.

Chemical Dimerizers and Cellular Signaling

Studies on the mechanisms of action of the immunosuppressive macrocyclic lactone lactams FK506 and rapamycin paved the way for the subsequent development of small molecule three-hybrid systems. FK506 and rapamycin are examples of small molecules, which have become termed chemical "dimerizers," meaning that by binding to two proteins simultaneously, they crosslink these proteins and promote the formation of a ternary

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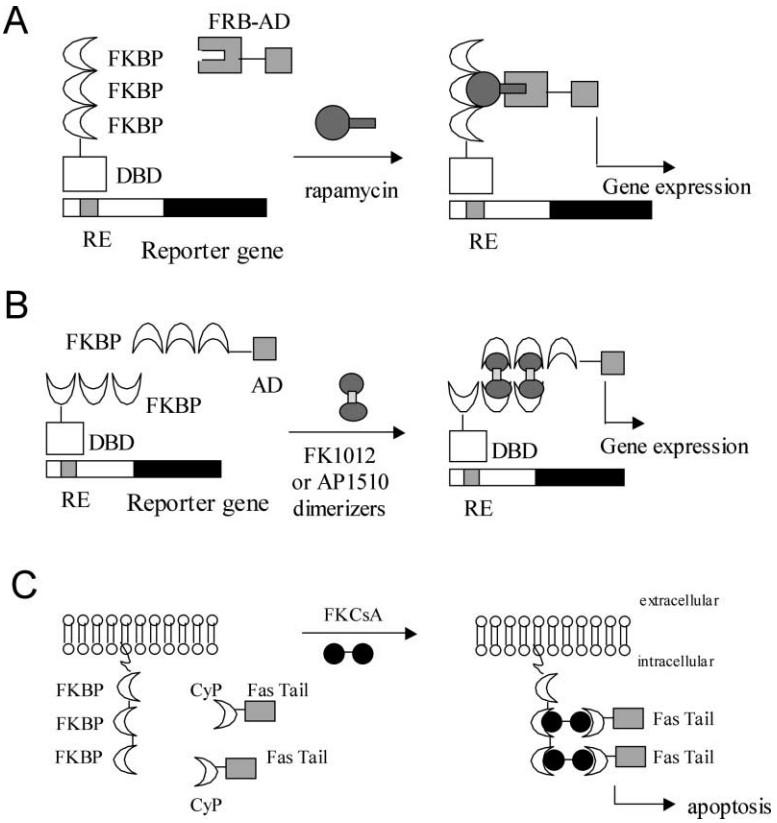

Figure 1. Chemical Inducers of Dimerization (CIDs) and Cell Signaling

Examples of uses of homo- and heterodimerizing CIDs.

(A) Induction of gene expression by rapamycin [25, 26, 30].

(B) Induction of gene expression by synthetic FK506-based dimerizers (FK1012 and AP1510) [30, 32].

(C) Recruitment of Fas tail to membrane-anchored FKBP and activation of apoptotic signal by the FKCsA (FK506-Cyclosporin A) heterodimer [33]. FRB, FKBP12-rapamycin binding domain of FRAP; CyP, cyclophilin; AD, transcription activation domain; DBD, DNA binding domain; RE, DNA response element.

complex. Important early findings concerning the mechanism of action of FK506 and rapamycin were that their biological effects required as an obligatory but not sufficient step the formation of a complex with the immunophilin FKBP12 (FK506 binding protein). Thus, FK506 binds to FKBP12; this binary complex is able to bind calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, thereby inhibiting its function [21]. The inactivation of calcineurin results in impaired signaling of the T cell antigen receptor (TCR) and subsequent immunosuppression. Similarly, rapamycin binds to FKBP12, and the resultant complex is able to bind FRAP (FKBP12-rapamycin-associated protein, also named RAFT1, RAPT1, or TOR) [22–25]. The inhibition of FRAP in T lymphocytes blocks interleukin 2 receptor signaling, which is thought to be the basis for the immunosuppressive actions of rapamycin. Another example of an immunosuppressant that acts in this manner is cyclosporin A, which forms a complex with cyclophilin, which then binds calcineurin [21].

