32 Protein Bundling to Enhance the Detection of Protein–Protein Interactions

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INTRODUCTION

Many key cellular processes, including transcriptional regulation, protein degradation, and signal transduction, are the outcome of the protein–protein interactions that occur in vivo. In almost all cases, the proteins that participate in these processes exist in multiprotein complexes, the formation of which is dependent on extensive protein–protein interactions. For example, the process of transcriptional activation of eukaryotic genes is dependent on the interaction of multiple transcriptional activators with dozens of proteins that are part of several multiprotein complexes, including TFIID, RNA polymerase, and chromatin-modifying complexes (Laemmli and Tjian 1996; Kingston 1999; Lemon and Tjian 2000). A large number of protein–protein interactions occurring in many biological processes have been identified in recent years, although they repre-

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sent only a small minority of the total protein–protein interactions that are thought to occur in eukaryotic cells. At present, a variety of in vitro and in vivo methods are used to study protein–protein interactions (Fields and Song 1989; Adams et al. 1991; Rossi et al. 1997; Li et al. 2001). Despite the widespread use of many of these methods, there is a growing realization that developing more sensitive and/or high-throughput methods is necessary to analyze the vast majority of protein–protein interactions that are yet to be identified. This chapter describes a method that is specifically designed to enhance the sensitivity of detection of protein–protein interactions in vivo.

Two-hybrid Interactions

The two-hybrid method is the most popular and widely used to study protein–protein interactions in vivo (Chien et al. 1991; Allen et al. 1995; Bai and Elledge 1996). The conceptual basis for the two-hybrid approach originated from the initial understanding of the mechanism of action of transcriptional activator proteins (discussed in Gill and Ptashne 1988; Mendelsohn and Brent 1994; Ptashne and Gann 1997; Keaveney and Struhl 1998). Transcriptional activators are usually composed of at least two functionally autonomous domains: a DNA-binding domain that is essential for binding to the regulatory elements in the promoter region in a sequence-specific manner and an activation domain that recruits the transcription machinery to the promoter region of a gene (Ptashne and Gann 1997). Earlier studies have shown that substituting the relatively weak activation domain of the yeast transcriptional activator protein GAL4 with the strong activation domain derived from the herpes simplex virus protein VP16 could greatly enhance its transcriptional activation potency without affecting its DNA-binding function (Sadowski et al. 1988). This simple yet elegant study showed that activation and DNA-binding domains are highly modular in nature. Importantly, studies that followed this work have shown that covalent attachment of the DNA-binding and activation domains is not essential, rather, a mere non-covalent linking of these domains is sufficient to induce the transcription of their target genes (discussed in Fields and Song 1989; Bemis et al. 1995; Estojak et al. 1995).

Separately expressed DNA-binding and activation domains can be brought together in a number of ways. As shown in a key paper by Fields and Song (1989), separately expressed activation domain and DNA-binding domain fusion proteins can be non-covalently linked by fusing them to proteins that possess an inherent affinity for each other. The presence of these fusion proteins in the same cell promotes their interaction, resulting in the reconstitution of the functional transcriptional activator and subsequent transcriptional activation of its target gene. In a different version of this method, instead of fusing the DNA-binding and activation domains to proteins that possess inherent affinity for each other, these domains were fused with proteins that are capable of interacting with a third protein or a chemical compound simultaneously (Belshaw et al. 1996; Rivera et al. 1996; Zhang and Lautar 1996; Senguptha et al. 1999). The presence of all three components within the same cell promotes the reconstruction of functional transcriptional activator molecules. These modifications of the basic two-hybrid system form the basis for the three-hybrid and dimerizer-dependent gene regulation system, respectively.

Problems in Two-hybrid Assays

Several problems associated with the expression of foreign proteins in eukaryotic cells may preclude the capture even of high-affinity interactions between proteins using the conventional two-hybrid system. It is common to find that the interaction between two-hybrid proteins cannot be detected simply because the expression of one or both hybrid proteins is lethal to the cells. Another frequently faced problem in the two-hybrid assay is that the hybrid proteins, instead of being lethal, may nevertheless be sufficiently toxic that the cells can tolerate only extremely low levels of these proteins (Tasset et al. 1990; Berger et al. 1992; Gilbert et al. 1993). In some cases,

these fusion proteins could reach levels so low that the interaction between the hybrid proteins can occur only at an extremely low frequency. In this situation, even if the hybrid proteins have relatively high affinity for each other, a sufficient number of reconstituted transcriptional activators may not be formed and therefore could not be delivered to the promoter of the reporter gene to induce its transcription. Another problem that may occur in two-hybrid assays derives from the fact that many protein–protein interactions that occur in vivo are highly transient. Consequently, the complexes formed may not be stable enough to recruit the protein complexes that are required for transcriptional activation of the target gene.

