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Two-hybrid-based systems: Powerful tools for investigation of membrane traffic machineries

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ABSTRACT

Protein–protein interactions regulate biological processes and are fundamental for cell functions. Recently, efforts have been made to define interactomes, which are maps of protein–protein interactions that are useful for understanding biological pathways and networks and for investigating how perturbations of these networks lead to diseases. Therefore, interactomes are becoming fundamental for establishing the molecular basis of human diseases and contributing to the discovery of effective therapies. Interactomes are constructed based on experimental data present in the literature and computational predictions of interactions. Several biochemical, genetic and biotechnological techniques have been used in the past to identify protein–protein interactions. The yeast two-hybrid system has beyond doubt represented a revolution in the field, being a versatile tool and allowing the immediate identification of the interacting proteins and isolation of the cDNA coding for the interacting peptide after *in vivo* screening. Recently, variants of the yeast two-hybrid assay have been developed, including high-throughput systems that promote the rapidly growing field of proteomics. In this review we will focus on the role of this technique in the discovery of Rab interacting proteins, highlighting the importance of high-throughput two-hybrid screening as a tool to study the complexity of membrane traffic machineries.

1. Introduction

Cellular events are modulated by functional interactions between specific proteins. A single protein can interact with different, and often numerous, partners in the cell, thus regulating different processes. Although the human genome has been sequenced, many uncharacterized genes remain whose functions are unknown. Thus, identifying new interactions helps to characterize these genes, new protein functions and, possibly, new molecular pathways. Indeed, the role and function of a specific protein in a cell can often be inferred by identifying its molecular partners. Furthermore, the discovery of previously unknown interactions between known and characterized proteins may reveal new and possibly unexpected roles for these proteins. Several techniques are available to analyze protein–protein interactions. Biochemical approaches, such as co-immunoprecipitation and affinity chromatography followed by mass spectrometry, have been widely used with success, although the identification of interactors with these techniques is usually time consuming and laborious. Moreover, after the difficult process of protein purification and identification, the cDNA still must be obtained. The yeast two-hybrid assay, developed in 1989 by Fields and Song, revolutionized the process of searching for interacting protein (Fields and Song, 1989). This technique allows rapid identification of several putative interacting proteins for a given protein of interest, enabling also the isolation of the cDNA associated with the interacting peptides (Chien et al., 1991). Similar versatility is present in the phage or virion display technique that was developed at the same time (McCafferty et al., 1990; Smith, 1985). In this case, cDNAs coding for peptides or for antibody fragments are inserted into viruses, and peptides or antibody fragments are expressed that are fused with viral coat proteins. In this manner, "displaying viruses" are generated and subsequently screened against proteins, peptides, DNA or other molecules, thus allowing for the detection of interactions (McCafferty et al., 1990; Smith, 1985). However, the great advantage of the two-hybrid system over the virion display and mass spectrometry techniques is that the screening is performed *in vivo* (Chien et al., 1991; Fields and Song, 1989). The two-hybrid system has beyond a doubt greatly contributed to interactome mapping (Parrish et al., 2006). Mass spectrometry and the

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two-hybrid systems present similar limitations, such as the detection of spurious and non-physiological interactions, or the detection of only a limited number of interactions. However, these two techniques address different aspects of protein–protein interactions, and thus they are considered complementary. Indeed, while the two-hybrid system mainly identifies direct binary interactions, mass spectrometry can identify the components of a complex. Thus, combination of data coming from both approaches allows for a more complete and reliable map of interactions. Notably, the yeast two-hybrid method has been improved greatly in recent years, and several variants of the technique have been described that can search for membrane proteins, DNA-binding proteins and RNA binding proteins (Causier and Davies, 2002; Petschnigg et al., 2014; Reece-Hoyes and Marian Walhout, 2012). Furthermore, reverse two-hybrid systems that search for molecules that disrupt interactions have been developed and have been proven to be extremely useful for drug discovery (Fetchko et al., 2003; Vidal et al., 1996a).

In this review we will focus on the yeast two-hybrid assay, describing the different variants that are now available, and will illustrate examples of its use. In particular, we will highlight the contribution of this technique to the identification of components of the membrane traffic machinery through the search of interactors of Rab GTPases.

