

Tools for Studying Protein-Protein Interactions: Pros and Cons

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After the publication of the draft of the human genome on Feb. 16, 2001 (Science and Nature), we realized that we need more than gene sequences to understand the complexity of their designated function(s). The actual complexity of organisms lies in proteins (products of these genes). Although a portion of the protein population are expected to function in isolation, the majority are expected to interact with other protein(s) forming protein complexes, which members may change according to specific function or in response to different stresses. Therefore, the identification of interactive stable or transiently interacting protein partner(s) in such complexes will help us better understand biological functions and provide us with a protein-protein interaction map of the cell. This information is valuable towards understanding the biology of the cell in general and specifically will enhance our knowledge of disease processes leading to the discovery of new drug targets.

Several in vitro and in vivo approaches have been introduced for the purpose of physical identification of protein-protein partners in a protein complex. Mostly utilized approaches depend on co-purification coupled with mass spectrometry including immunoprecipitation, pull down assays, tandem affinity purification (TAP), and yeast two-hybrid system.

Immunoprecipitation and Co-immunoprecipitation

If the antibody against a protein or its fusion is available, immunoprecipitation (IP) becomes a method of choice to in vitro co-immunoprecipitate (co-IP) proteins interacting with the target protein. Despite that co-IP has been key studying protein-protein interactions, if the protein(s) co-migrate on the SDS-PAGE gel with antibody subunits, the resolution and separation of co-IP proteins becomes problematic. The cross linking step of the IgG to protein A or G matrix has reduced this problem significantly, resulting in a co-IP profile without the interference of antibody subunits. However, the success of an IP or co-IP experiment depends on the quality of the antibody (i.e. purity and specificity), and sample preparation. In order to maintain the integrity of protein-protein interaction, it is always favorable to utilize a sample prepared under non-denaturing conditions, and binding conditions should be kept as close as possible to physiological pH. To overcome this problem, there is now a wide selection of commercially available IP kits with sample preparation reagents included (e.g. Sieze® IP kits from Pierce).

Pull-Down assay

When an antibody against a target protein is not available, a pull down assay can be utilized as an alternative to IP. Pull-down assay is an affinity chromatography method that involves using a tagged or labeled protein target (bait) (His, GST, FLAG, SNAP, Strep II, MBP, Biotin Calmodulin, Intein, T7 tag, Cellulose binding domain, Nus A, SUMO etc..) to create a specific affinity matrix that will enable binding and purification of interacting protein(s) (prey) from a lysate sample or other protein containing mixtures. If during expression the inclusion body issue is bypassed, and the tagged protein is expressed in a soluble and non denatured form, several points have to be considered before utilizing the purified protein in a pull-down assay, including:

- i- Purity of the tagged or labeled protein
- ii- Maintenance of the post-translational modification of the bait or prey protein
- iii- Native state of the interacting proteins
- iv- Bait protein concentration
- v- Interaction conditions
- vi- Stoichiometry of protein-protein interactions

These methods have generated a wealth of information about protein functions, and their protein partners, however, an assay is needed to simulate in vivo protein-protein interactions. The development of TAP provides a very promising tool towards this goal (1). The TAP-tag is produced by intrachromosomal tagging of bait proteins by protein A and calmodulin. The TAP tag has been specifically designed to express proteins at their naturally occurring level, under the control of their promoters. Therefore, proteins co-purifying with the bait protein closely simulate the in vivo situations. One potential application of TAP is studying the dynamics of protein complex formation including stable and transient partners contributing to a specific cellular function. However, under optimal conditions, the tagged construct should replace the endogenous wild-type gene, unfortunately, this is not always the case and often becomes laborious and time consuming.