Protein Bundles Enhance the Sensitivity of the Two-hybrid Assay: Focus on Mammalian Cells

Because a positive signal in the two-hybrid assay requires the assembly of a two-component transcription factor complex, both fusion proteins must be expressed at sufficient levels relative to their affinity for one another for enough of these complexes to form. In many cases, however, these fusion proteins are poorly expressed, or their affinity is below the detection threshold. These problems can be particularly acute when trying to unite a two-hybrid paradigm in mammalian cells. For example, although the interaction of the c-Src-SH3 domain and its partner c-CBL can be detected in yeast two-hybrid assays, we were unable to detect the interaction between these proteins in mammalian two-hybrid assays using conventional methods (Robertson et al. 1997; Ribon et al. 1998). Western blot assays of transfected mammalian cells suggested that the very poor expression of GAL4-cCbl (G-CBL) fusion protein caused the lack of transcriptional activation of the reporter gene. Therefore, we asked whether increasing the potency of p65 activation domain by attaching multiple copies to a c-Src-SH3 domain partner protein could overcome the negative effects of the low levels of G-CBL fusion protein on the reporter gene expression. To test this possibility, we coexpressed the G-CBL fusion protein with either SH3-S or SH3-4S (carrying a single copy or four copies of p65 activation domains, respectively) in HT1080B cells. We observed that neither combination of the hybrid proteins induced the reporter gene activity to detectable levels (Schmitz and Baeuerle 1991; Nateson et al. 1997). This finding suggested that SH3-S4 fusion proteins were either not recruited efficiently to the promoter of the target gene or, contrary to our assumption, an SH3-4S fusion protein carrying four copies of p65 activation domain is less potent than an SH3-S fusion protein that carries only a single copy of the p65 activation domain. Western blot analysis of the level of expression of hybrid proteins in the transfected cells showed that both GAL4-CBL and SH3-4S were expressed at extremely low levels and, perhaps for this reason, failed to function as potent activators of transcription in vivo (data not shown).

The inability of the hybrid proteins carrying reiterated activation domains to induce transcription robustly led us to develop an alternative method to deliver multiple activation domains to each activator-binding site in the promoter of the reporter gene. In this method, a tetramerization domain derived from the bacterial protein, lactose repressor (Friedman et al. 1995), was placed at the junction between the "target" protein and the p65 activation domain. We assumed that the presence of the tetramerization domain in the c-Src–SH3 fusion protein would allow the formation of protein "bundles" composed of four activation domains and four SH3 domains in vivo. We predicted that the bundled activation domain fusion protein, upon interacting with the G-CBL, could deliver at least a four-times-higher number of activation domains to the promoter of the responsive gene, which might result in the significant enhancement of its transcription (Fig. 1A).

To test this prediction, we coexpressed GAL4-CBL and SH3-S or its tetrameric version SH3-LS fusion protein and examined whether bundling SH3-S protein (Fig. 1B) could help to overcome the effect of very low expression of the GAL4–cCbl fusion protein. We observed that the use of bundled protein SH3-S in this assay led to an extremely strong signal from the reporter gene (Fig. 1C). This finding demonstrates that a simple modification, such as bundling the activation

