Biochemical Pharmacology, Vol. 60, pp. 1009–1013, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00394-4

Protein Recruitment Systems for the Analysis of Protein–Protein Interactions

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ABSTRACT. Following the completion of genome projects in a number of organisms, it is becoming evident that a relatively large proportion of the genes identified encode for proteins that have no sequence homology with known proteins. One possible approach towards understanding protein function is to identify the proteins with which a particular protein associates. Although very powerful, the most commonly used genetic method, the two-hybrid system, is limited in its ability to detect all possible protein–protein interactions. The development of novel approaches, such as the protein recruitment systems, provides attractive alternatives towards identification of protein–protein interactions where other methods have failed to function. BIOCHEM PHARMACOL **60**;8:1009–1013, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. protein recruitment; two-hybrid system; protein–protein interaction; AP-1 transcription, membrane proteins

Protein–protein interaction plays a major role in all biological processes. It is becoming evident that proteins act in large modular structures also referred to as protein machines. This has led to the establishment of a novel area of research known as "proteomics", i.e. the study of protein properties and function [1]. Until recently, the search for protein–protein interaction was limited to known protein targets in which novel protein partners were screened with the known target, using various genetic, molecular, and biochemical approaches. However, following the completion of the genome projects in lower organisms comprising relatively small size genomes and the rapid advance in the human genome project, it is becoming evident that many putative proteins encoded by the novel identified genes has no known function. For example, in the budding yeast *Saccharomyces cerevisiae* the genome project was completed in 1996 [2]. However, still today, one-third of the predicted open reading frames are classified as proteins with unknown function [3]. In human, the nucleotide sequence of chromosome 22, the second smallest human chromosome, was recently completed [4]. The sequence analysis revealed about 100 real genes that exhibit no sequence homology with any of the known proteins in the databank. Based on this analysis, it is estimated that by the completion of the human genome, about 2500–3000 novel genes will be identified whose encoded proteins will exhibit no sequence homology with known protein. Taking into consideration that proteins act as modular complexes, one possible approach to uncover the function of an unknown gene product is to identify its potential protein partner. The notion behind this approach is that identification of a known partner would provide a clue to the function of the unknown protein.

THE TWO-HYBRID SYSTEM

One of the most commonly used genetic systems of today is the two-hybrid system, which was originally described more than a decade ago by Fields and Song [5]. The system is based on the ability to split a transcription factor into two separable functional domains: a DNA-binding domain and a transcriptional activation domain. Each one when expressed separately is unable to activate transcription. These domains are used to generate hybrid proteins to be tested for potential protein–protein interaction. Once protein–protein interaction is generated, it reconstitutes a functional transcription factor that can be readily monitored using reporter gene assays in yeast. These assays can be either colorimetric or used to provide a yeast nutrient growth marker [6]. The two-hybrid system was shown to be a powerful method for the identification of protein–protein interaction between known protein partners as well as for identification of novel proteins using a library screening approach [7, 8]. Typically, the protein target ("the bait") is fused to either the LexA or the Gal4 DBD, whereas the protein partner ("the prey") or a cDNA library is fused to a strong transcription activation domain such as the VP16 or the Gal4.

Although the two-hybrid system is an efficient method

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for the analysis of protein–protein interaction, it still exhibits several limitations and inherent problems. First, since the readout of the system is mainly based on transcription, the protein to be studied should not exhibit a significant transcriptional activity. Although transcriptional activation is mainly attributed to transcription factors, numerous proteins of cytoplasmic origin may contain structural domains that exhibit transcriptional activity. Second, some protein targets, when fused to a heterologous DNA-binding domain and expressed in yeast, exhibit toxicity to the yeast host (e.g. homeobox proteins or cell cycle regulators). Third, the use of a library screening approach results in repetitive isolation of "false-positive" cDNAs encoding for proteins which in turn results in identification of a positive signal, although it turns out to be independent of the specific interaction with the corresponding bait. Moreover, the two hybrid approach requires that the protein–protein interaction should occur at the yeast nucleus. This is not perfectly appropriate for protein of cytoplasmic origin, especially integral membrane proteins such as G-protein-coupled receptor, transporters and membrane receptors. Indeed, a genome-wide effort to identify all possible pairwise interactions between yeast's 6000 proteins has succeeded in doing so only in a limited number of genes [3]. This strongly suggests that alternative approaches should be used to complement the two-hybrid approach where it has failed to detect protein–protein interaction.