In Vivo Biotinylation

Recently, biotinylation of tags has been utilized in the recovery and characterization of ribonucleoprotein complexes (2). This approach utilizes the co-transfection of the cell with two plasmids. One expresses an RNA binding protein (RNP) tagged with a biotin acceptor peptide (BAP), a protease recognition site is placed between RNP and BAP, the other plasmid expresses BirA enzyme. The biotinylation of the BAP tag only occurs in the presence of BirA upon the addition of exogenous biotin. RNP complexes with their corresponding RNA can be recovered by incubation with streptavidin-sepharose beads, followed by protease treatment. Although this approach has been employed for the recovery and quantification of relevant mRNAs, with a slight twist it can be employed to study protein-protein interactions. The co-expression of BirA with the bait protein tagged with BAP in cells deficient of the bait gene will cure for this deficiency. When coupled with streptavidin-sepharose, beads interacting proteins can be purified. Utilizing this approach for studying protein-protein interactions seems lucrative; however, the generation of a null mutant of the gene expressing for the bait protein will be a prerequisite and thus DNA manipulation is required for the study of non-essential genes.

Yeast-two hybrid system

The yeast-two hybrid system requires DNA manipulation to study protein-protein interactions. In the first step of the yeast two-hybrid system, the target protein is cloned into the "bait" vector (Fig. 1). In this way, the gene encoding the bait protein is placed into a plasmid next to the gene encoding a DNA-binding domain (DBD) from some transcription factor (e.g. LexA), thus generating the DBD-Bait fusion (depicted in red) (3). If a bait protein has no ability to activate per se the reporter genes in yeast, the yeast cell will not be able to survive on plates lacking histidine and will not turn blue (lacZ-). Separately, a second gene (or a library of cDNAs encoding potential interactors, collectively called "prey" depicted in green), is cloned in frame adjacent to an activation domain of the Gal4 yeast transcription factor. Since a prey protein has no ability to bind DBD-responsive elements, the yeast cell will again not be able to survive on plates lacking histidine and will not turn blue (lacZ-). If the two proteins fused to the DBD and AD physically interact, they will bring the DBD and the AD close enough together to restore a functional transcription factor. The reporter genes, e.g. a nutritional selection marker (HIS3) or lacZ, are transcribed resulting in histidine prototrophy and blue coloration of yeast cells. Thus, the interaction of two proteins is measured by the reconstitution of a hybrid transcription factor and the consequent activation of a set of specific reporter genes. As mentioned above, common reporter genes include an auxotrophic gene for growth selection and a secondary reporter gene for color selection. The power of the yeast two-hybrid system lies in this combined approach since growth selection enables sampling of highly complex cDNA libraries encoding millions of potential binding partners: only those clones which encode an interacting protein survive growth selection are analyzed further using a convenient color assay (e.g. BlueTech). The complexity of such analysis is time consuming and requires expertise, for this reason the yeast two-hybrid custom services market is expanding.

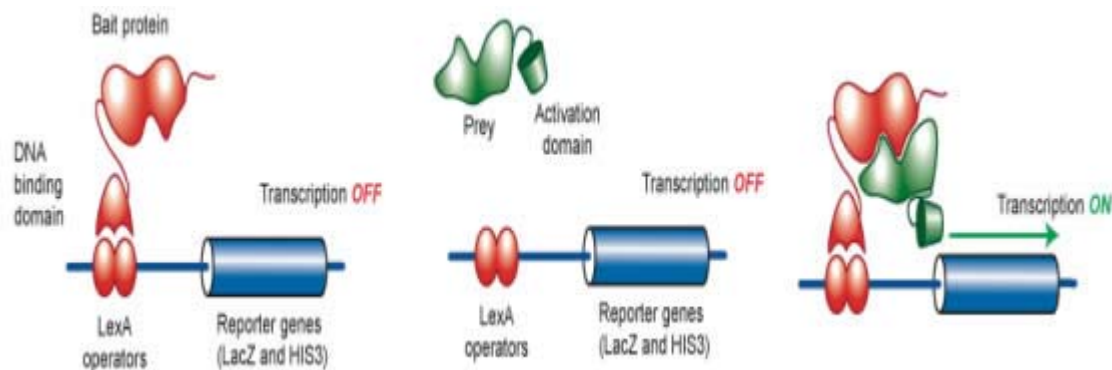


Fig.1. Yeast Two Hybrid System

Mapping of Membrane Protein Interactions by Yeast Two Hybrid System

An important advancement in the yeast two hybrid system is the introduction of a commercially available kit for mapping membrane protein-protein interactions. The DUALmembrane system kit is provided by Dualsystems Biotech, a Swiss based company. In the DUALmembrane system, the reporter protein is a fusion of the DNA-binding domain of the LexA protein and the Herpes simplex VP16 transactivator (Fig. 2).