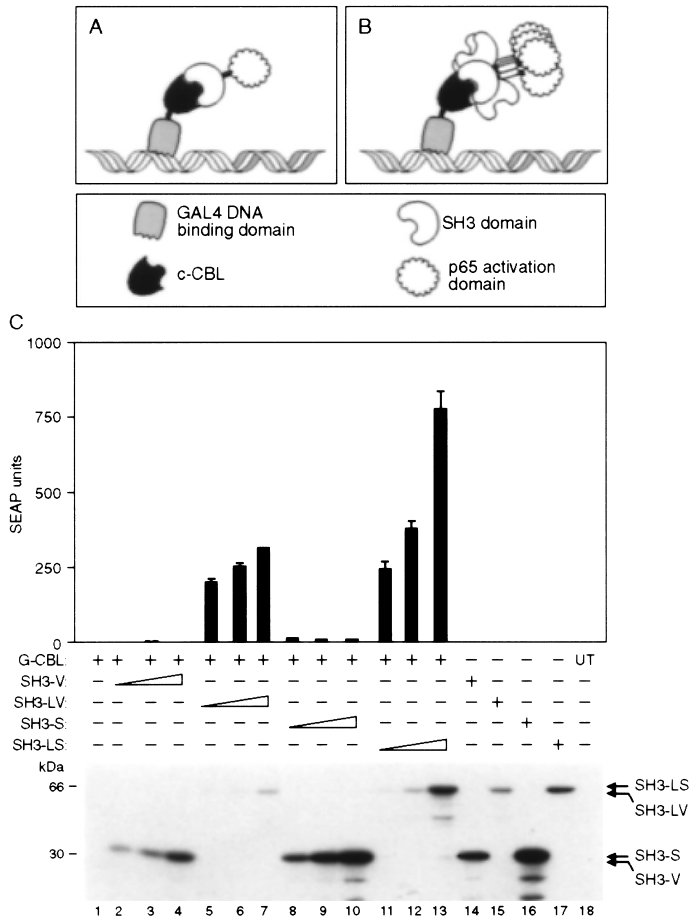

FIGURE 1. Non-covalent bundling of activation domain fusion protein enhances the detection of protein–protein interactions in mammalian cells. Diagrammatic representation of two-hybrid assays with bundled fusion protein containing the target and activation domains. (*A*) GAL4 DNA-binding domain fused to c-CBL (G-CBL in panel *C*) is shown interacting with its target protein SH3 fused to p65 or VP16 activation domain (SH3-S or SF3-V in panel *C*). (*B*) GAL4 DNA-binding domain fused to c-CBL is shown interacting with its target protein SH3 fused to lactose repressor tetramerization domain and p65 or VP16 activation domain sequences (SH3-LS or SH3-LV in panel *C*). (*C*) HT1080B cells carrying SEAP reporter genes placed under the control of five GAL-binding sites were transfected with 100 ng of indicated expression plasmids. The description of protein domains used in this experiment is as follows: G = Gal4 DNA-binding domain; S = p65 activation domain; V = VP16 activation domain; SH3 = SH3 domain from c-SRC protein; L = lactose repressor tetramerization domain; C = c-CBL protein. Mean values of SEAP activity secreted into the medium 24 hours after transfection are shown (± S.D.). Western blot analysis of extracts prepared from transiently transfected cells probed with anti-hemagglutinin antibody is also shown.

domain fusion protein, can improve the outcome in the two-hybrid assay very dramatically. Although it has not been tested extensively, we predict that the use of bundling strategy in general may permit the measurement of protein–protein interactions that escape detection in a conventional system.

Benefits of Bundling Hybrid Proteins in Two-hybrid Assays

The use of bundled activation domain fusion proteins in two-hybrid assays could enhance the detection of protein–protein interaction in two ways. First, unlike the conventional two-hybrid system in which an interaction between the DNA-binding domain and activation domain fusion

protein could deliver only a single activation domain to the promoter, bundling allows the delivery of multiple activation domains to the promoter per interaction event. This increases the sensitivity of the assay and allows the interactions between poorly expressed proteins or proteins that have weak affinity for each other to be detected in mammalian two-hybrid assays. Second, bundled fusion proteins may create an avidity effect for the protein–protein interactions and thus may greatly enhance the interaction itself and/or increase the sensitivity of the assay.

In addition to the two-hybrid system, the bundling approach described here can also be used in other assays that are designed to detect protein–protein interactions. For example, it should be possible to employ the bundling strategy in the fluorescence resonance energy transfer (FRET) or mammalian α-complementation-based protein–protein interaction methods (Adams et al. 1991; Li et al. 2001; see also relevant chapters in this volume). In these methods, bundled proteins could enhance the interaction affinity through the avidity effect and/or significantly increase the strength of the detection signal, which, in turn, should allow the detection of interactions that may not score positively in the conventional assays.

