Analysis of membrane protein interactions using yeast-based technologies

Igor Stagljar and Stanley Fields

Proteins associated with membranes total approximately a third of all proteins in a typical eukaryotic cell. However, the analysis of interactions between membrane proteins is difficult because of the hydrophobic nature of these proteins, and conventional biochemical and genetic assays are often of limited use. We summarize here recent yeast-based interaction technologies that can be applied to membrane proteins.

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In the past few years, the scientific community has generated an astounding amount of information about the genetic details of living organisms. The genome sequences of more than 100 organisms are now available in databases, with nearly 800 sequencing projects of other organisms currently under way [1]. Following from these sequencing projects, functional genomics approaches have begun to reveal the roles of the encoded proteins [2–5].

One striking feature of all complete genome sequences is that approximately a third of the predicted proteins of an organism are predicted to be anchored in the lipid bilayer [6,7]. These membrane-associated proteins perform a wide range of essential cellular functions. For example, pores, channels, pumps and transporters facilitate the exchange of membrane-impermeable molecules between cellular compartments and between a cell and its extracellular environment. Transmembrane receptors sense changes in the cellular environment and, typically through associated proteins, initiate specific cellular responses. Because of their accessibility and essential roles, membrane proteins are also of considerable diagnostic and therapeutic importance: 50% of currently known drug targets (~500) are either membrane receptors or ion channels [8]. Thus, understanding the physiology of membrane proteins and the means by which these proteins communicate in the cell is of crucial importance.

Protein interactions are involved in the regulation and execution of all biochemical pathways within the cell. Thus, the identification of binding partners is crucial for understanding the function of an uncharacterized protein, and when these partners turn out to have known function, deductions about the potential role of the uncharacterized protein can often be made. Traditionally, biochemical methods such as co-purification or co-immunoprecipitation have been used to investigate the composition of protein complexes. However, these methods generally require extensive optimization for each complex, making them unsuitable for simultaneous application to the tens of thousands of uncharacterized proteins predicted from genome sequences. Recently, though, a biochemical approach using standard affinity tagging methods combined with mass spectrometric (MS) analysis has proven sufficiently robust to apply to yeast protein complexes on a large scale [9,10]. Two groups carried out affinity purifications and analyses on ~10% of all yeast proteins, which had been expressed with an affinity tag. The associated proteins that co-purified with each tagged protein were identified using standard MS methods [9,10].

Genetic systems that are based on the detection of protein–protein interactions *in vivo* are valuable because they require little individual optimization and are well-suited to screening in a high-throughput format. With its powerful genetic and molecular approaches, a completely sequenced genome [6] and a collection of deletion strains [11], the budding yeast *Saccharomyces cerevisiae* has emerged as an important tool in the study of protein interactions [1,12]. In particular, the yeast two-hybrid system has the major advantage that interactions are detected in an *in vivo* setting by the reconstitution of separated domains of a transcription factor, without requiring *in vitro* handling of any protein molecules at all [13,14]. As the assay can be optimized for many different protein pairs, it can be readily automated. This approach has enabled the generation of large interaction networks within *S. cerevisiae* [4,15], the bacterium *Helicobacter pylori* [16] and the worm *Caenorhabditis elegans* [17].

The two-hybrid system, however, has limitations with respect to identifying partners for membrane proteins. Because a protein–protein interaction that leads to the reconstitution of an active transcription factor must occur on the promoter of the reporter gene, the interacting proteins have to be located in the nucleus to detect the interaction. For this reason, transmembrane proteins, which tend to be insoluble and form aggregates if not present within membranes, are poor candidates for this assay. Moreover, these proteins can undergo post-translational modifications

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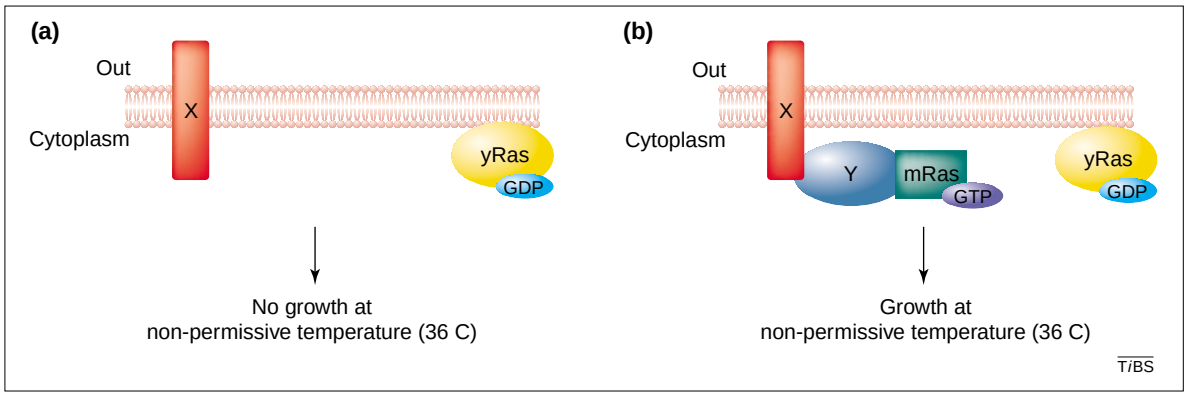

