23 Protein Interactions in Live Cells Monitored by β-Galactosidase Complementation

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INTRODUCTION

The characterization of protein interactions is important to the understanding of signal transduction pathways and cellular processes. Here we describe a method utilizing β-galactosidase (β-gal) complementation that can monitor protein interactions in live mammalian cells (Rossi et al. 1997, 2000; Blakely et al. 2000). In brief, the method involves expressing chimeric proteins consisting of two potentially interacting proteins of interest fused to complementing β-gal deletion mutants. When the two proteins of interest interact, the β-gal mutants complement, reconstituting an active β-gal enzyme. Some of the advantages of β-gal complementation are: (1) It is a direct assay, that is, a signal is generated at the site of the interaction, and activation of a reporter gene is not required; (2) it is a sensitive assay, due to enzymatic amplification of the product; (3) over-

expression is not required—interactions can be detected at physiological levels of expression; (4) β-gal enzyme activity can be measured using a variety of substrates that permit quantitative assays that can measure β-gal activity in single live cells, or that are amenable to high-throughput screening technologies.

A variety of biochemical methods is available for demonstrating the interaction of two proteins, the most common of which is immunoprecipitation followed by identification by immunoblotting. Such biochemical methods have proven reliable, but can be limited by the inability of some complexes to survive cellular disruption and immunoprecipitation. Complexes can be stabilized by chemical cross-linking before the cell is disrupted, but this step can introduce artifacts by cross-linking adjacent, but noninteracting, proteins. The yeast two-hybrid system has been the most powerful method for identifying novel protein interactions (Fields and Song 1989; Bai and Elledge 1996). However, proteins in this system must be capable of interacting in the yeast environment and must then translocate to the nucleus and activate transcription of a reporter gene.

An ideal system for studying protein interactions would generate a signal upon the interaction of the proteins of interest. This signal would be specific, and would be detectable in the cellular compartment in which it is generated, either directly or with minimal additional steps, such as addition of a substrate. Ideally, the signal would be detected in live cells, or the signal must be able to survive disruption of the cells. Several recent developments have come close to this goal. Fluorescence resonance energy transfer (FRET) has been used to study protein interactions via the in vitro labeling of two proteins of interest with fluorescent tags (Adams et al. 1991; Gadella and Jovin 1995; Chapter 10). However, the difficulties of introducing fluorescently labeled proteins into cells at sufficiently high concentrations to detect a signal can limit the utility of this method. An alternative FRET methodology involves expressing chimeric proteins that incorporate one partner of an interacting protein pair and a fluorescent protein, such as green fluorescent protein (GFP). By using two different GFPs, FRET analysis of the interacting chimeras is possible, but it remains to be seen whether this method will work well with a variety of interacting proteins (Miyawaki et al. 1997; Pollok and Heim 1999). Other non-FRET methods using interacting chimeric proteins depend on the complementation of two mutant protein fragments to reconstitute a functional protein. Complementation of dihydrofolate reductase (DHFR) has been used to examine protein interactions in mammalian cells, but this system is dependent on either a nonenzymatic substrate-binding assay that is quantitative only in cell lines that lack endogenous DHFR, or a nonquantitative enzymatic survival assay that only works in cells lacking endogenous DHFR (Pelletier et al. 1998; Remy et al. 1999). The complementation of bacterial β-gal, discussed in detail below, generates an enzymatically amplified signal that can be detected quantitatively by a variety of assays in many different cell types without overexpression of the proteins (Rossi et al. 1997, 2000; Blakely et al. 2000).

BACKGROUND

Intracistronic β-gal complementation is a phenomenon first observed by Jacob and Monod, in which two mutants of the bacterial enzyme β-gal that have inactivating deletions in different critical domains recreate an active enzyme by sharing the intact domains (Ullmann et al. 1965, 1967). β-Gal complementation has been used for decades as a marker for molecular cloning experiments in bacteria (blue-white colony selection). The basis of this system is the expression of a truncated inactive β-gal protein (ΔM15) by the host bacterium, which can be complemented by expression of a peptide (α peptide) from a plasmid cloning vector, but only if the α-peptide coding sequence is not disrupted by a cloned cDNA. β-gal was successfully complemented in mammalian cells using three different complementing peptides (Fig. 1): Δα, similar to the ΔM15 mutant used in bacteria, has a deletion near the amino terminus of the protein (amino acids 11–41); Δω, trun-

β-galactosidase

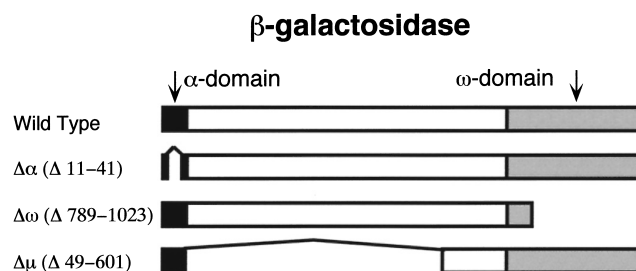

FIGURE 1. Schematic diagram of the β-gal deletion mutants. The α and ω domains, as defined by Jacobson et al. (1994), are represented by the black and gray boxes at the amino and carboxyl termini of the protein, respectively. Δα has a small deletion in the α domain, Δω has a large deletion of the carboxyl terminus, and Δμ has a large deletion in the region of the protein between the α and ω domains.

