Detecting Protein-Protein Interactions Using Renilla Luciferase Fusion Proteins

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ABSTRACT

We have developed a novel system designated the luciferase assay for protein detection (LAPD) to study protein-protein interactions. This method involves two protein fusions, a soluble reporter fusion and a fusion for immobilizing the target protein. The soluble reporter is an N-terminal Renilla luciferase fusion protein that exhibits high Renilla luciferase activity. Crude cleared lysates from transfected Cos1 cells that express the Renilla luciferase fusion protein can be used in binding assays with immobilized target proteins. Following incubation and washing, target-bound Renilla luciferase fusion proteins produce light from the coelenterazine substrate, indicating an interaction between the two proteins of interest. As proof of the principle, we reproduced known, transient protein-protein interactions between the Cdc42 GTPase and its effector proteins. GTPase Renilla fusion proteins produced in Cos1 cells were tested with immobilized recombinant GST-N-WASP and CEP5 effector proteins. Using this assay, we could detect specific interactions of Cdc42 with these effector proteins in approximately 50 min. The specificity of these interactions was demonstrated by showing that they were GTPase-specific and GTP-dependent and not seen with other unrelated target proteins. These results suggest that the LAPD method, which is both rapid and sensitive, may have research and practical applications.

INTRODUCTION

Protein-protein interactions occur in all biological processes (15). In signal transduction pathways, these interactions transmit signals that regulate cell morphology, cell growth, apoptosis, and transcription. A variety of standard techniques is currently available for studying these interactions; however, most of these assays are time consuming and involve labeling at least one component of the assay with radioactivity, antibodies, and/or reporter molecules (15). For example, affinity chromatography, co-immunoprecipitation, Western blot analysis, and filter binding assays are commonly used to detect protein-protein interactions. The yeast two-hybrid assay is extremely useful in discovering new interacting proteins but is less useful for testing known interactions. Other methods such as detecting altered surface plasmon resonance energy require relatively expensive instrumentation (15). These methods, which often can detect only long-lived protein-protein interac-
ations, usually are unable to detect directly or provide molecular-level information about the activated signaling intermediates that are generated via conformation-induced steric changes.

The *Renilla reniformis* luciferase gene expresses well in mammalian cells (11) and is commonly used as a control co-reporter with firefly luciferase constructs (5). Furthermore, *Renilla* luciferase and firefly luciferase have been used to study protein-protein interactions. For example, methods using fusion proteins of both *Renilla* luciferase and GFP can detect protein interactions using a modified fluorescence energy transfer method (20–22). Additional methods for using firefly luciferase include conjugation to protein-A (2), luciferase fusion proteins with biotin acceptor peptides (19), and a split luciferase based on RNA splicing (14).

Here we describe a novel protein fusion system based on *Renilla* luciferase for studying protein-protein interactions. This simple two-component binding assay, designated the luciferase assay for protein detection (LAPD), is rapid and sensitive. We have demonstrated the utility of this assay by examining interactions between the Cdc42 GTPase and its effector proteins.

**MATERIALS AND METHODS**

**Construction of Renilla Luciferase Fusion Proteins**

The sea pansy *R. reniformis* luciferase cDNA was amplified by PCR. Two linker primers, 5′-GAGGAATTCACTTCGAAAGTTTATGAT-3′ and 5′-GAGGTCGACTTGTTCATTTTTGAGAAC-3′, containing EcoRI and SalI sites, respectively, were used in PCR with a cytomegalovirus (CMV)-*Renilla* luciferase plasmid as a template (Promega, Madison, WI, USA). After amplification and EcoRI/SalI digestion, the 890-bp *Renilla* luciferase DNA fragment was subcloned into the EcoRI/XhoI site of pCAF2, a CMV mammalian epitope-tagged expression vector (7). The resulting vector, pREN, was used as the parental vector for all additional constructs.

Previously described plasmids of Cdc42-L61 (9), Cdc42-N17 (13), and RhoA-L61 (1) were used as templates for PCR to generate cDNAs for these mutant GTPases. DNA encoding amino acids 2–193 of the Rho mutant was amplified with BamHI and EcoRI linker primers 5′-GAGGATCCCGATCCGAGGAA-3′ and 5′-GAGGATCCCGAGGAA-3′. DNA encoding amino acids 2–177 of the Cdc42 mutants were amplified with BamHI and EcoRI linker primers 5′-GAGGATCCCGAGGAAATTAAGTG-3′ and 5′-GAGGATCCCGAGGAAATTAAGTG-3′. The PCR products were then digested with BamHI and EcoRI restriction enzymes and cloned in frame into the BamHI/EcoRI site of pREN between the FLAG® epitope tag and *Renilla* luciferase (Figure 1). The integrity of each of these constructs was verified by DNA sequencing.