Fig. 1. The reverse Ras recruitment system. (a) A membrane protein of interest (X) is expressed in a *cdc25–2* mutant yeast strain. This protein is not expected to induce yeast growth at the non-permissive temperature (36°C) because it will not recruit mammalian Ras to the membrane. (b) If an interacting partner (Y) is expressed as a membrane fusion with the mammalian Ras protein, the X–Y interaction can lead to the translocation of the Y–mRas fusion to the yeast plasma membrane and growth at the non-permissive temperature.

(e.g. glycosylation, disulfide bond formation), contain intra-membraneous ligand-binding pockets (as in receptors), or oligomerize through interactions between their transmembrane domains, which are not favorable for a nuclear-based assay. Genome-wide yeast two-hybrid screens have shown that the coverage of membrane protein interactions is poor [4,15]. Although certain membrane protein interactions have been detected successfully in the two-hybrid assay [18,19], the characterization of integral membrane proteins, as well as the identification of their interactions with cytoplasmic and/or other integral membrane proteins, are best carried out using other methodologies.

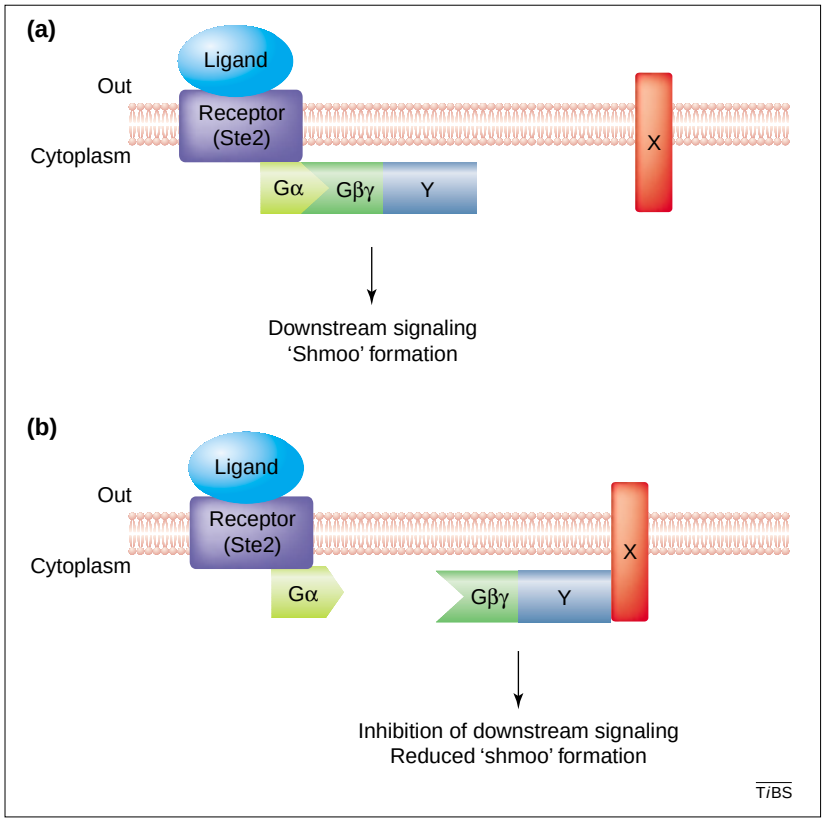

Fig. 2. The G-protein based yeast two-hybrid system. (a) One binding partner is an integral membrane protein (X), and the other (Y) is soluble but fused to a G-protein γ-subunit. If X and Y do not interact, G_{γ}–Y together with G_{α} and G_{β}, bind to the cytoplasmic domain of the yeast G-protein-coupled receptor Ste2. The ligand activation of Ste2 induces G-protein dissociation and downstream signaling by the $G_{\beta\gamma}$–Y complex. (b) If the binding partners X and Y interact in the yeast plasma membrane, the G_{γ}–Y fusion protein will be sequestered by X, thus leading to disruption of the G-protein signaling process. The yeast colonies containing two such interacting proteins can be identified by their different morphology.