cated at amino acid 788, lacks the carboxyl terminus of the protein; and Δμ has a deletion (amino acids 49–601) in the middle of the protein (Mohler and Blau 1996). The amino-terminal (α) and carboxy-terminal (ω) regions of the protein are critical to forming a functional enzyme (Villarejo et al. 1972; Jacobson et al. 1994). Δω functions as an "α donor" (analogous to but much longer than the α peptide used in bacterial complementation), Δα functions as an "ω donor," and Δμ can provide either region but lacks the middle region that is important for the structure of the active enzyme. Any two of these mutant proteins, when expressed in the same cell, result in formation of an active β-gal enzyme (Mohler and Blau 1996). Although native β-gal forms a functional enzyme as a homotetramer, it has not been definitively established whether the active complemented enzyme complex in mammalian cells requires eight mutant proteins.

β-Gal complementation in mammalian cells was first used to study the process of cell fusion in myoblast differentiation (Mohler and Blau 1996). Each of the deletion mutants was expressed in separate populations of myoblasts. When two of the different populations were cocultured in differentiation-inducing conditions, the myoblasts fused into multinucleated syncytia, or myotubes, and complementation of β-gal was observed. Measurement of β-gal activity provided a simple quantitative assay for myoblast fusion and thus provided a rapid method to measure the effects of genetic mutations and culture conditions of myoblast differentiation (Charlton et al. 1997).

By constructing chimeric proteins incorporating one of the β-gal deletion mutants, interactions between the non-β-gal components of the chimeras can be detected (Rossi et al. 1997). Coexpression of any two of the three different deletion mutants in the same cell results in β-gal complementation, but lower activity was usually observed when Δα was paired with Δω, compared to either of these mutants with Δμ (Mohler and Blau 1996). In a complementation assay for protein interactions, the interaction should be driven by the proteins of interest, rather than the β-gal mutants; therefore, the weakest complementing pair, Δα and Δω, were used for constructing chimeras for protein interaction assays. In the initial test of this system, Δα and Δω were linked to the rapamycin-binding proteins FRAP and FKBP12 (Rossi et al. 1997). β-Gal activity was very low in the absence of rapamycin but increased significantly upon addition of rapamycin. β-Gal activity was dependent on the dose of rapamycin and continued to increase throughout the time measured (Fig. 2, left).

Membrane receptor dimerization can also be quantitated using β-gal complementation (Blakely et al. 2000). By expressing chimeric proteins that link Δα or Δω to a truncated epidermal growth factor (EGF) receptor, EGF-induced receptor dimerization resulted in increased β-gal activity. β-Gal activity initially increased more rapidly than was observed with rapamycin-binding proteins; however, after increasing about eightfold in the first hour, the activity plateaued, as would be expected with membrane receptor dimerization (Fig. 2, right). Thus, the kinetics of β-
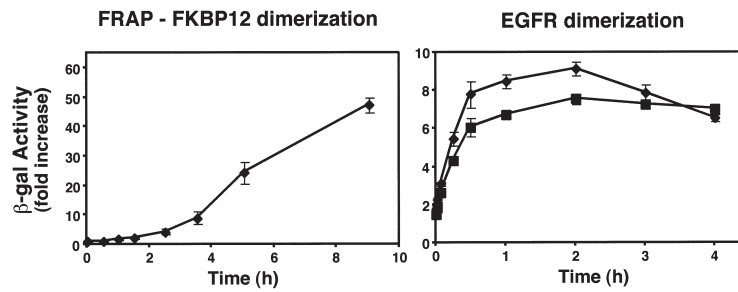
FIGURE 2. The kinetics of chimeric β-gal protein complementation depend on the non-β-gal portion of the chimera. The rapamycin-binding proteins FRAP and FKBP12, or the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR), are fused to β-gal deletion mutants and expressed in C2C12 cells. The left panel shows that β-gal activity increases over a 9-hour period following the addition of rapamycin to a population of cells expressing the FRAP and FKBP12–β-gal chimeras. The right panel shows that β-gal activity increases rapidly over the first 1–2 hours and then plateaus after the addition of EGF to two different clones expressing the EGFR–β-gal chimeras. The β-gal activity in each experiment reflects the dimerization kinetics of the wild-type rapamycin-binding proteins and EGFR, respectively.

gal activity reflect the kinetics of the non-β-gal components of the chimeric proteins. β-Gal complementation was used to characterize fully the effects of an anti-EGF receptor antibody on receptor dimerization, which previously had not been possible using biochemical methods. Furthermore, the levels of expression of the chimeric receptors used in these studies did not exceed the levels of the endogenous receptor in normal cells.