The ability of bundled activation domain fusion proteins to induce gene expression robustly in two-hybrid assays suggested that a similar strategy could also be useful in boosting the expression of both artificially introduced and endogenous genes. To test whether bundled activation domains could induce gene expression to higher levels compared to their unbundled counterparts, we used a modified rapamycin-regulated gene expression system (see Fig. 2) (Natesan et al. 1999). The basic system is composed of a GAL4 DNA-binding domain fused to a single copy of FKBP12 and a p65 activation domain fused to the FRB domain of FKBP12-rapamycin-associated protein. An immunosuppressive drug, rapamycin, binds simultaneously to both FKBP12 and FRB; therefore, in its presence, RS (FRB domain + p65 activation domain) fusion protein can be recruited to the DNA-binding fusion protein. Under these conditions, only a single copy of the p65 activation domain can be recruited by each GAL4 monomer. To increase the number of copies of p65 activation domain recruited by each GAL4 monomer, we constructed a chimeric activator that carried a tetramerization domain between the FRB domain and a single p65 activation domain, such that each GAL4 monomer can recruit a minimum of four activation domains in the presence of rapamycin. In theory, the resulting protein should exist in cells as a tetramer, carrying four FRB domains and four p65 activation domains. In this arrangement, a single molecule of rapamycin can recruit the entire tetrameric bundle of activation domain fusion protein to each GAL4 monomer. By fusing four copies of reiterated FKBP12 moieties to the GAL4 DNA-binding domain, up to 16 copies of the p65 activation domain can be recruited to a single GAL4 monomer (Fig. 2).

To examine whether bundled activators can function as robust inducers of transcription in this system, HT1080B cells were transfected with plasmids expressing various combinations of transcription factor fusion protein and treated with 10 nM rapamycin to reconstitute functional transcriptional activators. The data from this experiment showed that delivering a single copy of the p65 activation domain to each GAL4 monomer induced the reporter gene very poorly. In contrast, delivering RLS (FRB domain + lactose tetramerization domain + p65 activation domain) fusion protein containing four copies of the p65 activation domain to each GAL4 monomer induced the reporter gene very strongly. Western blot analysis indicated that the RS and RLS fusion proteins were expressed at similar levels in the transfected cells. In this experiment, by testing various combinations of fusion proteins, it is possible systematically to vary the number of activation domains delivered to the GAL4 DNA-binding domain from 1 to 16. Under these conditions, there was an excellent correlation between the number of activation domains delivered to the promoter and induced reporter gene activity. Furthermore, these findings also suggest that increasing the number of activation domains delivered to a target promoter leads to significant increases in gene expression (Fig. 3A).

We have also found that delivering bundled activators to the promoter of the reporter gene induced its transcription to much higher levels, whereas delivering tandemly reiterated activation

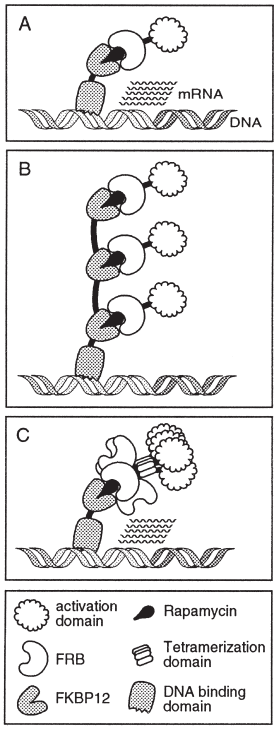

FIGURE 2. Diagram depicting the strategies used to increase the number of activation domains delivered to the promoter. (*A*) In the basic method, two fusion proteins, one containing a GAL4-DNA-binding domain fused to FKBP12 and the other composed of the p65 activation domain fused to FRB, are expressed in cells. Addition of rapamycin results in the reconstitution and subsequent recruitment of a single activation domain to each DNA-binding monomer (GF3 + RS, see panel *A*). (*B*) Fusion of multiple FKBP moieties to the DNA-binding domain allows rapamycin to recruit multiple activation domains to each DNA-binding monomer (GF3 + RS, see panel *B*). (*C*) Addition of the lactose repressor tetramerization domain to the FRB-activation domain fusion protein, producing RLS, allows rapamycin to recruit four activation domains to each FKBP fused to the DNA-binding domain. The number of activation domains recruited to the promoter can be increased by attaching more FKBP moieties to the DNA-binding domain and/or multiplying the number of binding sites for the activator. In theory, as many as 160 activation domains can be delivered to a promoter containing five GAL4-binding sites by treating the cells expressing GF4 and RLS fusion proteins with rapamycin. The actual number of activation domains delivered to the promoter of the reporter gene in vivo by using the bundling method described here has not been determined.