PROTEIN RECRUITMENT SYSTEMS

In view of the problems of the two-hybrid system, we have developed a complement system that intends to provide an alternative approach to the two-hybrid system and extend the ability to explore protein–protein interaction. The system employs translocation of an active molecule to its site of action via protein–protein interaction. The system is based on the observation that the Ras-GEF,* hSos, is activated by its recruitment to the plasma membrane [9, 10]. This mode of regulation is functional both in mammalian and in yeast cells [9]. In yeast, the activity of the Ras-GEF can be easily monitored in a yeast strain mutated in its Ras-GEF, Cdc25. One such mutant is the temperature-sensitive Cdc25-2 [11]. This strain is able to grow at the restrictive temperature of 36° depending on the expression of a functional GEF [12]. Alternatively, the mammalian Ras-GEF, hSos, is able to complement Cdc25-2 mutation only when expressed at the inner leaflet of the plasma membrane [9]. The requirement for Sos membrane localization can be achieved via the interaction between two-hybrid proteins [13]. In principle, the protein of interest is fused to Sos ("the bait"), whereas the protein partner ("the prey") is fused to a membrane localization signal such as the v-Src myristoylation signal. The prey expression plasmid is placed under the control of the Gal1 promoter [14]. Following a screening protocol, candidate colonies that exhibit efficient growth at the restrictive temperature when grown on galactose medium are selected. These colonies are then tested for their ability to grow at the restrictive temperature only when grown on a galactose-containing medium, thereby eliminating the isolation of revertant colonies. This system, designated SRS or Cytotrap (Stratagene), was shown to be efficient for identification of protein–protein interaction between known partners as well as identification of novel protein partners [13]. The SRS system greatly extends the use of genetic methods in yeast for the characterization of protein–protein interactions and serves as an attractive alternative to the two-hybrid system. The SRS system was proven useful in a number of cases [13, 15, 16], but was not without problems. First, following library screens it became apparent that one of the proteins that can bypass the requirement for a functional protein–protein interaction is the mammalian Ras protein. This is due to the fact that the yeast GTPases (Ira 1/2) are unable to inactivate the mammalian Ras function. The expression of the mGAP provided only a partial solution to this problem, since some of the Ras proteins that exhibited efficient growth only in the presence of hSos were mGAP-insensitive [17]. Second, about 30% of baits fused to Sos exhibit efficient growth at the restrictive temperature independently of the presence of a specific prey.† Third, the size of Sos used (150 kDa) is relatively large. This causes problems when small peptides are fused to Sos or alternatively when full-length proteins are to be used as baits. In addition, it resulted in technical problems in bait construction due to plasmid size. In order to overcome the above problems, we developed an improved method, designated RRS [18]. This system is based on the absolute requirement for Ras to be located at the plasma membrane for it to function [19, 20]. In principle, the cDNA encoding for the oncogenic Ras protein devoid of its membrane localization signal is used to fuse the bait protein. The use of Ras as an effector protein with its relatively small size makes the RRS system more user-friendly. This system was already proven useful for numerous baits [18, 21]. In addition, the use of the RRS system in a library screening approach completely eliminates the isolation of mammalian Ras that is predicted to be "false-positive", although the Sos proteins encoded by the library are able to complement the requirement for functional GEF independent of protein–protein interaction and therefore represent false-positive in this assay. In addition, since the Ras requirement for membrane localization is stricter compared to that of Sos, the background resulting from bait–Sos fusion was highly reduced when fused to the Ras protein. Consequently, the RRS system greatly improves the usefulness of the protein recruitment systems and serves an

* *Abbreviations:* GEF, guanyl nucleotide exchange factor; mGAP, mammalian GTPase activating protein; Sos, Son of sevenless; SRS, Sos recruitment system; RRS, Ras recruitment system; MAPK, mitogen-activated protein kinase; and AP-1, activating protein 1.

† Aronheim A, unpublished observation.

attractive alternative to identify protein–protein interaction in a cytoplasmic milieu.