The understanding of these mechanisms led to the use of rapamycin and FK506, and various analogs thereof, to effect at will the crosslinking ("dimerization") of hybrid proteins that have been designed to contain the appropriate binding sites for these small molecules, thereby controlling intracellular signaling events that are naturally or otherwise regulated by protein-protein interactions [1]. Thus, rapamycin was used to dimerize hybrid proteins containing FKBP12 and FRAP rapamycin binding domains [26–30] (Figure 1). Synthetic dimeric versions of FK506, such as bivalent FK506-FK506 (appropriately called FK1012) [31], FK1510 [32], or FK506-cyclosporin A [33], were used to homodimerize and heterodimerize various hybrid proteins (Figure 1). A further refinement in "dimerizer technology" was the generation of analogs of rapamycin [34] and FK506 [35] that recognized and bound only to appropriately mutated forms of FRAP or FKBP12, thereby also reducing their cytotoxicity. Chemical homo- and heterodimerizers have been used to control a variety of signal transduction events in cells engineered to express chimeric proteins encoding appropriate ligand binding domains and signaling domains, such as induction of membrane recruitment and activation of cytosolic signaling proteins [36–39], crosslinking of plasma membrane receptors and activation of receptor signaling [31, 40–44], induction of translocation of proteins into the nucleus [33], and activation of gene expression by recruitment of transcriptional activators to specific genes [29, 30, 32, 33], both exogenous transgenes and endogenous genes [45]. In this manner, cellular processes such as programmed cell death and cellular proliferation [33–35, 42, 46] or the expression and secretion of proteins with therapeutic potential were placed under the control of chemical dimerizers [26, 29, 30]. More recently, small molecule ligands have been employed to assemble as well as disassemble complexes of FKBP12, and to create cells that can secrete biologically active hormones under the control of designer FK506 analogs [47, 48]. The broad uses of CIDs may eventually constitute the basis for drugs that control fate or function of genetically modified cells in gene therapy treatments [29, 30].

While rapamycin and its analogs (also known as "rapalogs") have been widely used as chemical dimerizers,

it was the synthesis and use of the synthetic hybrid ligand/dimerizer FK1012 (dimeric FK506) [31] that marked the beginning of three-hybrid systems for either homo- or heterodimerization of chimeric proteins (i.e., the crosslinking of two hybrid proteins, same or different, by a hybrid/bivalent synthetic small molecule). FK1012 was used to induce aggregation of a T lymphocyte receptor fused to FKBP12 [31]. Alternatively, FK506-cyclosporin A was used to heterodimerize hybrid proteins [33]. Other dimeric analogs of FK506 that have been used in different settings include FK1510 [32] and AP1903 [35]. The latter recognizes specifically a mutant form of FKBP12. None of these studies, however, incorporated a small molecule test compound as a moiety of a hybrid ligand for the purpose of de novo identification of proteins that would bind such a test compound. Such an approach would require a robust screening system that would allow for expression of complex cDNA libraries and straightforward selection of productive interaction events. The Y2H system [49, 50] presented such an opportunity.

The Y2H system has been a most notable and widely used technology for the detection and identification of interactions of hybrid proteins in living cells on a large scale [16]. Since its inception by Fields and colleagues, the basic concept of Y2H has been applied and modified to create many approaches for measuring association or dissociation of macromolecules, including (1) RNA-protein interactions [51], (2) protein-peptide interactions [52], (3) protein-antibody interactions [53], (4) hormone-regulated association of protein-protein interactions [54, 55], (5) the screening for small molecules that disrupt or interfere with protein-protein interaction [56–58], (6) protein dimerization in response to the natural product rapamycin [25], and (7) protein crosslinking (dimerization) by synthetic chemical homo- or heterodimerizers (i.e., the Y3H system) [17]. Features of Y3H and other potential three-hybrid systems are described in more detail below.

Yeast Three-Hybrid Systems: Identification of Small Molecule Protein Targets

The basic elements of the Y3H system are schematically described in Figure 2. It is based on the use of three hybrid molecules: a hybrid protein that contains a DNA binding domain (DBD) fused to a first small molecule ligand binding domain (LBD), a hybrid protein that contains a transcriptional activation domain (AD) fused to a second ligand binding domain, and a bivalent hybrid molecule. The hybrid small molecule consists of an "anchor moiety" with known binding affinity for the LBD of the DBD-fusion protein, a "test compound" moiety, for which binding proteins are to be identified, and a linker, which covalently links the two distinct small molecule moieties to form a bivalent hybrid ligand. A productive interaction of the test compound with the ligand binding domain of the AD-fusion protein results in recruitment of this protein to DNA, an event that results in activation of the expression of a downstream reporter gene.

The first report on Y3H described the use of a dexamethasone (DEX)-FK506 heterodimer to crosslink two chimeric proteins, one displaying the glucocorticoid receptor (GR) fused to a DNA binding domain (LexA), and the other being FKBP12 fused to a transcriptional activation domain of the bacterial protein B42 [17]. The use of mutant forms of the GR, which displayed higher affinity for dexamethasone (nanomolar) than wild-type GR, was necessary for detection of the interaction. These findings suggest that affinities in at least the nanomolar range are most likely required for successful display of a synthetic hybrid ligand (e.g., DEX-FK506) by the DBD-fusion protein (e.g., LexA-GR). Importantly, Licitra and Liu showed that the DEX-FK506 heterodimer could be used to identify FKBP12 in a cDNA library screen, demonstrating that the Y3H system could, in principle, be used to screen complex cDNA libraries to identify drug receptors.