domains capable of delivering the same number of activation domains as the bundled activators induced the reporter gene very poorly. For example, when the DNA-binding fusion protein GF1 was expressed with either RS4 (FRB domain + four copies of p65 activation domain) or RLS and functional activators were reconstituted in vivo by adding rapamycin in the culture medium, only RLS bundles strongly induced the reporter expression (Fig. 3B). Because each delivery event is expected to bring the same number of activation domains to the promoter of the reporter gene, regardless of whether RLS or RS4 fusion protein was used in this assay, the inability of RS4 to induce gene expression is very likely due to its reduced delivery to the promoter. In support of this interpretation, western blot analysis of the extracts from transfected cells showed that RS4 fusion protein is expressed at very low levels and, perhaps for this reason, may not be delivered to the promoter efficiently. In contrast, the RLS fusion was produced at much higher levels in the transfected cells and induced the reporter gene expression very strongly. Thus, taken together, our data suggest that bundled activators are less toxic and delivered more efficiently to the promoter, leading to the strong activation of gene expression.

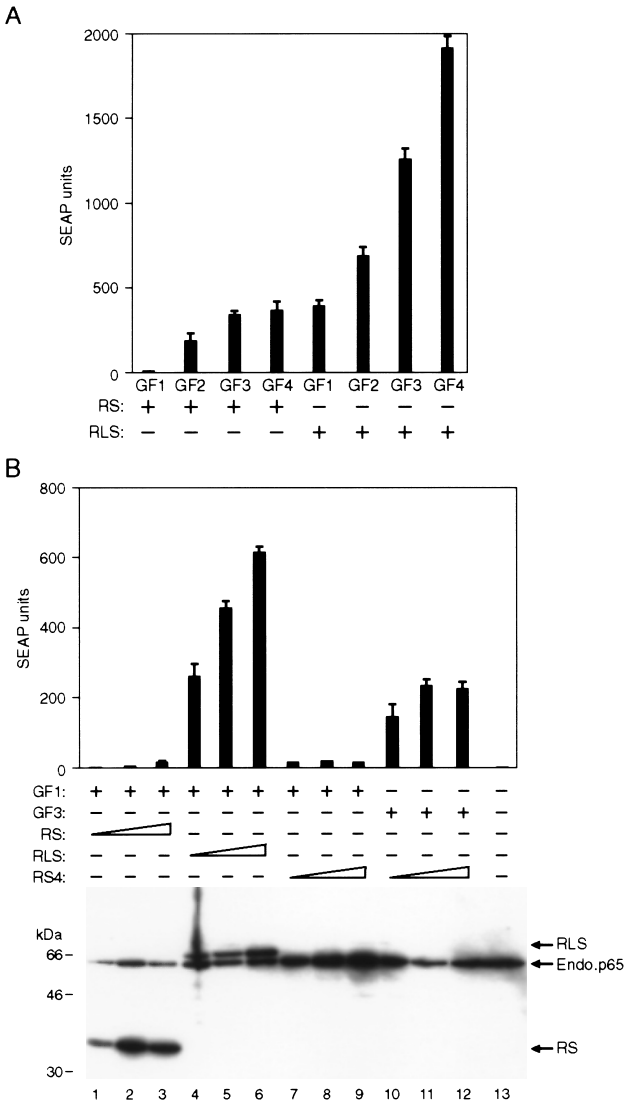

FIGURE 3. The level of expression of a stably integrated reporter gene correlates with the number and strength of the activation domains bound to its promoter. (*A*) The indicated DNA-binding domain and activation domain fusion proteins were transfected into HT1080B cells that carry a stably integrated SEAP reporter gene placed under the control of five GAL4-binding sites. In all cases, SEAP expression values are plotted for cultures receiving 100 ng of activation domain expression plasmid, which gives peak expression values in transiently transfected cells and slightly below-peak values in stably transfected cell lines. The background SEAP activity was subtracted from each value before plotting. In this experiment, expressing the GF1+RS combination of proteins produced four SEAP units above background levels in the presence of rapamycin. (*B*) The DNA-binding domain and activation domain expression plasmids were transfected into HT1080B cells. In all cases, mean values of SEAP activity secreted into the medium after the addition of 10 nM rapamycin are shown (± S.D.). Western blot analysis of the total cell lysates with anti-hemagglutinin antibody allowed an assessment of transcription factor component levels in cells.

