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Review

The Use of Yeast Two-Hybrid Screens in Studies of Protein:Protein Interactions Involved in Trafficking

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The yeast two-hybrid system has provided a convenient means to both screen for proteins that interact with a protein of interest and to characterise the known interaction between two proteins. Several groups with an interest in the molecular mechanisms that underlie discrete steps along trafficking pathways have exploited the yeast two-hybrid system. Here, we provide a brief background to the technology, attempt to point out some of the pitfalls and benefits of the different systems that can be employed, and mention some of the areas (within the trafficking field) where yeast two-hybrid interaction assays have been particularly informative.

Key words: Trafficking, two-hybrid, vesicular transport

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An Outline of the Yeast Two-Hybrid System

The yeast two-hybrid system is based on the modular nature of transcription factors, and provides a convenient assay for the study of protein:protein interactions occurring within eukaryotic cells. Transcription factors can be physically divided into two distinct domains, a DNA binding domain (DNA BD) and a transcription activating domain (AD). These domains are independently non-functional as a transcription factor but, when in very close proximity to one another (but not necessarily covalently associated), can reconstitute transcription activity. This phenomenon is exploited in the yeast two-hybrid system through the generation of independent fusion proteins incorporating these separate domains (i.e. one fusion protein incorporates the DNA binding domain and the other the transcription activating domain) (see Figure 1). Interaction of such polypeptide fusions generates a functional transcription factor, which initiates transcription of reporter genes that have previously been engineered into the genome of the yeast host. Typically, nutritional markers (e.g. the ability of yeast to grow on medium lacking histidine) and enzymatic reporters (e.g. the expression of β-galactosidase) are used in tandem for this analysis. Thus, yeast bearing appropriate reporter genes might be transfected with plasmid-based cDNA constructs encoding a library of independent fusions (the 'prey' molecules) with the transcription activation domain of the transcription factor GAL4. The fusion proteins encoded by these constructs will not, in isolation, be able to activate transcription of the reporter genes (because, although each one includes a transcription activation domain, none possesses a DNA binding domain). However, if a second plasmid, encoding a fusion between a protein of interest (the 'bait') and the DNA binding domain of GAL4, is introduced into the same yeast cells, expression of the reporter genes may occur. Such expression is dependent upon an interaction between the bait and the prey. Isolation of yeast clones in which a functional interaction has occurred (e.g. clones growing on medium lacking histidine) permits the subsequent isolation of the plasmid DNA encoding the GAL4 transcription activation domain fusion and thus identification (following sequence analysis and database searching) of the prey. An excellent overview of the yeast two-hybrid system is available elsewhere (1).

Comparison of Different Yeast Two-Hybrid Systems

There are several variants on the original two-hybrid system described by Fields and Song (2). The principle points of variation being the exact nature of the transcription factor used and the specific reporter systems used for analysis of putative interactions. The commonly used systems rely upon either the GAL4 or LexA DNA binding domains in combination with either a GAL4, VP16 or B42 transcription activation domain. Several of these alternatives are interchangeable (for example the GAL4 DNA BD can be used with the GAL4, VP16 or B42 AD). Each combination of DNA binding domain and transcription activation domain has associated advantages and disadvantages. For example, the fact that the VP16 transcription activation domain is an extremely strong transcription activator makes it suitable for the investigation of low affinity protein:protein interactions but also results in an increased proportion of 'false positive' interactions identified in library screens. The VP16 transcription activation domain can also be used in conjunction with many different DNA binding domains; these include those from the transcription factors GAL4 and LexA. An alternative to the VP16 transcription activation domain is B42, an heterologous 88-residue acidic peptide which is capable of activating transcription in yeast. A B42/LexA based system has been developed that allows the inducible expression of fusion proteins from the GAL1 promoter. Such inducible expression allows the analysis of protein:protein interactions that may otherwise prove inhibitory to yeast growth (through toxicity) and also provides a further control against false positive interactions.

The yeast strain used for the experiments is also an important factor to be considered when selecting a system for use. Strains constructed for two-hybrid assays contain differing numbers of copies of the upstream activating sequence to which the DNA binding domain can bind. More copies of the upstream activating sequence will mean a stronger activation of transcription when an active transcription factor complex is reconstituted. The strain L40 (3), commonly used with LexA based systems, contains 8 copies of the relevant upstream activating sequence upstream of the LacZ reporter cassette (driving expression of β-galactosidase). In contrast, the HF7c strain (4), commonly used for GAL4-based assays, has only three copies of the GAL4 upstream activating sequence upstream of the LacZ reporter. The inclusion of more copies of the relevant upstream activating sequence will enhance sensitivity, but will also lead to an increase in false positive signals.