Since the first report on Y3H, various types of synthetic ligands have been deployed for use in Y3H, using small molecules with known and high affinity for particular receptor proteins (DEX, FK506, and methotrexate, MTX). A recent report described the use of a DEX-MTX hybrid ligand in the identification of dihydrofolate reductase (DHFR), the known receptor for MTX, in a cDNA library screen [59]. Analogously, Cornish and colleagues demonstrated the use of MTX-DEX dimerizers in which MTX was used as the anchor moiety of the dimerizer [60–62]. MTX has a high affinity for its target, DHFR. Binding of MTX to DHFR follows a two-step mechanism, with isomerization to the second, high-affinity complex being the rate-determining step. Interestingly, although the inhibition constants (Ki) of MTX for both the *E. coli* DHFR (eDHFR) and murine DHFR (mDHFR) are indistinguishable, 1–20 pM depending on experimental conditions [63–67], the eDHFR monomeric protein has been shown to be significantly more effective in displaying MTX-DEX for binding to the glucocorticoid receptor [61]. It appears that the initial complex for mDHFR is 100 times less stable than for eDHFR and is characterized by a rapid off-rate (k_{off}). Whether this is the basis for the observed differences remains unclear. Whether MTX-based hybrid ligands would be suitable for the de novo identification of small molecule binding proteins using cDNA library screens also remained unclear. This was, however, demonstrated in a very recent study designed to explore the utility of Y3H in screening for targets of small molecule kinase inhibitors [78]. The results of this study showed that a LexA(DBD)-DHFR fusion protein could be used in conjunction with MTX-based hybrid ligands to identify known and novel targets of purine analogs. This study also shed light on how a Y3H system may perform in satisfying numerous criteria essential for an effective screening platform. For example, it was shown that library screens can be performed at high complexity and redundancy, the emergence of false positives can be controlled for and easily suppressed using appropriate genetic counterscreens and a combination of diverse hybrid ligands, interactions can be detected with a high degree of specificity, and interactions of components with known affinities in the micromolar range or higher can be detected with such a system. Furthermore, it became apparent that the Y3H system could be used to characterize the interaction of ATP-competitive active site kinase inhibitors with both serine/threonine kinases (including cyclin-dependent ki-