Finally, β-gal complementation, in addition to measuring cytoplasmic protein interactions and membrane protein interactions, can also measure the interaction of a cytoplasmic protein and a membrane receptor (e.g., TGF-β receptor 1 and FKBP12; T.S. Wehrman et al., unpubl.). Thus, β-gal complementation can measure three types of interactions that are key components of most signal transduction pathways (Fig. 3).

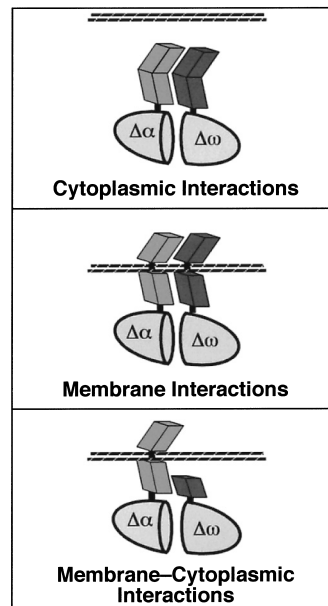**FIGURE 3.** β-Gal complementation can detect protein interactions in the cytoplasm or at the membrane. Two chimeric proteins containing different β-gal deletion mutants (labeled Δα or Δω) and proteins of interest (shaded rectangular boxes in figure) are expressed in cells. If the proteins of interest interact, the β-gal mutants will complement, and β-gal enzyme activity serves as a measure of the interaction.

β-Gal complementation can be used to confirm protein interactions and to characterize known interactions. A variety of assays is available to measure β-gal activity, further increasing the utility of this method. Of particular interest are a flow cytometry assay using a fluorogenic substrate for β-gal for the measurement of β-gal activity in single live cells and a chemiluminescent assay that enables the analysis of hundreds of samples in microtiter plates using high-throughput screening technologies. The simple, rapid assays available for β-gal make it possible to test the effect of a variety of conditions (e.g., inducers, inhibitors, concentrations, antibodies, cell backgrounds). β-Gal complementation will also be useful for screening for novel inducers and inhibitors of protein interactions using high-throughput screening methods (e.g., receptor agonists and antagonists) and potentially could be used as a "mammalian two-hybrid" screen for novel interactions.

OUTLINE OF PROCEDURE

The first step is constructing two chimeras each containing one of the β-gal deletion mutants and the protein of interest. These constructs must then be expressed in cells. We use retroviral vectors to express the chimeras to rapidly obtain cells that stably express the constructs while limiting the copy number of the construct and thus the expression levels. Once cells that express both constructs are obtained, the cells can be tested for β-gal activity in the presence and absence of an inducer of the desired interaction. In some cases, cloning the cells that express the chimeras may be desirable. Several different assays for β-gal activity are presented here.

Strategy for Construction and Expression of the Chimeric Proteins

Although our initial experiments were carried out with the β-gal deletion mutant linked to the carboxyl terminus of the protein of interest, we have subsequently found that, at least in the case of cytoplasmic rapamycin-binding proteins, complementation works equally well with the β-gal mutants at either end of the chimeric protein (T.S. Wehrman et al., unpubl.). In the case of membrane proteins, the β-gal mutant has only been placed at the carboxyl terminus, so that complementation occurs in the cytoplasm. The use of PCR and standard cloning techniques is required to place the protein of interest accurately in frame with the β-gal deletion mutant.

The use of retroviral vectors simplifies the expression of the chimeric proteins. We use two retroviral vectors, which contain either Δα or Δω under the transcriptional control of the viral long terminal repeat (LTR) (Fig. 4) (Rossi et al. 1997; Blakely et al. 2000). An intracistronic ribosome entry site (IRES) and a drug-resistance marker are located downstream of the β-gal mutant. After the protein of interest is cloned in frame with the deletion mutant, the plasmid containing the retroviral vector is transiently transfected into the Phoenix-E packaging cell line using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Indiana) according to the manufacturer's instructions. Although calcium phosphate transfection protocols can sometimes result in transfection of a higher percentage of the cells, we consistently obtain higher viral titers using FuGENE 6, possibly because of the absence of toxic side effects. Virus supernatant is used immediately to infect the target cells, or it can be frozen at –70°C with a resulting small drop in titer. Although many cell lines can be infected with nearly 100% efficiency, any uninfected cells can be eliminated through the use of the selectable marker in the vector.