Reverse Ras recruitment system

Over the past few years, researchers have developed alternative yeast-based systems that retain the advantages of the original yeast two-hybrid system and that are also capable of detecting interactions involving membrane proteins. The reverse Ras recruitment system (reverse RRS) is based on the Ras pathway in yeast, which requires Ras localization to the plasma membrane for its function [20,21]. When localized at the plasma membrane, the yeast-essential Ras guanyl exchange factor Cdc25 stimulates guanyl nucleotide exchange on Ras and promotes downstream signaling events that ultimately lead to cell growth [20]. The reverse RRS uses a mutant *cdc25–2* yeast strain that is able to grow at the permissive temperature of 23°C but fails to grow when shifted to 36°C. Importantly, mammalian Ras (mRas), when membrane-bound, complements the *cdc25–2* mutation in yeast. In the reverse RRS system (Fig. 1), a membrane protein of interest (X) is expressed in the membrane, and its interaction partner (Y) is fused to a mutant of mRas that is cytoplasmic. The interaction of proteins X and Y brings mRas to the membrane, resulting in efficient growth of the *cdc25–2* mutant at the non-permissive temperature (36°C). This system has been used to isolate two novel interaction partners of the small GTPase Chp [21]. However, a drawback of this technology is that fusions of integral membrane proteins or membrane-associated proteins to mRas will lead to cell growth independent of a protein–protein interaction. Although the use of inducible promoters circumvents this problem, it also complicates a potential adaptation of the RRS system for high-throughput screens.

G-protein fusion technology

In another yeast membrane-based interaction approach, inactivation of a G-protein signaling pathway serves as the reporter readout [22]. In this experimental system, a protein under investigation (X) is an integral membrane protein and the other protein (Y) is soluble but fused to a G-protein

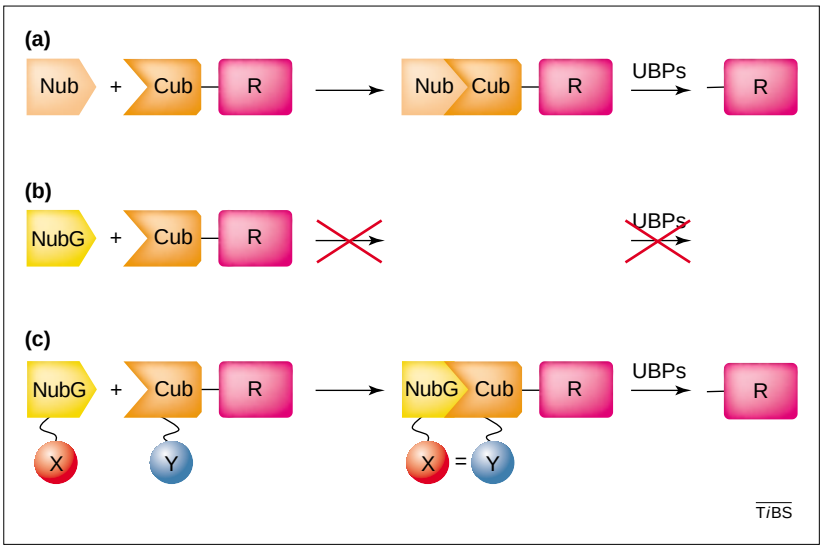

Fig. 3. The split-ubiquitin assay. (a) Ubiquitin can be expressed as an N-terminal (Nub) half as well as a C-terminal (Cub) half which is fused to a reporter protein (R). The two halves retain affinity for each other and spontaneously reassemble to form the so-called split-ubiquitin. (b) A point mutation in the N-terminal half of ubiquitin (NubG) completely abolishes the affinity of the two halves for each other, and as the separate NubG and Cub parts are not recognized by ubiquitin-specific proteases (UBPs), no detectable cleavage of the attached reporter takes place. (C) NubG and Cub are fused to the interacting proteins X and Y. The X–Y interaction brings the NubG and Cub domains close enough together to reconstitute ubiquitin, resulting in the release of the reporter protein by the action of the UBPs.