**Bacterially Produced GST Fusion Proteins**

Recombinant GST, GST-N-WASP, a fragment of N-WASP containing amino acids 151–277 (17), and GST-CEP5 (7) were produced using the pGEX-4T3 bacterial expression vector (Amersham Biosciences, Piscataway, NJ, USA), purified, and left immobilized on glutathione-agarose beads in buffer A (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl$_2$) as previously described (7). The amount of each protein immobilized to the glutathione-agarose beads was estimated by SDS-PAGE.

**Production of Recombinant Renilla Fusion Proteins in Cos1 Cells**

Cos1 cells were grown in DMEM containing 10% FCS and were split to 60% confluence one day before transfection. The pREN expression vector constructs (2 µg) were transfected into...
Cos1 cells by FuGENE™ 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) as previously described (7). Forty-eight hours after transfection, the cells were washed once with PBS and harvested by scraping into 1.2 mL buffer A containing 0.5% Triton® X-100 and protease inhibitors (0.5 mM PMSF, 25 µg/mL aprotinin, 50 µg/mL pepstatin, and 25 µg/mL leupeptin). After centrifugation at 13 000× g for 2 min, the supernatants were collected and used directly to determine the total number of light units in each of the lysates from the transfected cells. Luciferase activity was measured essentially as described in the Dual Luciferase Reagent Kit (Promega), except that the steps involving the detection of firefly luciferase were omitted (16). Briefly, 100 µL Stop and Glow® buffer (Promega) and 2 µL coelenterazine substrate were added to each 12 × 75-mm glass tube. Renilla luciferase assays were initiated by the addition of 5 µL Renilla containing supernatant to the substrate. These mixtures were then vortex-mixed and immediately measured for light units with a model LB9501 Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany).

LAPD

LAPD is simple to perform. Because of intrinsic GTP of the Renilla GTPase fusion proteins, the assays were performed fresh, within 1 h of harvesting the cell lysates. Extracts containing equal amounts of Renilla luciferase activity (1 million light units) of each of the fusion proteins were added to 4 µg immobilized GST, GST-N-WASP, or GST-CEP5 fusion proteins in buffer A containing 0.5% Triton X-100 and protease inhibitors in a final volume of 100 µL. After a 20-min incubation on ice, unbound and loosely bound proteins were removed by washing the beads four times in the same buffer, followed by centrifugation at 13 000× g. Approximately 70%, 16%, 2.5%, and 2.2%, respectively, of the total unbound Renilla activity was removed after the first, second, third, and fourth washes, respectively. After the fourth wash, the beads and associated proteins were resuspended in 30 µL buffer A. The amount of Renilla luciferase bound to the beads was determined by adding 15 µL bead suspension to 100 µL Stop and Glow buffer containing 2 µL coelenterazine substrate and immediately measuring the light units.

RESULTS

N-terminal Fusion Proteins with Renilla Luciferase Activity

A cDNA-encoding Renilla lucife-
rase was amplified by PCR and cloned into the EcoRI/XhoI site of a CMV mammalian expression vector. The resulting vector, pREN, contains, starting from the 5'-end, a Kozak consensus start methionine sequence, an N-terminal FLAG epitope, unique BamHI and EcoRI restriction sites, and the Renilla luciferase enzyme (Figure 1). Cos1 cells transfected with pREN could also be detected with immunofluorescence by the epitope tag (data not shown). Forty-eight hours after transfection, the cells were harvested and lysed by scraping in buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, and 0.5% Triton X-100. Transfection with 2 µg pREN vector typically yielded extracts containing 2–8 × 10⁹ light units/100 mm² plate of Cos1 cells.

DNA encoding three mutant GTPases, constitutively active Cdc42-L61, dominant negative Cdc42-N17, and constitutively active Rho-L63 mutants were cloned in frame between the BamHI and EcoRI sites of the pREN vector (Figure 1). The corresponding plasmids were transfected into Cos1 cells, and the fusion proteins were harvested 48 hours later. Using this protocol, all three mutant GTPase-Renilla fusion proteins had robust luciferase activity that was similar to the pREN control vector. Additional plasmid constructs with other N-terminal Renilla fusion proteins, including a fragment of B2 cyclin, also produced active luciferase fusion proteins with roughly similar levels of luciferase activity (data not shown). These results suggest that Renilla luciferase fusion proteins can maintain most of their luciferase enzymatic activity if N-terminal fusions are used as described here.