Although one can infect cells with both vectors (the Δα and Δω constructs) simultaneously, we usually infect the cells sequentially. It can be useful to have cells on hand expressing only one of the vectors in case future experiments call for changing one of the interacting proteins. Furthermore, if there are problems with expression of the chimeric proteins, it may be easier to determine which of the constructs is defective if they are singly expressed, especially if the two
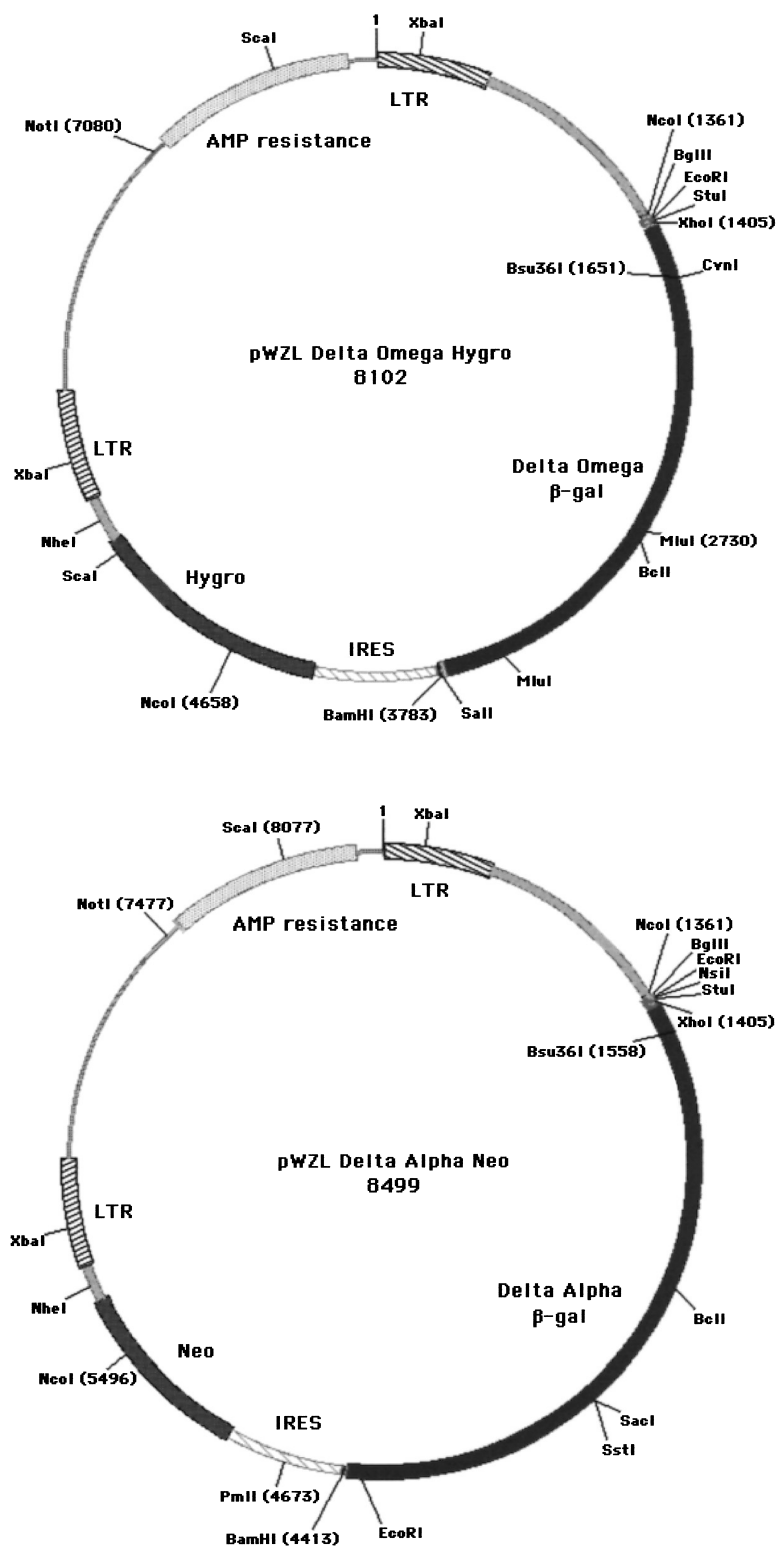
FIGURE 4. Maps of the β-gal complementation vectors. Using the plasmids shown, chimeric proteins can be engineered by using PCR and restriction digests to clone cDNAs of interest in frame with either the Δα or Δω mutants of bacterial β-gal. When transfected into packaging cell lines, retroviruses are produced (lacking the ampicillin-resistance gene and other bacterial sequences between the LTRs) that express the chimeric protein and a selectable marker (resistance to G418 or hygromycin).

chimeric proteins are similar in size. Selection with antibiotics such as G418 and hygromycin should be carried out at the lowest dose that effectively kills uninfected cells, because selection with higher doses can lead to overexpression of the constructs.

Assays for β-Gal

Chemiluminescent Assay for β-Gal

The chemiluminescent assay for β-gal is the quickest and simplest method for assaying a large number of samples. In brief, cells are plated and treated with reagents that will induce the protein interaction of interest. Gal-Screen reagent is added to the cells, resulting in the prolonged production of photons ("glow" kinetics, not "flash" kinetics) in the presence of active β-gal. The light emitted by the sample is then measured in a luminometer. Cells are plated on a 96-well plate, a format that permits the use of high-throughput screening instrumentation if desired. This format greatly simplifies the assay because cells are plated and assayed on the same dish. However, if a microplate luminometer is not available, the samples can be transferred to a tube for use in a tube luminometer.