PROTEIN RECRUITMENT SYSTEM SCREENING PROTOCOL

The basic library screening protocol is depicted in Fig. 1. In principle, the cDNA encoding for the bait protein is fused to the cDNA encoding for the oncogenic Ras devoid of its farnesylation sequence {Ras(61)ΔF}. Fusion can be performed to either its amino- or carboxy-terminus. The bait plasmid is co-transfected with a mammalian GAP expression plasmid into Cdc25-2 cells. The transformants are grown on glucose plates lacking the amino acids leucine and tryptophan, nutrient markers that are provided by the bait and GAP expression plasmids, respectively, and are incubated at the permissive temperature. Transformants are selected and further transformed with the Yes-M-library plasmid encoding for random cDNAs fused to the v-Src membrane localization signal. The expression of the cDNA library is designed under the control of the Gal1-inducible promoter. The expression of the cDNA is induced in the presence of galactose in the medium and strongly repressed in the presence of glucose [22]. The plasmid also provides the growth selection marker for uracyl. The transfected yeast cells are spread on plates at a density of about 10,000 transformants on a 10-cm plate. Plates are incubated for 5–7 days at the permissive temperature of 24° and are subsequently replica-plated onto galactose medium containing the appropriate amino acids and bases and then incubated at the restrictive temperature of 36°. Colonies that exhibit efficient growth in the following 5–7 days are selected and grown on appropriate glucose plates for two days. Subsequently, a galactose-dependency test is performed by replica-plating the plates onto medium containing either galactose or glucose. Colonies that exhibit efficient cell growth in the presence of galactose in the medium, but not glucose, are further analyzed. Plasmid DNA is then extracted and the library plasmid identified by restriction digest analysis. Identified library plasmids are reintroduced into Cdc25-2 cells with either the specific or non-specific bait to examine the specificity. Only plasmids that result in reintroduction of the phenotypic growth in the presence of the bait plasmid are considered promising candidates and are further subjected to DNA sequence analysis.

MAMMALIAN PROTEIN RECRUITMENT SYSTEM

One of the main disadvantages of the protein recruitment systems is their inability to quantitate the "strength" of interaction; instead, they provide a qualitative signal, i.e. growth/no-growth. Towards this end, the mammalian Ras recruitment system was developed. In mammalian cells, the Ras-MAPK cascade results in the phosphorylation of transcription factors at their transcriptional activation domain.