The increased potency of bundled activator proteins has other practical applications. For example, we have found that even highly potent chimeric activator proteins such as GAL4–p65 and GAL4–VP16 fail to induce transcription of stably integrated reporter genes robustly when they are placed under the control of a single GAL4-binding site. However, stably integrated reporter genes placed under the control of a single GAL4-binding site in the promoter region can be stimulated to high levels by using bundled p65 or VP16 activation domains (S. Nateson,

unpubl.). Bundled activators may also be useful in other scenarios in which the level of activator protein in the cell is too low to support target gene activation or in cell lines recalcitrant to transfection or when the messenger RNAs encoded by the target gene are highly unstable.

To test the ability of bundled activators under at least one such scenario, we generated stable reporter cell lines in which expression of the chimeric transcription factors was deliberately limited by placing the activator expression under the control a relatively weak Rous sarcoma virus promoter instead of a strong cytomegalovirus promoter (Nateson et al. 1999). One pool of stable cell lines (HT34) expressed the bundled activator, whereas the other pool (HT35) expressed the conventional activator RS. The two pools differed dramatically in their responsiveness to rapamycin; HT34 responded robustly, whereas HT35 did not respond at all. In contrast, the levels of expression of RS and RLS fusion proteins are the same in the two pools. Thus, bundled activators can robustly induce gene expression under conditions in which the conventional activators cannot (Natesan et al. 1999).

Many gene therapy applications can benefit from high-level expression of therapeutic genes. Bundled activation domain fusion proteins are well tolerated in mammalian cell types; therefore, they could induce high-level expression of therapeutic genes in many gene therapy procedures. Activation domain bundles should also allow the rapamycin-regulated system to function robustly, even when the transcriptional activators are expressed at low levels—a likely situation in many gene therapy situations. Perhaps the most important benefit of bundling in this system is that it shifts the dose response of rapamycin activation of gene expression by at least tenfold, indicating that the use of bundled activation domains could improve the practicality of regulated gene therapies by substantially reducing the level of drug required. In theory, bundled activation domains could also be used in other small-molecule-regulated gene expression systems, including the tetracycline and steroid hormone-regulated gene expression systems (Gossen and Bujard 1992; No et al. 1996; Wang et al. 1997).

The regulation of expression of an endogenous gene is dependent on the recruitment of several transcription factors to their binding sites in its promoter region. The ability to regulate the expression of endogenous genes with a single transcription factor could be highly beneficial in many gene therapy applications. At present, methods developed for this purpose use synthetic transcription factors capable of binding to specific sites in the promoter region of the gene of interest to induce or repress the expression of the endogenous gene (Beerli et al. 2000; Zhang et al. 2000). For example, it has been recently shown that it is possible to induce the expression of vascular endothelial growth factor (VEGF) in cells that normally do not produce this protein by expressing zinc-finger–VP16 activator proteins that are specifically designed to bind to the promoter of the VEGF gene (Zhang et al. 2000). It is likely that bundling these synthetic zinc-finger transcription factors could lead to a significant enhancement in the level of expression of the endogenous genes. Alternatively, the synthetic zinc fingers or the DNA-binding domains from natural transcription factors can be used in the modified rapamycin-regulated gene expression system that utilizes the bundled activation domains to regulate the expression of therapeutically relevant genes. The ability of bundled activators to induce high levels of gene expression without apparent cellular toxicity may be critical for the success of these approaches.

OUTLINE OF PROCEDURE

DNA-binding Domains and DNA-binding Domain Fusion Proteins

In theory, any sequence-specific DNA-binding domain with a modest or high affinity for its binding site can be used in two-hybrid assays. However, the vast majority of two-hybrid screens were done using DNA-binding domains derived from yeast and bacterial transcription factors, GAL4

(amino acids 1–94) and LexA, respectively. Recently, synthetic DNA-binding domains, such as ZFHD (Rivera et al. 1996), have been used in small-molecule-regulated gene expression systems; these DNA-binding domain proteins can also be used in the two-hybrid system. Plasmids carrying GAL4 or LexA coding regions are commercially available from such suppliers as Invitrogen, Stratagene, and Clontech. These sequences can easily be amplified by PCR with appropriate restriction sites, and the digested fragments can be cloned into the vector of choice. The synthetic DNA-binding domains can be obtained from either academic laboratories or biotechnology companies that make these reagents available through their Web sites (for example, Ariad Pharmaceuticals).