The microtiter plate used in this assay should be of the type recommended by the manufacturer of the luminometer. For many instruments, this will be a white plate with either a white (opaque) or clear bottom. However, some instruments may specify the use of black plates. Although opaque plates are ideal, clear-bottomed plates, which permit observation under a microscope, can be used until the experimenter is comfortable that he is obtaining a subconfluent, evenly plated culture. Chemiluminescence can spill from one well to another through the clear bottom of the plate, which will be noticeable if a well producing thousands of induced light units is adjacent to a negative control or uninduced sample.

We use a Tropix TR717 microplate luminometer, although other instruments also work well, including the following instruments that we have tested: EG&G Wallac Berthold LB96V (which is nearly identical to the Tropix instrument), Turner Designs Reporter, and the EG&G Wallac MicroBeta Plus (current version is MicroBeta Trilux; MicroBeta units with the automatic injector may have reduced sensitivity). One disadvantage of the MicroBeta is that it takes 5–10 minutes to count a plate, compared to less than 2 minutes for the others. Some instruments may not have sufficient sensitivity, and this should be considered if β-gal activity is not detected.

Flow Cytometry Assay for β-Gal

By assaying the product of the fluorogenic β-gal substrate fluorescein di-β-D-galactopyranoside (FdG) using a flow cytometer, β-gal activity can be measured in single live cells. This is useful for determining changes in β-gal activity in a population of cells and thus determining whether or not cloning of the cells is necessary to achieve a uniform response. Furthermore, like the chemiluminescent assay, the FdG assay is quantitative and can be used to study the kinetics and dose response of the interaction. The fluorescence-activated cell sorter (FACS) can also be used to isolate subpopulations of cells or to clone cells that exhibit the desired response.

The protocol given here is based on that published by Nolan et al. (1988) and has been modified largely to accommodate the processing of large numbers of samples. Using this method, one person can readily assay 50–100 samples, and with two persons working together, as many as 200 samples can be assayed within a few hours. If only a few samples are to be analyzed, the assay can be completed in less than half an hour. The FdG substrate used in this assay is not cell-permeable and must be introduced into the cells by a hypotonic shock. Cleavage by active β-gal produces free fluorescein, which is also unable to cross the plasma membrane and remains inside the cells that have complemented β-gal. Following the analysis, most cells remain viable and can be returned to culture. In populations or clones that exhibit homogeneous changes in β-gal activity, the mean fluorescence of each sample is a reliable indicator of the interaction.

X-Gal and Fluor-X-Gal Assays for β-Gal

The X-gal assay is a simple chromogenic assay that requires no specialized instrumentation. In the presence of active β-gal, the substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), and a potassium ferricyanide buffer, a blue precipitate that can be observed by microscopy forms in fixed cells. This assay is not quantitative and is not particularly sensitive, but it can be used to detect β-gal complementation if the induction of complementation is sufficiently robust (greater than about fivefold) (Rossi et al. 1997).

Because the X-gal assay product quenches fluorescence, the Fluor-X-gal assay was developed so that β-gal activity could be assayed in cells that had been immunofluorescently labeled for other proteins (Mohler and Blau 1996). This assay combines an azo dye, Fast Red Violet LB, with either X-gal or 5-bromo-6-chloro-3-indolyl β-D-galactopyranoside (5-6 X-gal) to form a fluorescent precipitate in the presence of active β-gal. Fluor-X-gal is more sensitive than X-gal, but like X-gal, is not quantitative and requires fixation of the cells.

Preparation of Retrovirus and Infection of Target Cells

Retroviral vectors are used here to express the chimeras and obtain cells that express the constructs.

MATERIALS

Buffers and Reagents

Dulbecco's modified Eagle medium (DMEM), high glucose formulation (450 g/liter)
Fetal bovine serum (FBS; HyClone)
Polybrene (8 mg/ml) (1000x) in H_2O, filter-sterilized (Sigma)

Vectors

FuGENE 6 (Roche Molecular Biochemicals)
> The retroviral vector should not exceed 9 kb in length to ensure efficient packaging of the vector. This limit does not include the portions of the plasmid required for plasmid propagation which are not incorporated into the retrovirus.

Plasmids and Cells

Phoenix-E cells
> For information on obtaining these cells, see Dr. Garry Nolan's Web site at http://www.stanford.edu/group/nolan/mtas.html).