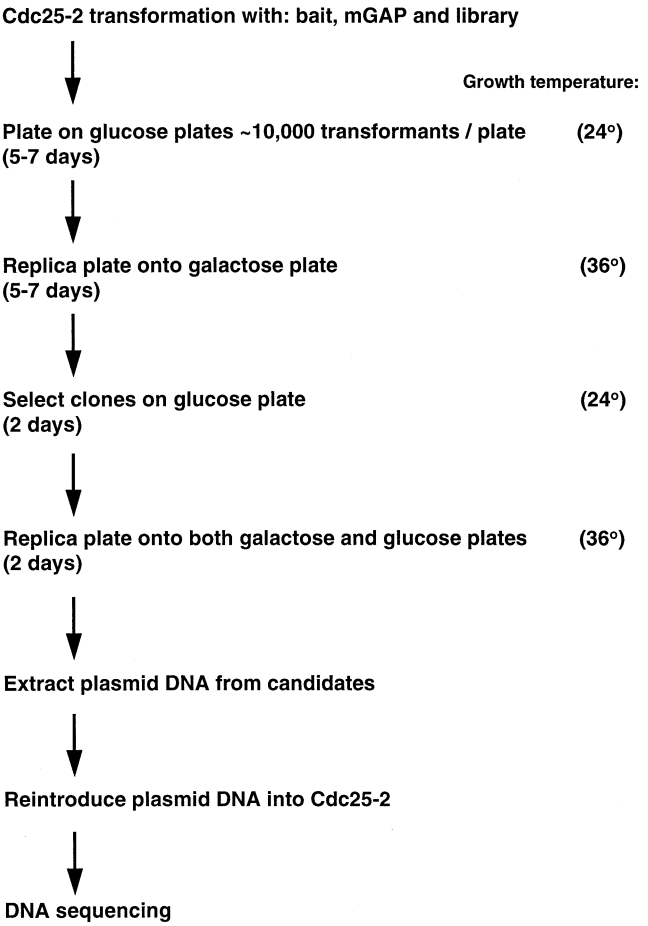

FIG. 1. Library screening flow chart using the protein recruitment systems. Cdc25-2 are transfected with three expression plasmids encoding for: 1. the bait plasmid fused to either activated Ras devoid of its farnesylation sequence Ras(61)ΔF or truncated hSos (5′Sos). The bait is expressed under alcohol dehydrogenase promoter in an ADNS-based plasmid which provides complementation of leucine auxotrophy (LEU); 2. the mGAP expression plasmid designed under the control of the GAL1 promoter in Yes2-derived expression plasmid (Invitrogen) which complements tryptophan auxotrophy (TRP); and 3. the prey expression plasmid fused to the v-Src myristoylation sequence designed under the control of GAL1 promoter in a Yes2-derived expression plasmid (Invitrogen) which complements uracyl auxotrophy (URA). Transformants are selected on an appropriate medium grown at 24° for 5–7 days. The plates are used to replica plate to a galactose-containing medium incubated at 36°. Colonies that exhibit efficient growth in the following 5–7 days are selected and allowed to grow at the permissive temperature for 2 days followed by replica-plating to either a galactose or a glucose-containing medium (YPD) incubated at 36°. Clones exhibiting selective growth on galactose-containing medium are considered "candidates" and are further analyzed. Plasmid DNA is extracted and the identified library plasmids are used to test specificity of interaction with either the specific bait or a non-relevant bait. DNA plasmids extracted from a candidate clone that exhibits growth in the presence of the specific bait are further identified by DNA sequence analysis.

This phosphorylation results in potentiation of their transcriptional activity. One example is the potentiation of the activity of the Elk-1 transcription factor [23], resulting in

c-Fos transcription and activation of the member of the basic leucine zipper transcription factor, AP-1 [24]. Using a mammalian cell host and a luciferase reporter gene assay, one can monitor the activity resulting from the activation of the MAPK cascade. In order to provide a quantitative dimension to the RRS, the system was transferred to a mammalian host in which protein–protein interaction can be correlated with MAPK activation. The expression of either the cytoplasmic-activated Ras bait or the corresponding prey protein in mammalian cells does not result in activation of the AP-1-dependent reporter gene. However, co-expression of the bait and prey proteins results in significant potentiation of reporter activity [25]. Although not all protein pairs can be used in the mammalian assay since they may affect the AP-1 activity independently, this assay may be used to confirm protein–protein interaction initially identified in yeast in a number of cases [25]. In addition, protein–protein interaction that can be identified using the mammalian RRS system can be used in a drug screening approach to disrupt the specific protein–protein interaction [26], with the objective that the toxicity and permeability of the drug can be tested directly within the target host cells.

PERSPECTIVE

The protein recruitment systems provide excellent methods to identify and characterize protein–protein interaction within the yeast cytoplasm. These systems complement the limitations and inherent problems of the classical two-hybrid approach and together may cover a wide range of protein–protein interactions to be explored. Whereas the two-hybrid system fails to detect interaction with transcriptional activation domains, the protein recruitment systems, which are not based on a transcriptional readout, can do so. Conceivably, the protein recruitment systems cannot detect protein–protein interaction with a bait encoding for a membrane protein or a protein that associates with the yeast membrane. For example, protein-containing pleckstrin homology (PH) domains [27] may associate with membrane phospholipid, thereby resulting in Ras translocation to the membrane independent of additional prey expression. On the one hand, this limits the use of the protein recruitment system to either nuclear or cytoplasmic proteins, while on the other, it may provide an approach to identify and map domains within proteins that would result in translocation of proteins to the plasma membrane.

The screening of protein–protein interaction using the methods mentioned above may result in identification of interactions which, although reproducible and specific, are not easily assessed. This is mainly due to the fact that proteins are misexpressed in a compartment from which they are typically excluded and therefore may be exposed to a repertoire of proteins that are not accessible in their natural situations. In addition, the fusion of a protein or a protein fragment to an effector protein such as the Gal4 DNA-binding domain or Ras, used in the two-hybrid or protein recruitment approaches respectively, may result in misfolding of the protein and exposure of protein surfaces that are typically not accessible for association with other proteins. Therefore, the method of choice for a specific bait should take into consideration the localization where the interaction should occur *in vivo*. In addition, the fusion of the gene to the effector protein should preserve the structure and functionality of the protein. One area of research surely in need of further development of novel approaches is the study of protein–protein interaction with membrane proteins, in particular membrane receptors, transporters, and ion channels, which span the membrane multiple times and fold into a special structure involving non-linear peptide loops. In order to preserve their three-dimensional structure and functionality, one would be interested in using these proteins directly as bait with no fusion at the plasma membrane. The Ras recruitment system can be used as an attractive approach for the identification of protein–protein interactions with such membrane baits. This requires the fusion of a cDNA encoding for either a known protein partner for the membrane bait to Ras or a random cDNA library when a screening approach is to be employed. Further development will be required to facilitate the selection between bait-specific and non-bait-specific Ras membrane translocation events. Such an approach will undoubtedly greatly extend the usefulness of the protein recruitment system for integral membrane proteins to explore new territories.

This research was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities-Charles H. Revson Foundation. A.A. is a recipient of an academic lectureship from Samuel and Miriam Wein.

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