2. Principle of the yeast two-hybrid assay

The yeast two-hybrid assay is based on the modular nature of eukaryotic transcription factors. Indeed, transcription factors are structurally composed of domains: the DNA-binding domain (BD), which binds DNA sequences (called response elements) mediating the recruitment of the transcription factor on specific genome DNA sequences, the activation domain (AD), or trans-activating domain (TAD), which is responsible for recruitment of the transcription machinery, and an optional signal sensing domain (SSD), which is able to sense external signals and regulate the transcription complex (Latchman, 1997). The first two domains together are sufficient to initiate transcription, and it has been established that they do not need to be present within the same protein in order to function, although they have to be in close proximity (Ma and Ptashne, 1988). To develop the first two-hybrid systems, the properties of both domains were used, taking advantage of the fact that eukaryotic transcription factors are separable in BD and AD domains (Causier, 2004; Fields and Song, 1989). The system takes its name from the two hybrid proteins, one containing the BD domain and the other containing the AD domain, that are used to test the interaction. Indeed, if the two proteins interact, the AD and the BD components will be brought together, thus reconstituting the transcription factor and activating transcription of the reporter gene (Fig. 1A). Fields and Song developed the method using the BD and AD of the yeast GAL4 protein; however, domains from other transcription factors can also be used. Furthermore, the system can also function by combining BD and AD domains from different transcription factors (Chien et al., 1991; Fields and Song, 1989). For instance, the BD of the bacterial repressor protein LexA can be used with the AD of yeast GAL4, or with the AD of the *Escherichia coli* B42 transcription factor (Gyuris et al., 1993).

2.1. Monitoring interactions and selecting clones containing interacting proteins

Normally, in the two-hybrid assay, the cDNA sequence encoding the protein of interest (called protein X) is sub-cloned into a plasmid vector that express the protein fused to the BD of a yeast transcription factor. This fusion protein is then used as a "bait" to screen a cDNA library in order to identify protein X-interacting proteins called "preys". The cDNA library is cloned into a plasmid that encodes the AD, leading to the expression of putative prey proteins (Fig. 1A). If protein X interacts with a prey, the BD and the AD domains reconstitute an active transcription factor, thus initiating transcription at reporter genes. The reporter genes used for this assay are genes that are required for the biosynthesis of amino acid and/or nucleotide precursors. The yeast strains used in the two-hybrid system are genetically engineered in a manner that the biosynthesis of certain nutrients is lacking. In particular, reporter genes have modified promoters that are recognized by the BD of the bait, and their transcription should begin only if the bait and the prey interact, thus reconstituting a transcription factor. The absence of interaction, and thus of transcription of these genes, prevents yeast growth as these metabolites are fundamental for growth and are not included in the culture medium. Instead, the physical interaction between bait and prey activates transcription and promotes the expression of the reporter gene that encode enzymes necessary for the synthesis of amino acid and/or nucleotide precursors. Common reporter genes include HIS3, LEU2, URA3, TRP1, LYS2 and ADE2. Therefore, the use of minimal media lacking these metabolites allows growth, and consequently selection, of the yeast clones where the transcription of the reporter(s) gene(s) was activated, possibly by bait–prey interaction. For example, expression of a HIS3 reporter is monitored by the growth of cell colonies on minimal medium lacking histidine (Causier, 2004; Coates and Hall, 2003). Often, in the two-hybrid system, multiple reporter genes under the control of promoters with similar response elements, but located in different part of the genome, are used in order to reduce the number of false positives. Another reporter gene used to further decrease false positives in the two-hybrid system is *LacZ*. This gene encodes the enzyme β-galactosidase and it is not a nutritional selectable marker, but is rather a neutral screenable marker. Clones selected with nutritional markers are then tested for β-galactosidase activity, thus lowering the number of false positives (Bartel et al., 1993; Bartel and Fields, 1995). Furthermore, the use of this reporter gene allows quantification of the interaction if a colorimetric and spectrophotometric substrate for the β-galactosidase enzyme, which is able to measure enzyme activity, is used (Bartel et al., 1993; Bartel and Fields, 1995).

2.2. Limits of the system

The two main limitations of the system are the possibility of having false negatives and false positives. Regarding false negatives, all proteins expressed in yeast have to fold properly, have to be correctly post-translationally modified, have to reach the nucleus and should not be toxic for yeast. Otherwise, not being able to interact with their partners, they will originate false negatives. For instance, the construction of a fusion protein leading to a different tertiary structure could mask or modify interacting domains, resulting in a false negative.

However, the high number of false positive clones represents the main problem with the two-hybrid screening. Spurious activation of the reporter gene can occur for different reasons. For example, presence of preys able to bind DNA or able to activate reporter genes are the more frequent causes and can be counteracted by the use of more than one reporter gene and checked by swapping the BD and AD domains in the two proteins. Another frequent cause of false positives is due to interactions that cannot be confirmed in other experimental systems and, in particular, in the correct cell environment. The presence of these false positives can be due to the different environment where the interaction takes place (yeast *versus* mammalian, nucleus *versus* cytosol, for instance), or to the fact that proteins that normally are localized within different compartments and do not physiologically ever

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