The reporter is fused to the Cub moiety which in turn is fused to an integral membrane protein (depicted in red, collectively called Cub-TF) (3). A transmembrane prey protein (depicted in green) is fused to the NubG moiety. The only requirement is that both Cub and NubG are located on the cytoplasmic face of the membrane. Co-expression of Bait-Cub-TF with a non-interacting Prey-NubG does not lead to the formation of split-ubiquitin nor cleavage by UBPs, resulting in cells that are His³- and lacZ⁻. If the bait and prey interact, Cub and NubG are brought into close proximity, where they will form split-ubiquitin, resulting in cleavage and liberation of the TF reporter. The reporter is now free to enter the nucleus, where it will bind and activate the reporter genes to result in cells that are His³+ and that turn blue in a β-galactosidase assay (3). The system is the first genetic screening system for integral membrane proteins and membrane-associated proteins. It also can be utilized as a tool for screening cDNA libraries to find novel proteins interacting with the membrane protein of interest (3).

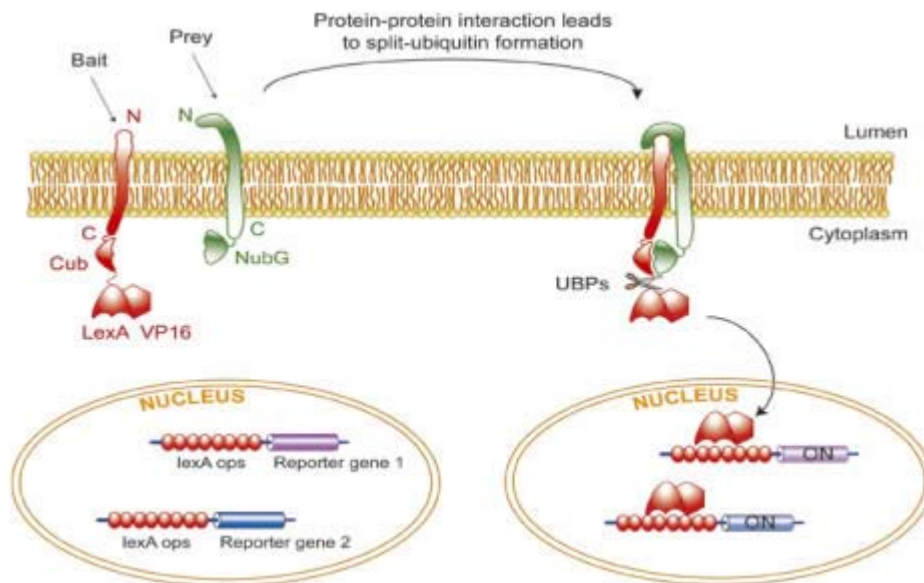


Fig. 2. Membrane Protein Interactions by Yeast Two Hybrid System

Conclusion

The combination of several techniques would be the ideal strategy for the confirmation and validation of protein interactions including *in vivo* protein localization and co-localization (multiplexing). However, sample preparation plays a major role in this process where many protein interactions require either their native or post-translationally modified form. Therefore, a great deal of attention to sample preparation will increase the chances of purifying significant proteins interacting in a complex. In recognition of such need several leading biotech companies have launched sample prep kits (e.g. Pierce, Active Motif). Increasing evidence is showing that protein function is regulated by one or multiple post-translational modifications (e.g. Abgent). The list of identified post-translational modifications are growing, thereby an intriguing need exists for the development of sample prep kits that preserve significantly important post-translational modifications. The cumulative information of protein-protein interactions mapping in their native and post-translationally modified situation will provide initial connection between proteins and their regulatory networks (e.g. the kinome), and will excel our understanding of disease processes towards the discovery of new drug targets.

References

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