The more recent generation of two-hybrid systems employs a third or even fourth selection marker designed to further reduce the numbers of false positive interactions detected. Using an additional nutritional marker, such as the *ade2* gene, provides a further selection step without the need for any additional time-consuming assays. These additional reporters are engineered downstream of differing upstream activating sequences and TATA box sequences. They should therefore eliminate the identification of false positives which arise due to direct interaction between the transcription activation domain fusion and upstream activating sequences (i.e. give rise to expression of the reporter gene in the absence of interaction between the fusion partners in the transcription activation domain and DNA binding domain constructs). These newer developments are of particular relevance to the application of the two-hybrid system to blind screening of cDNA libraries where elimination of false positives at the earliest stage possible is not only desirable but essential.

Quantitative Two-Hybrid Assays

Two-hybrid assays are often considered to be a quantitative measure of protein interactions. This is true to only a limited extent. Two-hybrid assays are often employed to provide a comparative analysis of the interaction affinity of one protein with a number of mutant versions of a known interactor. In this type of experiment, LacZ reporter assays (in which a quantitative colorimetric assay is used as the basis for the measurement of β-galactosidase activity) can indeed be considered quantitative as long as the same system is used for all assays and as long as all assays are performed in parallel from a pool of yeast transformants. It is particularly important for all yeast two-hybrid analyses that experiments are conducted on pools of transformants rather than on cells from a single colony. This is in order that artefactual results arising from the analysis of a single yeast colony might be avoided. Different proteins will be expressed at different levels in different yeast, vectors will be present at different copy

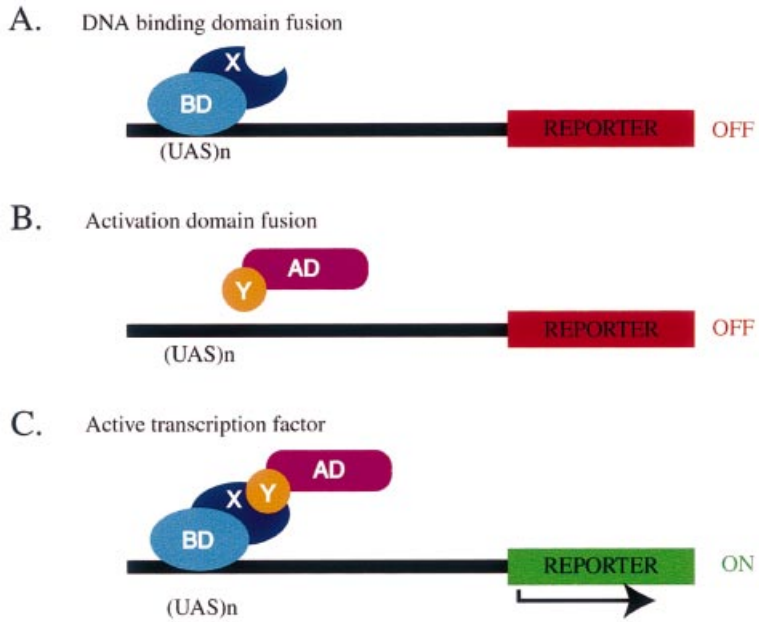

Figure 1: Principle of the two-hybrid system. DNA binding domain and transcription activation domain fusions are expressed in yeast. A. In the absence of an activation domain, the DNA binding domain (BD) (expressed as a fusion with protein X) is capable of binding to the upstream activator sequences (UAS) but is not itself capable of activating transcription. B. The transcription activation domain (AD) (expressed as a fusion with protein Y) is similarly incapable of activating transcription in the absence of a DNA binding domain. C. Interaction between the two fusion proteins (by virtue of an interaction between X and Y) results in reconstitution of an active transcription factor and subsequent transcription of a reporter gene (e.g. LacZ or HIS3) providing a means to assay the interaction between the two fusion proteins.

numbers in different colonies and the use of many different two-hybrid systems in different laboratories, coupled with differing protocols for growth and analysis means that comparison of data from one laboratory with those from another is not valid. Another facile way to compare the interaction of different pairs of proteins in the yeast two-hybrid system is by the use of liquid growth assays. Thus, in a system that requires a functional interaction for growth to occur in the absence of histidine, for example, the rate of growth in liquid medium lacking histidine (as measured by an increase in optical density of the culture) will provide an indication of the affinity of the interaction between the two interacting proteins (i.e. those that interact with higher affinity will give rise to faster growth of the yeast culture).