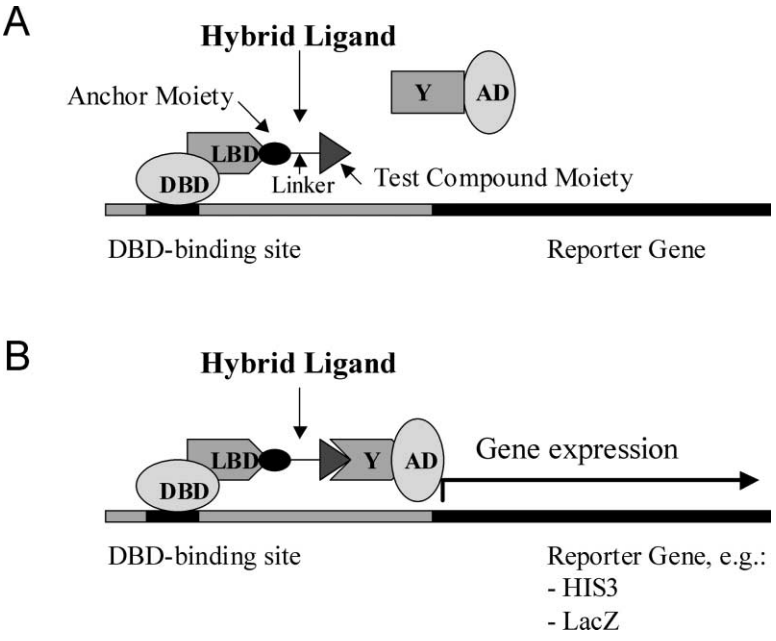

Figure 2. The Yeast Three-Hybrid (Y3H) System

Y3H is a three-hybrid system that exploits the modular characteristics of transcriptional activators [17]. Two chimeric proteins are expressed within the same yeast cell. One chimera is composed of a DNA binding domain (DBD, e.g., derived from bacterial LexA or yeast GAL4 transcriptions factors) and a ligand binding domain with known characteristics (LBD, e.g., DHFR). The second chimera is composed of a transcriptional activation domain (AD, e.g., derived from the bacterial protein B42 or yeast GAL4) fused to a polypeptide (protein Y) of interest. The small molecule heterodimer is composed of an "anchor moiety" capable of binding to the LBD portion of the DBD-LBD protein (e.g., MTX, which binds DHFR) and a linker which tethers it to a test compound of interest. Interaction of the test compound with protein Y promotes a ligand-dependent dimerization of the chimeric proteins, resulting in the reconstitution of a macromolecular complex with transcription-activating function. Recruitment of the complex to specific DNA binding sites (response elements, RE) upstream of a promoter region results in activation of a downstream reporter gene. Thus, induction of reporter expression reflects ligand-dependent dimerization of the chimeric proteins. In a yeast three-hybrid system, a reporter gene may, for instance, encode an auxotrophic marker (e.g., HIS3, induction of which enables yeast growth in the absence of histidine in the growth medium), an enzymatic marker (e.g., LacZ, detected in colorimetric assays), or a fluorescent marker (e.g., GFP). Random screening for small molecule targets can be performed on a proteome-wide scale by transforming a suitable yeast strain with an AD-fusion protein-encoding cDNA library.

nases) and tyrosine kinases (including receptor tyrosine kinases). Kinases constitute an important class of therapeutic targets, and the availability of a screening system for profiling the target space (kinase and nonkinase) for such inhibitors should prove useful across different areas of biomedical research. The findings that interactions with affinities in a range that is of pharmacological interest/relevance can be detected with Y3H also suggest that Y3H may be used in the context of a broader range of types of synthetic small molecules.

As discussed above, a chemical dimerizer is composed of essentially three parts, with a linker connecting an anchor moiety (e.g., MTX) and a test compound of interest. Choice of linker type may be governed by a number of considerations: linker length should be easily variable, as this may affect the performance of the hybrid ligand. Coupling of the linker to the test compound should be feasible using different chemistries such as to facilitate the synthesis of a broad range of CIDs with different linkages. The number of synthesis steps in generating a CID should be minimal with an overall acceptable yield. Finally, linkers should ideally improve solubility and cell permeability of a hybrid ligand. The use of straight-chain aliphatic linkers of variable length has been reported for the synthesis of MTX-DEX CIDs [61]. A three-methylene linker did not result in a functional CID. However, five-, eight- and ten-methylene linkers performed nearly equally well. Thus, a minimal linker length appears necessary for these CIDs to work effectively. The synthesis required nine steps with an overall yield of 2%–5%. Alternatively, a recent study made use of polyethyleneglycol (PEG) linkers [78]. PEG linkers have been used previously in coupling small molecules to solid-phase surfaces for protein purification [68]. Thus, integrating PEG linkers in the synthesis of a CID can yield derivatives of test compounds for parallel and straightforward use in standard biochemical assays. PEG linkers of variable lengths have been used in the synthesis of heterodimeric ligands [78], which have in general shown good solubility properties and, surprisingly, were able to penetrate yeast cells well. The rationale for this is not well understood, but the findings hold promise for the use of Y3H with a broad range of small molecules. At GPC Biotech, we have analyzed over 50 MTX-PEG-based heterodimeric molecules to date and have not seen any significant drop-out due to lack of compound uptake (although variability in uptake is observed). Figure 3 depicts a general strategy that has been pursued in the synthesis of MTX-based heterodimers with PEG linkers. Similar strategies should be possible with other types of anchor molecules, such as FK506 and its analogs.

Toward High-Throughput Y3H Screening Systems

Over the past decade, the scientific community has made broad use of the Y2H system. In recent years, the emergence of large-scale genomics and proteomics approaches has changed ways in which certain technologies are developed and deployed, including Y2H. Thus, Y2H has been used in large-scale screening formats to map protein-protein interactions and potential signaling networks at the proteome level, as exemplified by studies using model systems such as yeast, *C. elegans*, and *D. melanogaster* [16]. Similarly, the use of yeast in Y3H should also facilitate larger-scale screening for small molecule-protein interactions.