γ subunit. The interaction between X and Y brings G_γ to the membrane, where it sequesters the G_β subunit and forms a complex, thus inhibiting G-protein-coupled-receptor signaling in yeast. Interacting colonies are identified either by measuring the activity of an *Escherichia coli lacZ* reporter gene that is induced by the mating pathway or by assaying the sensitivity of the yeast cells to α-factor. In the latter case, interaction between X and Y leads to growth arrest in yeast and the resultant formation of elongated cells known as 'shmoos' (Fig. 2). This system was able to detect known interactions between syntaxin 1a and neuronal Sec1 (nSec1); and between fibroblast-derived growth factor receptor 3 (FGFR3) and SNT-1 [22]. In addition, this technology has been successfully applied to screen and isolate mutant forms of the Sec1 protein that are no longer able to bind syntaxin 1 [22].

Split-ubiquitin assay systems

The split-ubiquitin system provides another approach to study membrane protein interactions [23,24]. Ubiquitin (Ub) is a small, highly conserved protein that is attached to lysine residues of other proteins to tag them for proteasomal degradation [25]. Ubiquitin-tagged proteins are recognized by ubiquitin-specific proteases (UBPs) that cleave after the C-terminal residue (Gly76) of Ub and the first residue of the target protein, allowing release of the protein for degradation by the 26S proteasome. Johnsson and Varshavsky [26] found that native ubiquitin can be split into an N-terminal (Nub) and a C-terminal (Cub) half. The two halves retain a basic affinity for each other and reassemble spontaneously to form quasi-native ubiquitin. If a reporter protein is fused to the C-terminus of Cub, it will be cleaved off by UBPs upon assembly of the Nub and Cub moieties (Fig. 3a). A point mutation in the N-terminal domain of ubiquitin (NubG) abolishes the affinity of the two halves for each other, such that NubG and Cub fail to refold into split-ubiquitin when co-expressed in yeast (Fig. 3b). However, if the two ubiquitin halves are fused to the interacting proteins X and Y, this interaction brings the NubG and Cub moieties close enough together to reconstitute quasi-native Ub, resulting in the release of the reporter protein by the UBPs (Fig. 3c).