Plasmids containing β-gal deletion mutants fused in frame to the protein of interest

Special Equipment

Benchtop centrifuge with microplate carriers

METHOD

1. Transfect the plasmid containing the retroviral construct into the Phoenix-E packaging cell line using FuGENE 6. (The procedure is outlined here, but it is strongly recommended that the manufacturer's instructions be followed.)

   a. Plate 1.5×10^6 to 2×10^6 Phoenix-E cells per 60-mm dish in DMEM containing 450 g/liter glucose (high glucose formulation) and 10% FBS the day before transfection (or 3×10^6 cells on the day of transfection). Cells should be 50–80% confluent at transfection.

   b. Mix the following components in a microcentrifuge tube in the order given: serum-free DMEM, 6 µl of FuGENE 6 (vortex stock tube first, then add reagent directly to the medi-

um, not to the side of the tube), 2 µg of plasmid DNA. The total volume should be 100 µl. Mix the components by gently flicking the tube; do not vortex the tube. Incubate at room temperature for 15–30 minutes. The volumes can be scaled for multiple plates receiving the same plasmid, or for different-sized plates.

   c. Add the 100-µl FuGENE–DNA mixture dropwise to the plate of Phoenix-E cells. It is not necessary to change the medium first or to use serum-free medium on the cells.

2. Incubate overnight (18–24 hours), and then refeed the cells with 2–4 ml of DME (high glucose) + 10% FBS.

3. At least 30 hours after transfection and at least 6 hours after refeeding, remove the medium (viral supernatant) from the dish and filter through a 0.45-µm syringe filter.

4. Add Polybrene to a final concentration of 8 µg/ml to the viral supernatant, or immediately freeze the viral supernatant at –80°C.

5. Aspirate medium from a subconfluent dish of target cells and replace with sufficient viral supernatant containing Polybrene to cover the surface of the dish. If the target cells cannot survive in the Phoenix-E media, dilute the Phoenix-E media 1:1 or more in target cell media.

6. *Optional:* To increase viral infection efficiency, centrifuge the plates (up to 100 mm in diameter) containing the target cells in a benchtop centrifuge equipped with microplate platforms at 2500 rpm (Beckman GS-6 centrifuge or equivalent) for 30 minutes.

   Place dishes carefully in the center of the microplate carrier and make sure that the rotor is balanced. Sealing the plate with Parafilm will help the dish "stick" to the center of the carrier.

7. *Optional:* Continue to harvest retrovirus from the producer cells every 6–12 hours up to 72 hours after transfection, and then discard them. Either use the harvested supernatant for additional rounds of infection or freeze immediately at –80°C.

8. Refeed the target cells 6 hours after adding retrovirus, or, to increase infection efficiency, add another aliquot of viral supernatant containing Polybrene as above. A third round of infection may be carried out after another 6 hours if desired.

   Although centrifugation and multiple rounds of infection can increase the infection frequency, it is not desirable to have multiple copies of the retrovirus in a cell, as this can lead to overexpression. To ensure single-copy transduction in most of the cells, an infection efficiency of 15% is desired. Thus, the viral supernatant may have to be diluted. Infection efficiency can be measured using any retroviral vector expressing β-gal, GFP, or any readily observable marker. However, many of the experiments described here were accomplished without precise titration of the virus.

9. Refeed the target cells with medium containing the appropriate antibiotic to select for infected cells 24 hours after the final round of infection. The time required for uninfected cells to die depends on the cell line used. Cells should continue to be cultured in the selective antibiotics throughout the experiment to prevent a loss of expression of the chimeric proteins.

Chemiluminescent Assay for β-Gal

This protocol is the quickest and simplest method for assaying a large number of samples.

MATERIALS

Buffers and Reagents

> Cells from Protocol 1
> Gal-Screen reagent (Tropix)
> Phosphate-buffered saline (PBS)

Special Equipment

> Microplate luminometer
> Plates (96-well) appropriate for the microplate luminometer used

METHOD

1. Plate cells in 96-well plates the day before the assay at a density of 10,000 cells per well (number established for C2C12 or C2F3 mouse myoblasts) in a volume of 100 μl in medium appropriate for the cell line used. Cells should be subconfluent at the time of the assay.

2. Treat cells with reagents appropriate to induce or modify the interaction under study. Typically, each treatment is carried out on triplicate or quadruplicate wells.

3. At the end of the treatment period, aspirate the medium from the wells and add 200 μl of Gal-Screen reagent prepared according to the manufacturer's instructions (substrate is diluted 1:25 immediately before use with Gal-Screen buffer B equilibrated to room temperature) but additionally diluted 1:1 with 1x PBS.

   Removing the medium is not recommended by the manufacturer's instructions, but this is necessary if, because of kinetic analysis, some of the wells were refed with fresh medium at different time points. Removal of the medium also leads to a slight improvement in the signal-to-noise ratio. The Gal-Screen reagent is additionally diluted 1:1 with PBS to compensate for the removal of the medium from the plate.

4. Incubate the plate for 45 minutes to 1 hour at 26–28°C. Alternatively, incubate at room temperature for a period of time sufficient for the Gal-Screen reaction to reach a plateau.