The hybrid protein composed of a DNA-binding domain of choice and the "bait" protein can be expressed from any episomal or viral mammalian expression vector (see Fig. 4A). It is important that the chosen expression vector contain a selectable marker such as neomycin- or zeomycin-resistance genes, so that, if necessary, stable cell lines expressing this fusion protein can be generated. It is preferable to use retroviruses to express this chimeric protein to reduce the copy number of the chimeric gene in the genome and/or toxic effects of the fusion proteins in the recipient cells. Specially designed retroviral vectors capable of simultaneously expressing multiple recombinant proteins have been published recently (Pollock et al. 2000).

Activation Domains and Activation Domain Fusion Proteins

The potency of the activation domain is a critical determinant of the outcome in the two-hybrid screens. A large number of published two-hybrid screens have used the potent activation domain derived from the herpes simplex virus protein VP16 (amino acids 410–490). We have found that the activation domain derived from the p65 subunit of NF-κB protein (amino acids 361–551) induced the transcription of the reporter gene to higher levels compared to the VP16 activation domain in a mammalian two-hybrid assay (unpublished data). Plasmids carrying the VP16 or p65 activation domain can be obtained from commercial sources such as Clontech, Invitrogen, Stratagene, and Ariad Pharmaceuticals.

Either a plasmid or viral vector can be used to express the recombinant gene encoding a fusion protein composed of the "target" protein and the activation domain (see Fig. 4B). It is not necessary that these vectors contain a selectable marker. Traditionally, plasmid-based libraries carrying cDNAs fused with the activation domain of choice have been used in the two-hybrid assays. However, a wide variety of retroviral or adenoviral vectors that can be used for this purpose are currently available from both academic and commercial institutions (Clontech and Ariad Pharmaceuticals) (Pollock et al. 2000).

Bundling Domains

In theory, any multimerization domain placed between the activation domain and the target partner protein should enhance the detection of protein–protein interactions. We have tested a limited number of multimerization domains and found that the tetramerization domain derived from the bacterial transcription factor lactose repressor (amino acids 46–360) generated the best outcome in both the two-hybrid and regulated gene expression systems. A 30-amino-acid tetramerization domain in the carboxy-terminal region of lactose repressor proteins (amino acids 330–360) appears to function as well as the entire lactose repressor protein without its DNA-binding domain (S. Natesan and E. Molinari, unpubl.). A general description of a plasmid vector carrying the coding sequences for a bundled activation domain fusion protein is shown in Figure 4C.

Reporter Genes and Reporter Plasmids

A wide variety of reporter genes can be used in two-hybrid and other types of assays for analyzing protein–protein interactions. The use of EGFP, EYFP, and CD8 reporter genes provides the