**LAPD**

Next, we tested whether these Renilla fusion proteins could be used to detect protein-protein interactions. A two-component binding assay (LAPD) was devised in which the binding of a soluble Renilla fusion protein to an immobilized target was detected by measuring luciferase activity. Briefly, the cell lysates that contained the Renilla fusion protein bind to target proteins immobilized on glutathione-agarose beads. After incubation and washing, the protein-protein interaction was detected by the ability of the bound Renilla fusion protein to produce chemiluminescence after the addition of a luciferase substrate. In these assays, the amount of light produced was proportional to the amount of soluble fusion protein bound to the glutathione beads because previous studies have shown that Renilla luciferase shows a linear range of luciferase activity over seven orders of protein concentration (16).

As a test system of LAPD, we tested whether it could detect the GTP-dependent interaction of Cdc42 with its effector proteins, N-WASP (12) and CEP5 (7). GST, GSN-WASP, and GST-CEP5 fusion proteins were produced in bacteria and left immobilized to the glutathione-agarose beads after purification. Lysates from the four Renilla fusion proteins were measured for luciferase activity, and an equal amount of light units (1 million) from each of the lysates was added to 4 µg GST, GST-N-WASP, or GST-CEP5 immobilized beads. Following incubation for 20 min and four washes, a 15-µL suspension of the residual beads was then added to the coelenterazine substrate, and the light units were measured. Under these experimental conditions, we found that the Renilla control protein did not bind to either GST or GST-N-WASP (Figure 2). In contrast, Cdc42-L61-Renilla fusion proteins bound to both GST-N-WASP and GST-CEP5 but not to GST (Figure 2). We also tested a dominant negative mutant of Cdc42 (Cdc42-N17) that should not interact with GST-N-WASP or GST-CEP5 because it does not bind GTP. As expected, no significant binding of the dominant negative Cdc42-N17-Renilla was found with GST, GST-N-WASP, or GST-CEP5 (Figure 2). Rho-L63-Renilla fusion extracts also did not show GST, GST-N-WASP, or GST-CEP5 binding (Figure 2). Collectively, these results show that LAPD can easily reproduce known protein-protein interactions between Cdc42 and its effector proteins.

**DISCUSSION**

In summary, we have developed LAPD, a simple, rapid method to detect protein-protein interactions using Renilla luciferase fusion proteins as the detection system. If these fusion proteins bind immobilized target proteins, then a larger visible signal is generated following the addition of a luciferase chemiluminescence substrate. Note that LAPD is rapid and that qualitative protein-protein interactions can be detected or excluded in less than 50 min once both sets of the recombinant proteins are available. Renilla luciferase is an ideal reporter for LAPD because it is small (36 kDa), highly sensitive (10), and retains most of its activity as a fusion protein while allowing the other component of the fusion to also remain biologically active. Based on these characteristics, we used Renilla fusion proteins to reproduce known protein-protein interactions. Several additional aspects of this assay system should be noted. First, the ease, sensitivity, and rapidity of the assay depend on the remarkable activity of the Renilla luciferase reporter enzyme so that as little as 0.001 pg can be detected (10). Second, by fusing the Renilla reporter into

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Figure 2. Detection of Cdc42 GTPase interactions with GST-N-WASP and GST-CEP5 by LAPD. Lysates from Cos1 cells expressing Renilla (lanes 1, 5, and 9), Cdc42-L61-Renilla (lanes 2, 6, and 10), Cdc42-N17-Renilla (lanes 3, 7, and 11), or RhoA-L63-Renilla (lanes 4, 8, and 12) was prepared 48 h after transfection. Equal amounts (1 million light units) of luciferase activity from each of the different lysates were added to the immobilized GST, GST-N-WASP, or GST-CEP5. Following a 20-min incubation, the beads were washed four times, and then an aliquot was added to the coelenterazine substrate and measured for light units. The mean of three independent experiments is represented. Error bars indicate standard deviation.
the C-terminus of probe proteins, almost all of the recombinant molecules are labeled. It is also likely that N-terminal fusion proteins may also be active and useful in the LAPD system. Third, LAPD offers the advantage of rapidly detecting protein-protein interactions without any additional amplification steps or the use of fluorescence or radioactivity. Finally, as with any assay, LAPD requires some optimization to determine the conditions that maximize detection sensitivity.