'False-Positives'

Mention has already been made of 'false-positives'. These may occur due to a variety of reasons, e.g. direct interaction between the prey component of the transcription activation domain fusion and the upstream activating sequences in the reporter construct, an irrelevant interaction between a prey peptide fragment and the bait; some proteins appear to be inherently 'sticky' and frequently turn up as 'false-positives' in two-hybrid library screens (a summary of false positive interactions found in early two-hybrid screens, and common to many subsequent screens, can be found at: http://www.fccc.edu/research/labs/golemis/main_false.html).
Thus, it is imperative that interactions that have been identified in a yeast two-hybrid screen are confirmed by at least one alternative, non-yeast-based assay. A common initial check is to reverse the positions of the prey and bait (i.e. if the prey was originally fused to the transcription activation domain, put it on the DNA binding domain and vice-versa). Clearly, genuine interactors should still interact following this switch. Another common check is to perform a 'pull-down' assay using two recombinant fusion proteins produced in *Escherichia coli* (e.g. the prey expressed as a glutathione-S-transferase, GST, fusion and the bait as a maltose binding protein, MBP, fusion); if the bait and prey interact when incubated together, it should be possible to isolate the complex on a glutathione-agarose column, for example. However, this type of assay does little (if anything) to extend the yeast two-hybrid interaction assay, since mixing together of two recombinant proteins at relatively high concentration does not accurately reflect the situation that occurs within eukaryotic cells. The efficiency of the 'pull-down' is also clearly important, one is likely to have greater belief in the validity of the interaction if the GST fusion pulls down stoichiometric amounts of the MBP fusion than if it only pulls down a minor fraction of the MBP fusion. Whilst co-localisation of the putative interacting proteins in cells in culture (by immunofluorescence microscopy or immunogold electron microscopy) would be supportive of a 'genuine' interaction, the optimal method for confirming the validity of a protein:protein interaction which has been identified by a yeast two-hybrid screen is undoubtedly the co-immunoprecipitation of the interacting endogenous proteins from cells in culture (or from a tissue sample). However, this is frequently a technically difficult task to perform (due to expression levels, availability of antibodies, affinity of interaction etc.) and a common compromise is the co-immunoprecipitation of the interacting proteins from cells in which one of the partner proteins (prey or bait) has been over-expressed as an epitope-tagged recombinant molecule.

Yeast Two-Hybrid Assays and Protein:Protein Interactions Involved in Trafficking

The yeast two-hybrid system has been used to investigate protein:protein interactions at various stages along different membrane trafficking pathways within eukaryotic cells. It is beyond the scope of this short review to cover any of these areas in more than the most cursory manner: we have chosen to concentrate upon some of the two-hybrid assays which have been used to study protein:protein interactions in the early stages of the endocytic pathway and hope that these will serve as an example of what has been (and will be) performed in studies of other membrane traffic pathways. Some of these interactions, and the role played by two-hybrid assays in their characterisation, are illustrated in Figure 2.

The use of the yeast two-hybrid system to demonstrate that tyrosine-based internalisation motifs corresponding to the generic form YXXΦ (where Φ represents a bulky hydrophobic residue) interact with the medium subunit (μ2) of the AP-2 adaptor complex (5) precipitated a series of two-hybrid-based experiments designed to investigate: a) the relationship between tyrosine-based trafficking motifs and adaptor subunits (6–9); b) the interactions between different adaptor subunits (10,11); and c) possible novel interactions between adaptor subunits and other proteins (12). Thus, whilst the original report from Ohno et al. (5) identified YXXΦ as the core motif required for interaction with μ2, subsequent two-hybrid interaction assays demonstrated that the local environment of the YXXΦ motif and precise residues within the YXXΦ motif affect the relative affinity of that motif for medium chain subunits from different adaptor complexes (i.e. μ1, μ2, μ3A, μ3B from AP-1, AP-2 and AP-3) (6–9). Furthermore, it has proved possible, using the liquid growth assays mentioned above, to use two-hybrid interaction assays to monitor the effects of putative inhibitors of the interaction between different adaptor complex medium chain subunits and their cognate YXXΦ motifs (13). Aguilar et al. (14) have also used a combination of yeast two-hybrid interaction assays and proteolytic digestion to show that the N-termini of the μ1 and μ2 adaptor subunits are involved in interactions with the rest of the adaptor complex whilst the C-termini are involved in the interactions outlined above [the latter fact being borne out by structural data (15)]. Yeast two-hybrid interaction assays have also been used to map interactions within adaptor complexes, thus Page and Robinson (10) demonstrated interactions between the β/β′ adaptins and the α/γ adaptins, between the β/β′ adaptins and the μ2/μ1 adaptor complex subunits, between α adaptin and the σ2 subunit and be-