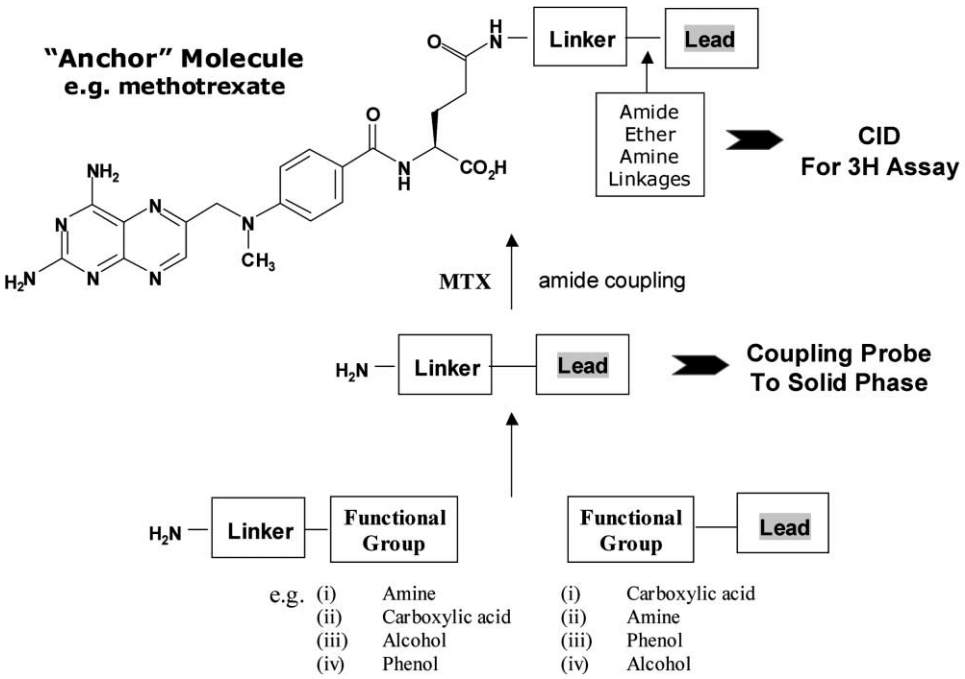

Figure 3. A Strategy for the Synthesis of Chemical Dimerizers

A general strategy for the synthesis of methotrexate-based chemical dimerizers (CIDs), which has been applied to the synthesis of heterodimers with polyethylene glycol (PEG) linkers [78], is shown. En route to synthesis of the dimer, a PEGylated derivative of the test compound is generated, which can be used in diverse biochemical assays requiring coupling of the test compound to solid phase. A diversity of chemical reactions could be applied when using different functional groups for coupling reactions (e.g., amide, amine, ether linkages).

Important benefits that derive from using yeast as a host system for analyzing macromolecular interactions extend beyond its easy manipulation, both genetically and from an experimental perspective, and the reasonable costs of high complexity screening. Yeast can also be manipulated easily using modern robotics and automation technology. Yeast cells may be easily arrayed and cultured on agar plates or membranes. Multiple replicas of yeast arrays can be generated in this manner, each of which may be subjected simultaneously to different selection criteria (e.g., growth conditions) or interrogated with a diverse set of hybrid ligands (see Figure 4). Besides improving data output quality, array analyses have greatly increased the flexibility with which yeast systems may be deployed. In the case of Y3H, the implementation of yeast cell arrays was found to be extremely useful for generating high quality data on small molecule-protein interactions, whether used to "validate" interactions identified in cDNA library screens or in direct screening of selected genes/proteins [78]. Not surprisingly, array-based analysis also seems to be more sensitive than complex cDNA library screening. In an array screening format, each potential small molecule-protein interaction is tested individually; hence, no competitive growth selection is taking place, and weak interactions can be detected more easily. Another important benefit of array-based analyses is that defined ORF (open reading frame) collections, e.g., a gene family of interest, may be rapidly screened (within days) with a set of small molecules of interest. Furthermore, each recombinant yeast cell that expresses a selected gene/hybrid protein of interest, once established, is a permanent resource for screening, with each experiment increasing the information content about ligand binding properties of that particular expressed protein(s). Since probing for ligand binding in a Y3H system does not require a priori knowledge of the biochemical activity of the protein or proteins of interest, no individual assay system is required for each protein. Hence, three-hybrid gene family screening may include members whose biological functions are unknown. An example of an array analysis would be the use of comprehensive "kinase arrays" in selectivity profiling of kinase inhibitors.

Figure 4 depicts how two-hybrid and three-hybrid based technologies, in conjunction with other technological and experimental approaches to small molecule mode-of-action studies (e.g., gene expression), could lead to a more comprehensive picture of molecular events underlying drug effects. Integration of diverse technological approaches with mode-of-action studies will remain an ongoing challenge but will benefit the drug discovery process.