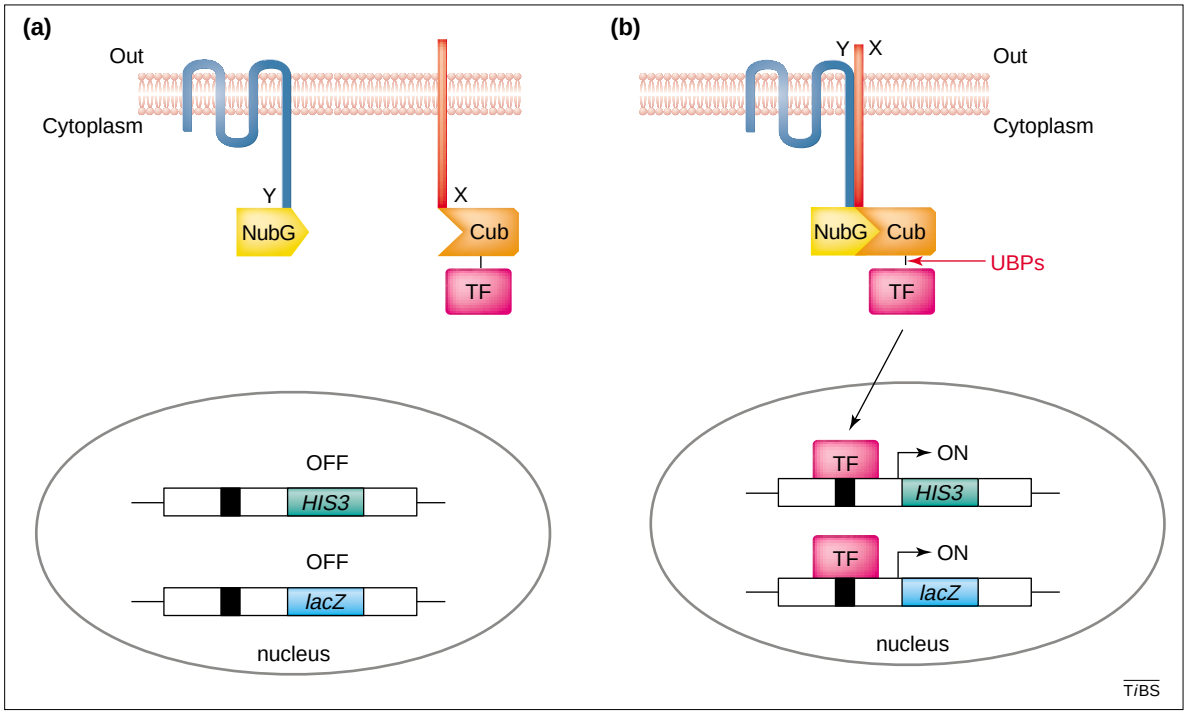

Fig. 4. The transctivator-based membrane yeast two-hybrid system. (a) A membrane protein of interest X is fused to Cub followed by an artificial transcription factor (TF), while another membrane (or cytoplasmic) protein Y is fused to the NubG domain (Y–NubG). Co-expression of X–Cub–TF with a non-interacting Y–NubG does not lead to the formation of split-ubiquitin nor cleavage by UBPs. (b) On interaction of the X and Y proteins, ubiquitin reconstitution occurs, leading to proteolytic cleavage and the subsequent release of the transcription factor. This factor activates reporter genes to result in cells that are histidine prototrophs and that turn blue in a β-galactosidase assay.

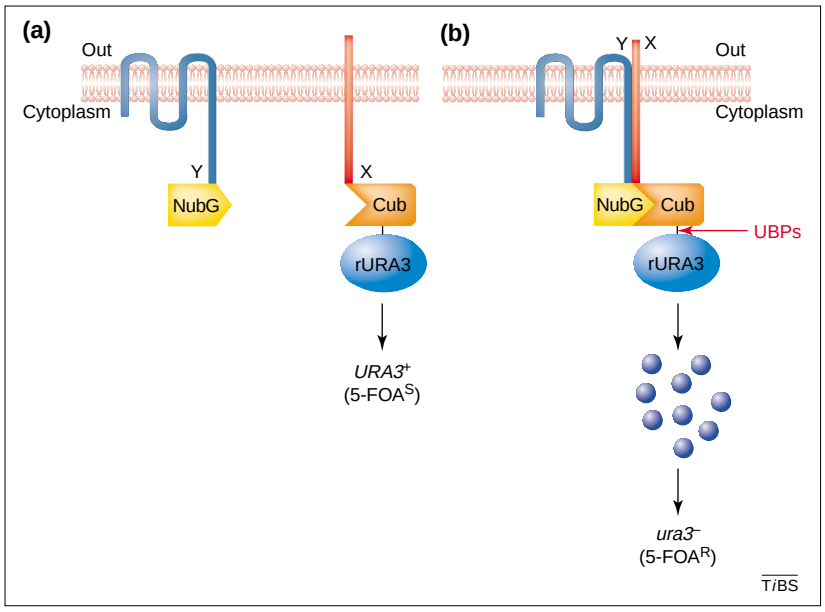

Fig. 5. The rUra3-based membrane yeast two-hybrid system. (a) A membrane protein (X) under investigation is expressed as a fusion to the Cub domain, which is fused to a destabilized version of the Ura3 protein (rUra3). The NubG domain is linked to the membrane protein Y. If X and Y do not interact, there is no ubiquitin reconstitution and thus no UBP-mediated cleavage, resulting in yeast cells that contain Ura3 activity and thus die (5-FOA^S) on medium containing 5-fluoro-orotic acid (5-FOA), a toxic metabolite of the Ura3 enzyme. (b) If the X and Y proteins interact, the Cub and NubG domains are brought into close proximity, where they reconstitute an active ubiquitin. Cleavage by UBPs then releases rUra3 from the Cub fusion. The cleaved rUra3 is targeted for rapid destruction by the enzymes of the N-end rule to yield cells that are uracil auxotrophs (*ura3*) and 5-FOA resistant (5-FOA^R).

The versatility of transcriptional activation of reporter genes in yeast was used to convert the split-ubiquitin system into a genetic assay for the *in vivo* detection of membrane protein interactions [23]. In this membrane-based yeast two-hybrid assay (Fig. 4), an artificial transcription factor (TF) consisting of the bacterial LexA protein and the *Herpes simplex* VP16 transactivator protein is fused to the Cub moiety. An integral membrane protein (X) is expressed as a fusion to the Cub–LexA–VP16 reporter cassette, with this cassette attached either to the N- or C-terminus of the transmembrane protein, depending on the orientation in the membrane of this protein. The second protein under investigation (Y), either another transmembrane protein or a cytoplasmic protein, is expressed as a fusion to NubG. If interaction between the X and Y proteins occurs, a split-ubiquitin molecule can be reconstituted, leading to the proteolytic release of the transcription factor to activate a reporter gene [23]. Thus, the re-association event initiated by the protein interaction is converted into a transcriptional output that can be detected easily. This assay has been used to investigate the influence of mutations on the assembly of fragments of presenilin (a protein implicated in Alzheimer disease) [27], to characterize the interaction between the yeast α1,2-mannosidase Mns1p and Rer1p in the endoplasmic reticulum [28], and to study intra- and intermolecular interactions between plant sucrose transporters [29]. This approach has also been extended to detect novel protein–protein interactions by screening libraries of NubG-fused inserts, from yeast and mammalian sources, against membrane proteins (S. Thaminy *et al*. unpublished).