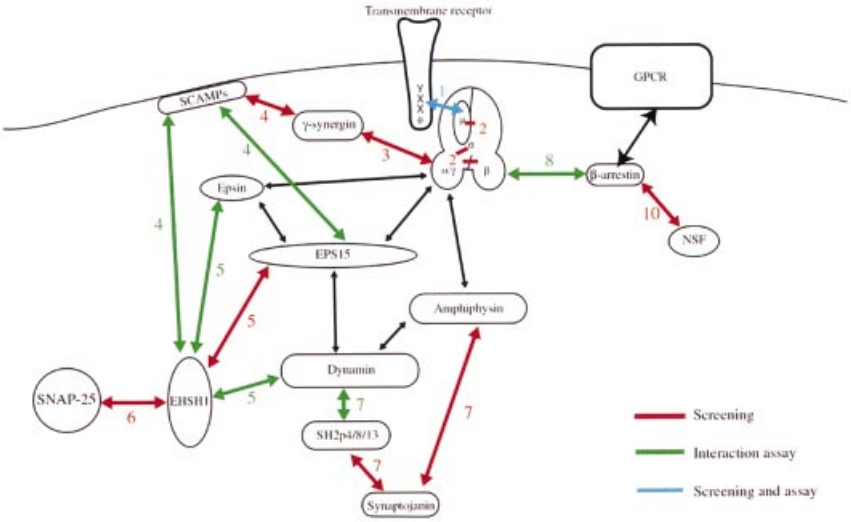

Figure 2: Application of the two-hybrid system to analysis of the endocytic pathway. Interactions have been detected either from *de novo* two-hybrid screens (red arrows) or through application of the two-hybrid technique to a systematic assay of protein interactions (green arrows). In the case of receptor–adaptor interactions, the technique was used to initially identify this interaction and subsequently used to provide a robust assay system (blue arrow). Black arrows indicate interactions determined using other approaches. Some interactions, e.g. eps 15 with synaptojanin, have been omitted for clarity. Numbers refer to citations as follows: 1 (5,8); 2 (10); 3 (12); 4 (26); 5 (17); 6 (16); 7 (27); 8 (28); 9 (29).

tween γ adaptin and the σ1 subunit. Furthermore, Hirst and colleagues (11) used a combination of co-immunoprecipitation and yeast two-hybrid interaction assays to characterise the subunit composition (ε, β4, μ4, σ4) of the novel adaptor complex AP-4. The same group used the γ adaptin subunit of the AP-1 adaptor complex as bait in a yeast two-hybrid library screen and identified the novel interacting protein γ synergin (12), thereby extending the cast of cytosolic proteins known to play a role in governing trafficking pathways. Many further candidate molecules involved in these processes have been identified using conventional biochemical methods and subsequently characterised, or identified independently, using two-hybrid systems. A good example of this is the identification of a protein called EHSH1 (for EH domain/SH3 domain-containing protein) which was identified in *Drosophila* (and named DAP160) by Roos and Kelly (30) and also in *Xenopus* (and named intersectin) by Yamabhai et al. (31). Immunoprecipitation analysis showed that dynamin, epsin and SNAP-25 are complexed to EHSH1/intersectin in brain and implicated EHSH1/intersectin as an adaptor, or scaffold protein that couples endocytic membrane traffic to exocytosis. This protein was subsequently identified following a yeast two-hybrid screen for proteins that bind to SNAP-25 (16). Likewise, Sengar and colleagues used yeast two-hybrid interaction assays to confirm that the protein Ese1 (the mouse homologue of intersectin) binds both epsin family proteins and dynamin (17).