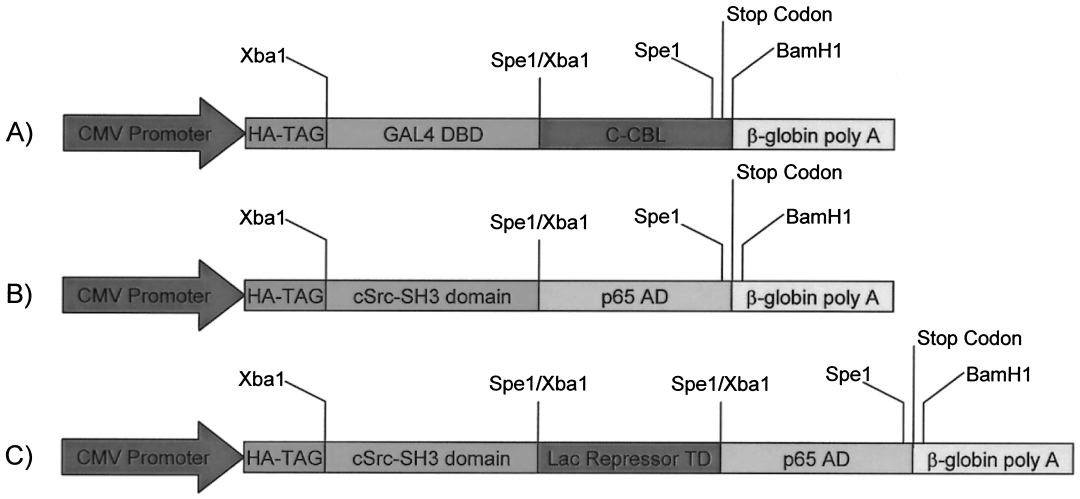

FIGURE 4. Diagram showing the components contained in the plasmids used in protein–protein interaction experiments. Plasmid pGCBL carries coding regions for the GAL4 DNA-binding domain (G) fused to c-CBL (CBL). Plasmid pSH3S carries coding sequences for the c-Src-SH3 (SH3) domain fused to p65 activation domain (S). Plasmid SH3-LS carries coding sequences for c-Src-SH3 domain (SH3) fused to the lactose repressor domain that lacks the DNA-binding domain (L) and p65 activation domain (S). The recombinant genes are under the control of CMV promoter and flanked by the hemagglutinin (HA) tag in the amino-terminal region and a poly(A) region from rabbit β-globin gene at the carboxy-terminal region.

choice of detecting and selecting cells that score positively by the fluorescence-activated cell sorting (FACS) method. If the purpose of the experiment is to map the protein–protein interaction domains, reporter genes, such as SEAP and luciferase, could be used. In general, the reporter gene is placed under the control of two or more copies of GAL4- or LexA-binding sites. For composite DNA-binding domains such as ZFHD, we have placed as many as 12 of its binding sites upstream of the reporter gene to obtain maximal transcriptional activation. However, placing multiple copies of transcription-factor-binding sites upstream of the reporter gene is not necessary when bundled p65 activator fusion proteins are utilized as they generally induce reporter genes driven by one or two copies of the transcription-factor-binding sites to high levels (unpublished data).

Like the DNA-binding and activation domain fusion proteins, the reporter gene can be delivered either in a plasmid or viral vector. The number of copies of the reporter gene integrated in the genome can be minimized or reduced to one by delivering it through retroviral vectors. Chimeric transcriptional activator proteins capable of binding to the promoter region of the reporter genes can be transiently expressed in cells to measure the degree of responsiveness of the stably integrated reporter gene.

Cell Line

The choice of cell line plays a very critical role in the outcome of the analysis of protein–protein interactions in vivo. Because many mammalian cell lines are recalcitrant to standard transient transfection protocols, the method of delivery of recombinant genes and cDNA libraries into mammalian cells needs to be optimized before undertaking the screen. If viral vectors are used for introducing recombinant genes into cells, the level of expression of the genes placed in the viral vector in the cell line of interest should be determined prior to generating custom cDNA libraries for the screen. In mammalian cells, it is not uncommon to find a steady decrease in the expression of stably integrated genes or responsiveness of the reporter genes placed under the control of reporter genes. Therefore, it is essential that the stable cell lines generated for the screen be tested periodically for their robust response. The level of reporter gene activity induced by the expres-

sion of GAL4–VP16 protein can be used to measure the responsiveness of the stably integrated reporter driven by GAL4-binding sites. The level of expression of the DNA-binding fusion protein can be measured by western blot analysis.

Controls

It is well known that the two-hybrid assays have the potential to generate a large number of false positives. Therefore, it is essential to use as many controls as possible to distinguish between the false and true positives scored in this assay. A commonly used control to eliminate the false positives is a reporter gene construct that is identical to the wild-type reporter gene, except that the binding site for the transcriptional activator protein is substituted with the mutant sites. Generally, in mammalian two-hybrid assays, we test all of the positives scored in the primary screen for their ability to induce transcription of the reporter genes driven by both the wild-type and mutant GAL4- or LexA-binding sites; only those that specifically induce the reporter gene driven by wild-type GAL4 or LexA sites are considered for further analysis. The positives isolated in this type of assay can be tested using a nonspecific bait protein attached to the DNA-binding domain used in the screen.

Methods

Analyzing the interactions between proteins and multiprotein complexes requires proficiency in several techniques, including transient transfection, stable cell line generation, reporter gene activity assays, western and immunoprecipitation assays, and general cloning procedures. See Sambrook and Russell 2001, *Molecular Cloning* for detailed protocols for all of these techniques. Also see other chapters in this volume describing two-hybrid and mammalian two-component systems for specific approaches.

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