The sensitivity of LAPD for detecting the GTP-dependent interaction of Cdc42 with N-WASP was comparable to standard techniques such as radioactive filter binding assays and GST-capture experiments with these GT-Pases (3, 6). While the affinity of interactions between Cdc42-GTP and CRIB-containing effector proteins have a Ka in the range of 4–0.8 μM (18, 24), it should be noted that under these conditions only a small percentage of the input Cdc42-L61-Renilla (2%–3%) bound to the immobilized GST-N-WASP or GST-CEP5. The reason for this low binding is the fact that these interactions are dependent on GTP-bound Cdc42. Consistent with this idea, additional experiments have revealed that prolonged incubation of the extracts (more than 3 h) show decreased binding activity with the effector proteins, presumably due to GTP-hydrolysis because no alteration in total Renilla luciferase activity was observed (unpublished data). In light of this, we expect the LAPD system might be useful in studying the activity of GT-Pase regulatory proteins and signaling pathways that alter the levels of GTP-bound GT-Pases. Furthermore, the LAPD system is likely to be suitable for the detection of other types of either stable or transient protein-protein interactions and might be simplified by producing both fusion proteins in bacteria rather than in mammalian cells, which would provide greater amounts of components that could be more easily standardized. It is already known that Renilla fusion proteins produced in bacteria retain their luciferase activity (Reference 8 and unpublished data). The LAPD system may be useful for drug discovery by facilitating high-throughput screens for agents that block known protein-protein interactions of medical interest. Finally, the recent ability to express (23) and optically image Renilla luciferase in live animals (4) may present the possibility of using variations of the LAPD system to study protein-protein interactions in vivo.

REFERENCES


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Negative Purification Method for the Selection of Specific Antibodies from Polyclonal Antisera

ABSTRACT

We developed a protocol to remove non-specific antibodies from polyclonal antisera by adsorption on non-target antigens immobilized on nitrocellulose membranes. This ‘negative’ purification method is simple and provides better immunoreagents than the blocking of nonspecific antibodies in solution or the enrichment of specific antibodies on nitrocellulose membranes. For routine applications, this method is quicker and cheaper than the purification protocols based on selective precipitations and affinity chromatography.

INTRODUCTION

Antibodies are instrumental in protocols for immunolocalization, immunopurification, Western blot analysis, EMSA super-shifts, or the screening of cDNA expression libraries—not to mention their use in detection kits. Each of these applications has its own requirements in terms of the purity of the antibodies. Although monoclonal antibodies are free from interfering host immunoglobulins, their production is expensive and requires technical knowledge and animal cell culture facilities. Alternatives such as the phage display technology and the in vitro production of recombinant antibodies suffer from similar limitations.

Thus, the use of polyclonal antisera remains very popular but necessitates the removal of nonspecific antibodies. Purification typically begins with a combination of ammonium sulfate/caprylic acid precipitation or ammonium sulfate/DEAE chromatography (5), and nearly pure antibodies are recovered, although frequently at low titers because of their limited solubilization or inactivation (2). They are typically submitted to an additional affinity purification. Protein A, protein G, or anti-IgG affinity chromatography of IgG from ascites or polyclonal antisera (1) is a straightforward method but not always suitable for all species and antibody subclasses (4), and nonspecific IgG antibodies still end up in the final eluate.

Antigen affinity chromatography is the best technique to obtain highly purified and specific antibodies (3) but often requires harsh conditions (e.g., extreme pH, chaotropic and denaturing agents, etc.) to elute the antigen, causing a loss of activity (2,3). Finally, whichever protocol is used, it has to be optimized for each antigen, which is hardly conceivable for the routine production of sera against a significant number of the proteins present on a 2-D electrophoresis gel.

Quicker protocols have been used to obtain partially purified preparations. These consist simply of the centrifugation of the nonspecific antibodies inhibited by blocking mixtures (8) or of the adsorption of specific antibodies on nitrocellulose-bound antigens and their subsequent recovery (7).

Here we discuss what we call a negative antibody purification in which we remove nonspecific antibodies by incubating polyclonal antisera with non-target proteins that are adsorbed on a membrane. The efficiency of each method is evaluated using Western blot analysis.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli BL21 (DE3)® (Novagen, Madison, WI, USA) clones were grown in liquid LB medium at 37°C under constant agitation in the presence of 100 µg/mL ampicillin. Two bacterial test clones were used. The pET15b-GMD clone contained the pET15b® plasmid (Novagen) with a 1122-bp NdeI/XhoI cDNA fragment of the Arabidopsis thaliana GDP-D-mannose-4,6-dehydratase gene (GenBank® accession no. U81805) and the pET21c-GE clone contained the pET21c® plasmid (Novagen) with a 1056-bp NdeI/SalI cDNA fragment corresponding to the A. thaliana UDP-glucose-4-epimerase gene (GE) (accession no. Z54214).