In another split-ubiquitin-based approach, Johnsson and colleagues [24] have fused a destabilized version of the yeast Ura3 protein, termed rUra3, to the Cub moiety. An integral membrane protein (X) is expressed as a fusion to the Cub–rUra3 cassette. When cells expressing the fusion protein are grown on medium containing the compound 5-fluoro-orotic acid (5-FOA), they die because the rUra3 protein converts 5-FOA into a toxic product. However, if the cells co-express an interacting protein (Y) fused to NubG, the Cub and NubG moieties can be forced into close proximity by the X–Y interaction and associate to form split-ubiquitin. This association, in turn, leads to UBP-mediated cleavage at the C-terminus of Cub and the release of the rUra3 fusion protein into the cytosol. Because the newly created N-terminal residue of the rUra3 protein is destabilizing in the N-end rule pathway of protein degradation [30], the entire fusion protein is degraded by the 26S proteasome, leading to cells that can grow on medium containing 5-FOA [24]. In this way, cells expressing two interacting proteins can be identified by their ability to survive selection on 5-FOA plates (Fig. 5). The rUra3 based split-ubiquitin method was used to map the interactions between several *S. cerevisiae* integral membrane proteins [24] and to analyze changes in protein conformation and stability of the *S. cerevisiae* protein Sec62, a component of the translocation machinery in the membrane of the endoplasmic reticulum [31].

Other assays

Genetic assays based on complementation of proteins or protein fragments have also been developed [32,33] in organisms other than yeast that allow the monitoring of membrane protein interactions in real time, including ones amenable in mammalian cells. In the intracistronic β-galactosidase complementation assay [32], interacting proteins are fused to weakly complementing β-galactosidase mutants that are expressed at low levels. The protein interaction physically positions the β-galactosidase mutants such that complementation occurs and there is an increase in β-galactosidase activity. With respect to membrane proteins, this system has been used to monitor the dimerization of epidermal growth factor receptor (EGFR) [34]. Another assay is based on the enzyme dihydrofolate reductase (DHFR), in which DHFR complementary fragments are fused to two partner proteins. Folding of DHFR from its fragments is catalyzed by the protein interaction and detected as the reconstitution of enzyme activity [33]. Remy and Michnick [33] showed that this strategy can be applied to study membrane protein receptors, demonstrating dose-dependent activation of the erythropoietin receptor by ligands. A similar assay is based on complementation of β-lactamase fragments [35]. Although still in the early stages of their development, these assays hold promise for applications that allow

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screens for proteins that interact with a membrane protein of interest. In addition, such assays can be applied to screen for agonists or antagonists of specific protein–protein interactions.

Conclusion

The development of the yeast two-hybrid system and its variations has provided a genetic means to identify proteins that interact physically *in vivo*. The recent application of these technologies to membrane proteins is a step forward in the analysis of how membrane proteins interact in a cell. It is probable that, in the near future, numerous genomic and cDNA libraries will be generated to enable these membrane-based systems to be used in screens for protein partners of a given membrane protein. In this manner, it will be possible to uncover novel protein interactions that were not possible to detect using the traditional yeast two-hybrid assay. Furthermore, the eventual adaptation of such methods to a high-throughput format and their use in combination with automated screens should help in elucidating protein–protein interactions on a genome-wide scale.

In addition, a logical extension of the use of membrane-based yeast technologies will probably be for pharmacological purposes. Efforts directed towards elucidating signal transduction pathways, ion channels, mechanisms leading to neurodegeneration, and interactions between viral and host proteins have identified numerous membrane protein interactions that are crucial to cellular regulation. Thus, it could be possible to design selection systems that can identify peptides, small molecules or antibodies that specifically inhibit some of these interactions. The continuing development of membrane-based technologies should expand their utility and broaden the areas of experimental research to which they can be applied.

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