Toward Mammalian Three-Hybrid Systems

As compared to Y2H, the Y3H system greatly benefits from the fact that the DBD-fusion protein for a given system (e.g., LexA-DHFR) remains invariant and that multiple control CIDs can be used simultaneously to rapidly sort for bona fide interaction events. The Y3H system, however, does share some limitations inherent to the Y2H system. Thus, it is limited to the analysis of proteins that can be expressed and translocate into the

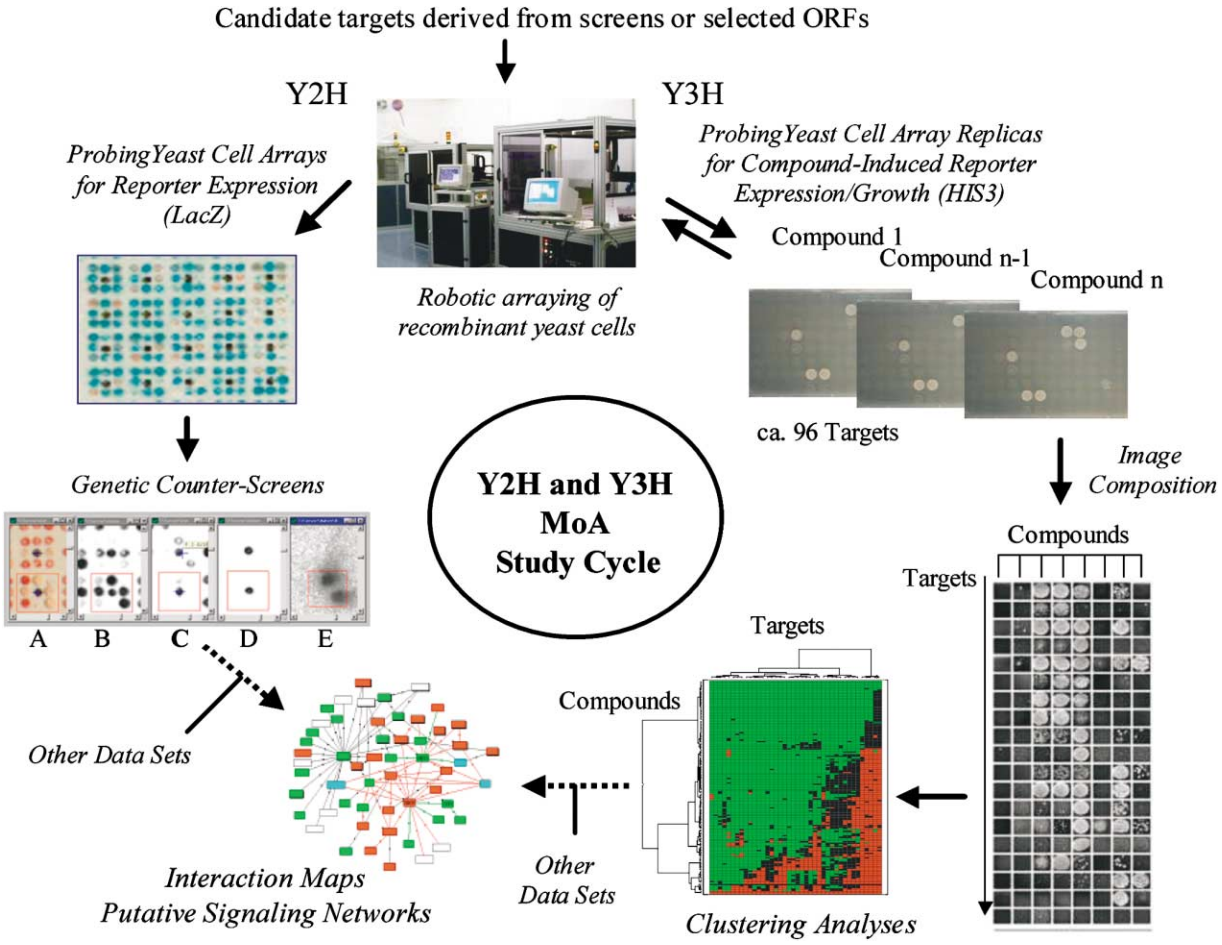

Figure 4. Y3H and Y2H Array Screening in Drug Mode-of-Action Studies

Screening scenarios that utilize the arraying of recombinant yeast cells for the analysis of either small molecule-protein (Y3H) or protein-protein interactions (Y2H) are shown. The Y3H workflow highlights robotic replica arraying of recombinant yeast cells in 96-well type array formats (i.e., 96 proteins may be assayed simultaneously) for parallel interrogation with compounds. Recombinant cells may be generated with a set of genes of interest (e.g., gene families or genes previously identified in random cDNA library screens). Composite interaction images can be generated rapidly and hierarchical clustering analyses performed to reveal spectra of proteins that may interact with a given small molecule or spectra of small molecules that a given protein interacts with. Targets identified using Y3H may subsequently be subjected to Y2H studies for mapping of signaling pathways. Robotic arraying of recombinant yeast cells can be used to generate complex arrays for the analysis of reporter gene expression (e.g., LacZ, as shown) in response to two-hybrid protein-protein interactions. Replica arrays can also be used to interrogate such arrays under different selection criteria (genetic counter screens) and testing for the dependence of an interaction event on the presence of both hybrid proteins (e.g., panel A, growth of arrayed yeast cells [cells are spotted in duplicate]; panel B, activation of LacZ reporter when both hybrid proteins are present; panel C, no LacZ reporter activation under conditions that deselect for presence of one hybrid protein; panel D, no LacZ reporter activation when the other hybrid protein is deselected; panel E, replica filters can be generated for hybridization with DNA probes). This type of array analysis increases Y2H data output quality. The integration of data sets resulting from use of Y2H, Y3H, and other experimental approaches (e.g., gene expression studies) can lead to the construction of putative signaling network maps that can be visualized using bioinformatic visualization tools. Visualization of composite data sets may drive the generation of new hypotheses and experimental strategies for more comprehensive mode-of-action studies, leading to a better understanding of the molecular basis of drug action.

nucleus of yeast cells. Additionally, it is not suitable for the analysis of full-length membrane proteins (although it can work with domains of membrane proteins) or small molecule-protein interactions that require accessory proteins or certain posttranslational modifications of the target proteins that are not compensated for by endogenous yeast proteins. Yeast cells are also generally less permeable to small molecules than mammalian cells, with the previously noted interesting exception, it seems, of MTX-heterodimers. The variable and generally less favorable uptake of small molecules by yeast cells limits the use of Y3H in "competition" experiments comparing the interaction of a parent test molecule with a hybrid ligand incorporating that small molecule with a target protein. Genetically modified yeast strains with improved permeability to small molecules have recently been used in screening for compounds that disrupt Y2H protein-protein interactions [58]. Analogously, modified yeast strains could potentially be used in some Y3H applications. Alternatively, one could use a different host system with better permeability to small molecules in particular mammalian cells. Access to a mammalian sys-

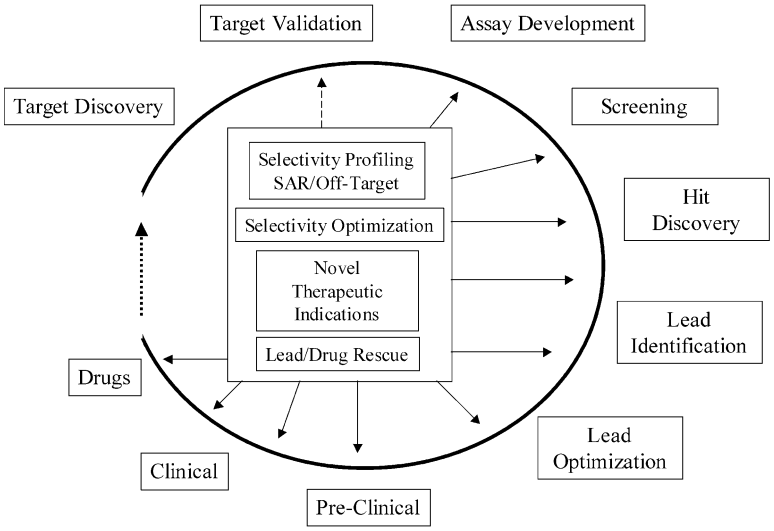

Figure 5. Three-Hybrid Applications in Drug Discovery

Three-hybrid systems can, in principle, be used to identify protein targets of small molecules or, alternatively, novel small molecules for a specific protein(s) of interest and, as a consequence, have the potential of impacting the drug discovery process at multiple stages. A broader understanding of the molecular basis of drug action should help guide medicinal chemistry efforts in lead optimization, reveal targets that may be associated with adverse side effects, identify targets for known leads/drugs that could point toward novel therapeutic uses, and provide information that may lead to reactivation of failed drug discovery programs (drug rescue).

tem(s) could conceivably also provide an additional and powerful platform for interaction screening.