In other studies, Trommsdorff and colleagues used yeast two-hybrid interaction assays to show that the neuronal adaptor proteins FE65 and Disabled bind to the cytosolic domains of the low density lipoprotein (LDL) receptor and the LDL-receptor related protein (LRP) potentially forming a molecular scaffold for the assembly of higher order cytosolic complexes (18), whilst the actin binding protein neurabin has been identified independently in yeast two-hybrid screens by two groups and implicated in playing a role as a scaffold protein linking the cytosolic domains of more than one integral membrane protein with both the actin cytoskeleton and intracellular signalling complexes (19,20).

However, the use of a yeast two-hybrid library screen to identify PACS-1 as a protein that interacts with the acidic cluster in the cytosolic domain of furin (21) remains one of the major successes in the trafficking field as far as *de novo* screening of two-hybrid libraries is concerned. This is because subsequent experiments clearly demonstrated that PACS-1 is involved in the trafficking of furin and other integral membrane proteins with acidic regions in their cytosolic domains (21). It should be noted that the success in identifying PACS-1 was in no small way due to the careful and intelligent design of the screening strategy employed. Advantage was taken of the fact that reversible phosphorylation of the furin cytosolic domain plays a role in the intracellular trafficking of the protein. Thus, only proteins that interacted with a bait corresponding to a constitutively phosphorylated form of the furin cytosolic domain (i.e. containing the sequence DDDEEDE), but that failed to interact with a bait corresponding to a constitutively non-phosphorylated form of the furin cytosolic domain (i.e. containing the sequence ADAEEDE), were selected for study. A similarly successful yeast two-hybrid screen led to the identification of TIP47 as a mannose-6-phosphate receptor interacting protein (23). Subsequent biochemical characterisation implicated TIP47 as a mediator of signal mediated endosome to TGN sorting. A key aspect of this work was the strategy of using sequential

screens for proteins interacting with both the cation-independent and, structurally unrelated, cation-dependent mannose-6-phosphate receptors.

The value of incorporating such functional relevance into two-hybrid screens is further illustrated by the work of Goud and coworkers in identifying components of Rab6-dependent trafficking machinery (24,32,33). Rab6 is a small GTP binding protein that, like other Rab proteins, regulates membrane traffic processes through the hydrolysis of GTP. This enabled the use of mutant Rab6 proteins, constitutively 'locked' in either the GTP or GDP-bound state, to screen two-hybrid libraries for interacting proteins. Some of the proteins identified in these screens, for example the guanine nucleotide dissociation inhibitor protein (Rab GDI), were found to interact with wild-type but not GTP-bound Rab6 (32). In contrast, Rabkinesin6 was found to interact with the wild-type, or GTP-locked, forms of Rab6 but not the GDP-locked form (24). This functional relevance, directly incorporated into the screening approach, clearly provides an intrinsic control for the biological importance of any candidate interaction identified through two-hybrid screening.

Thus, the yeast two-hybrid system has been used to great effect to elucidate the array of protein:protein interactions involved in endocytic and other, e.g. ER-to-Golgi (22) membrane traffic pathways. Together, these results demonstrate the wide applicability of the technology towards a fuller understanding of the molecular machinery of membrane traffic.

The Future

Yeast two-hybrid interaction assays will clearly continue to provide an important tool for the dissection and further characterisation of known protein:protein interactions. In addition, it will remain tempting to undertake screens of yeast two-hybrid libraries in searches for novel interactors. However, as mentioned above, it is preferable that functional relevance is built into the screening strategy and it is imperative that interactions identified by yeast two-hybrid screens are confirmed by an alternative approach before they can be considered to be genuine and/or to have any physiological relevance. The future undoubtedly also holds the prospect of more industrial scale yeast two-hybrid library screens. A comprehensive analysis of protein:protein interactions within the yeast *S. cerevisiae* has already been reported (25) (and see http://portal.curagen.com/extpc/com.curagen.portal.servlet.Yeast). This led to the detection of 957 putative interactions involving 1004 *S. cerevisiae* proteins; a significant number of these interactions involve proteins known to play a role in trafficking pathways in *S. cerevisiae*. As more genomes are sequenced, such protein interaction maps will be generated for more organisms and will undoubtedly provide us with a plethora of information about candidate protein:protein interactions involved in trafficking pathways, interactions that we will have to confirm by other means.

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