5. Read the plate using a microplate luminometer, measuring each well for 1 second or for a time appropriate for the instrument used.

6. Analyze data using appropriate software.

Flow Cytometry Assay for β-Gal

Flow cytometric assay of FdG measures β-gal activity in live cells so that a determination can be made of whether or not cloning of the cells is needed. In addition, the FdG assay is quantitative and can be used to study the kinetics and dose response of the interaction.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

Dimethylsulfoxide (Sigma) <!>
Fluorescein di-β-D-galactopyranoside (FdG, Molecular Probes) <!>
Phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS)
Propidium iodide (10 mg/ml in H_2O) (Sigma) <!>

Special Equipment

Benchtop centrifuge with adapters for holding 5-ml tubes
Flow cytometer
Pipetman P-1000
Polystyrene tubes (5-ml round-bottomed; Falcon 2058)

METHOD

1. Plate cells in appropriate medium the day before the assay in 24-well plates at a density such that the culture will be subconfluent at the time of the assay (50,000 cells per well in 0.5 ml of medium for C2C12 or C2F3 mouse myoblasts).

2. Treat cells with reagents appropriate to induce or modify the interaction under study. Typically, different treatments are carried out on duplicate or triplicate samples.

3. At the end of the treatment period, aspirate the medium from the wells and rinse once with PBS.

4. Add two drops of trypsin solution and incubate (room temperature or 37°C) for 5 minutes or until a firm tap on the side of the dish dislodges most cells.

5. Add 1 ml of PBS + 5% FBS to each well. Using a Pipetman P-1000 or equivalent, triturate the cell suspension and rinse the bottom of the well to remove all cells. Transfer cell suspension to 5-ml clear polystyrene tubes (Falcon 2058) appropriate for use on a flow cytometer.

6. Pellet cells by spinning in a benchtop centrifuge at about 1500 rpm for 5 minutes.

7. Remove the supernatant by inverting tubes (if possible, invert entire centrifuge bucket adapter assembly with the tubes still in it). While tubes are still inverted, aspirate the remaining drop of liquid from the lip of each tube.

   Beckman sells an insert that can be added to their adapter for 5-ml tubes that prevents the tubes from falling out when the adapter is inverted.

8. Add 100 μl of PBS + 5% FBS (room temperature) to each tube. Vortex to resuspend the cells (vortex the entire centrifuge bucket adapter assembly with tubes).

9. Prepare a 100× stock of substrate by adding 100 μl of dimethylsulfoxide to a 100-μg vial of FdG. Dilute appropriate amount of substrate in sterile deionized H_2O.

   After preparation, the stock can be stored for several weeks at –20°C protected from light.

10. Add 100 μl of substrate to each tube of cells (hypotonic shock). Incubate for 3 minutes at room temperature.

11. Stop the uptake of FdG by adding 2.0–2.5 ml of ice-cold PBS + 5% FBS + 1 μg/ml propidium iodide to each tube. Pellet cells at about 1500 rpm for 5 minutes.

    Propidium iodide is a fluorescent compound that accumulates in dead cells. Thus, cells with propidium iodide fluorescence can be excluded from the data analysis and sorting.

12. Remove most of the supernatant by gently inverting the tubes. If possible, invert the entire centrifuge bucket adapter assembly with tubes still in it. DO NOT shake tubes or allow liquid that clings to lip of tube to come out. Return the tubes to an upright position and vortex the entire centrifuge bucket adapter assembly with tubes. Sufficient liquid should have been retained to resuspend the cells in a volume appropriate for use on the flow cytometer (about 200 μl).

13. Place tubes on ice. Analyze the cells by flow cytometry.

X-Gal Assay for β-Gal

This assay, which is not quantitative or especially sensitive, can be used to detect β-gal complementation if the induction of complementation is sufficiently robust.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

Dimethylformamide (Sigma)
Magnesium chloride ($MgCl_2$) <!>
Paraformaldehyde (4%) <!> in PBS
Potassium ferricyanide ($K_3Fe(CN)_6$) <!>
Potassium ferrocyanide ($K_4Fe(CN)_6$) <!>
X-Gal (Sigma)

METHOD

1. Plate the cells on tissue culture dishes or on glass coverslips at subconfluent densities.

2. Treat the cells with reagents appropriate to induce or modify the interaction under study.

3. Fix the cells for 4 minutes with cold (4°C) 4% paraformaldehyde in PBS. Rinse with PBS twice for 5 minutes.

4. Prepare the substrate by diluting a stock solution of X-gal (40 mg/ml in dimethylformamide, stored at –20°C protected from light) to a final concentration of 1 mg/ml in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS.

5. Add the diluted X-gal to cells (sufficient volume to cover cells). Incubate overnight at 37°C (shorter times are sufficient for high levels of β-gal activity; monitor the reaction under a microscope).