Driven by the limitations of the Y2H system, various complementary two-hybrid systems have been developed over the past years, all of which could, in principle, be adapted for use in a three-hybrid fashion in mammalian cells. Indeed, making various two-hybrid systems applicable for cDNA library screening purposes should be easier for three-hybrid applications, given the aforementioned fact that one of the two chimeric proteins remains invariant (e.g., the DBD-LBD chimera in Y3H). Two-hybrid systems for mammalian cells include (1) the ubiquitin-split-protein-sensor (USPS) technology [69, 70]; (2) two-component protein fragment complementation assays (PCAs), e.g., systems based on reconstitution of split-DHFR [71] or split-β-lactamase [72, 73] (these have already been shown to support dimerizer-induced interactions); and (3) interaction technologies based on resonance energy transfer between reporter proteins with fluorescent or bioluminescent properties, i.e., FRET (fluorescence resonance energy transfer) and BRET (bioluminescence resonance energy transfer). It is important to note, however, that although these systems have been used to detect defined protein-protein interactions in mammalian cells, none of these has yet been utilized successfully in complex cDNA library screens for the discovery of novel interactions. The reason for this may be that the detection of interactions in these systems is subject to more stringent steric and spatial requirements. In contrast, MAPPIT, a recently reported two-hybrid-based technology established for mammalian cells, has been used successfully in library screens [74]. MAPPIT distinguishes itself from the other aforementioned systems in that it exhibits properties of a "protein recruitment" system, in which a signaling protein-protein complex is activated upon recruitment to a "docking station" (i.e., an intracellular receptor domain). In that regard, MAPPIT shares some similarity to Y2H, in which an AD-fusion protein is recruited to DNA through protein complex formation. Thus, MAPPIT could be a suitable system for the development of a mammalian three-hybrid screening system. It should also support the detection of some interactions that require post-translational modifications of the protein target as well as be suitable for rapid testing of interactions between parent test compounds and protein targets ("competition experiments").

Three-Hybrid System Screening Modalities

The yeast three-hybrid system can be used in different ways to study the interaction of small molecules with target proteins. As discussed earlier, it may be employed in the screening of cDNA libraries for targets of small molecules or in a more focused analysis of defined target classes. Both approaches are useful in determining the target space of small molecules and in performing more comprehensive structure-activity relationship (SAR) studies. Alternatively, as previously suggested [17], Y3H could be used to rapidly test for mutations in proteins that either positively or negatively affect binding to a small molecule. An analogous approach has been used to identify FKBP or FRAP mutants that bind specific analogs of FK506 and rapamycin [34, 35]. Similarly, such an approach could be used to identify drug-resistant mutant target proteins that could conceivably retain biological activity and be used to explore the role of a target in mediating a pharmacological effect of a compound of interest. Alternatively, the Y3H system could be used to screen for compounds that interfere with binding of a particular hybrid ligand to a selected target. The design of a reverse-Y3H system, analogous to the reverse-Y2H system [56], could be useful for such a purpose, although, as indicated earlier, a mammalian three-hybrid system would be more desirable. Finally, focused small molecule libraries, synthesized on scaffolds of anchor moieties (e.g., MTX) could be used to identify small molecules with interesting interaction profiles across a specific gene family. Verdine and colleagues have recently reported the synthesis of AP1867 (a FK506 analog) based combinatorial libraries [75]. It is conceivable that such types of compound libraries could be used to screen for phenotypic effects of small molecules in cells, followed by direct identification of targets mediating such effects using a three-hybrid system.

Recently, Cornish and colleagues reported yet another functional cloning application of a three-hybrid system [76]. In this case, the three-hybrid system was used to assay for an enzymatic activity of a protein expressed in yeast cells that could cleave the linker moiety of a defined dimerizer (MTX-linker-dexamethasone). Previously, a system in bacteria had been described based on AraC chimera dimerizer-regulated transcription to screen for dehydratase activity [77]. These approaches may prove useful in protein engineering and in screening for proteins with novel enzymatic activities. In summary, it is conceivable that as the spectrum of three-hybrid systems increases, multiple assay formats will be used in the analysis of small molecule-protein interactions and in the functional cloning of proteins.

Uses of Three-Hybrid Systems in Drug Discovery

Although only a few reports to date have described the use of three-hybrid systems for the identification and analysis of small molecule-protein interactions, these reports hold promise for the more widespread use of such systems in the future. As discussed in this review, many diverse technological advances have contributed to the recent development of more streamlined and productive screening paradigms, revealing more clearly the potential for three-hybrid systems. The increasing availability of cloned genes should further facilitate, accelerate, and diversify the use of currently available as well as future three-hybrid systems.

It is reasonable to assume that a better understanding of the pharmacological basis of drug action will not only improve our understanding of biological processes affected by small molecules, but will also benefit the drug discovery process at multiple stages (Figure 5). These include lead selection and optimization processes, prediction of potential dose-limiting adverse side effects, and revelation of unexpected new therapeutic applications of a drug candidate or drug. Ultimately, improving the rational basis for drug discovery should help in reducing attrition rates and costs associated with drug development. Three-hybrid systems should become increasingly useful tools to be added to the arsenal of drug discovery technologies designed to address major bottlenecks in today's drug discovery efforts.

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