6. Examine the dish or coverslip microscopically for blue cells.

Fluor-X-gal Assay for β-Gal

This assay determines β-gal activity in cells that were immunofluorescently labeled for other proteins.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Reagents

4′,6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) <!>
Fast Red Violet LB (Sigma)
Paraformaldehyde (4%) <!> in PBS
5-6 X-gal (Fluka)

Special Equipment

Epifluorescence microscope

METHOD

1. Plate the cells on glass coverslips at subconfluent densities.

2. Treat the cells with reagents appropriate to induce or modify the interaction under study.

3. Fix the cells for 4 minutes with cold (4°C) 4% paraformaldehyde in PBS. Rinse with PBS twice for 5 minutes.

4. If cells are to be immunofluorescently labeled with antibody as well as Fluor X-gal, carry out all immunolabeling procedures at this point at 4°C.

5. Prepare the reagent by diluting into PBS a stock solution of Fast Red Violet LB (50 mg/ml in dimethylformamide and store at –20°C. The substrate will not completely dissolve at this concentration) to a final concentration of 100 μg/ml, and a stock solution of 5-6 X-gal (50 mg/ml in dimethylformamide; store at –20°C. The solution will change from pale blue to yellow after exposure to light, but this does not appear to affect activity) to a final concentration of up to 25 μg/ml (decrease the concentration of 5-6 X-gal if β-gal activity is strong). Filter through a 0.45-μm syringe filter to remove any precipitate.

6. Add the mixture of diluted Fast Red Violet LB and 5-6 X-gal to cells (sufficient volume to cover cells). Incubate for 60–90 minutes at 37°C.

7. Rinse in PBS for 30 minutes at room temperature.

8. *Optional:* Nuclei may be stained by diluting DAPI in PBS to a final concentration of 100 ng/ml and incubating cells for 10 minutes at room temperature, followed by two rinses in PBS for 5 minutes.

9. Mount the coverslips in PBS and seal with nail polish.

10. Detect Fluor X-gal staining with either the fluorescein (FITC) or rhodamine (TRITC) filter sets of an epifluorescence microscope. The FITC channel gives a better signal-to-background ratio for weak signals, but strong signals appear to be quenched. Therefore, Fluor X-gal stain is best viewed with TRITC filters.

In some experiments, the initial population of cells expressing the chimeric constructs will have low β-gal activity in the absence of the interaction of interest and will exhibit a severalfold increase in β-gal activity when an inducer of the interaction is present. However, in other experiments, no induction or very poor induction may be observed. Low β-gal activity accompanied by a lack of induction can be caused by a failure of one of the constructs to properly express the chimeric protein. Constitutive β-gal activity can be caused by overexpression of one or both of the constructs.

No β-gal Activity Is Observed

If no β-gal activity is observed, the expression of the chimeric constructs must be confirmed. This is best accomplished by immunoblotting cell extracts using antibodies to either β-gal or the non-β-gal component of the chimera. If an immunoreactive protein of the appropriate size is observed, the chimera is being expressed. No immunoreactive band indicates a lack of expression, and a band that is too small indicates that the protein is likely terminated at the wrong residue. Such errors can often be identified by sequencing the cDNA for the chimeric protein as well. If antibodies for immunoblotting are not available for the non-β-gal component of the chimera, commercial antibodies for β-gal are available. We have had mixed success at immunoblotting β-gal chimeras expressed in mammalian cells using anti-β-gal antibodies. The best immunoblots have been obtained using a cocktail of the following antibodies: Calbiochem (La Jolla, California) anti-β-gal monoclonal antibody (OB02-100); Sigma (St. Louis, Missouri) anti-β-gal polyclonal antisera (G4644); Sigma anti-β-gal monoclonal (G6282); each was diluted 1:1000. However, if good antibodies to the non-β-gal protein are not available, we recommend adding a peptide tag to the chimeric protein.

Constitutive β-gal Activity Is Observed

Inducible interactions will ideally have low β-gal activity in the absence of the inducer or ligand. If constitutive β-gal activity is observed, several steps can be taken to reduce the uninduced levels of β-gal activity. First, it is important to avoid overexpressing the β-gal constructs. One significant advantage of this system is that the signal is enzymatically amplified and therefore the constructs do not have to be overexpressed (Blakely et al. 2000). Overexpression can result from selection at excessively high concentrations of drug. For example, we have observed higher levels of expression in C2C12 cells selected at 1 mg/ml of G418 or hygromycin rather than at 300 μg/ml, which is the minimum amount of drug required for selection in this cell line. The use of retroviral vectors limits the number of copies of the construct to a few per cell, which can also help to avoid overexpression. To ensure that most cells receive only a single copy, it is necessary to titrate the virus so that only 15% of cells are infected, according to the Poisson distribution.

In cases where there is a high level of uninduced β-gal activity or poor induction (less than a severalfold increase) of β-gal activity, it is helpful to examine the population of cells on a flow cytometer by assaying for β-gal activity using the fluorogenic substrate FdG (Blakely et al. 2000). If uninduced β-gal activity in the population is not uniform (both high- and low-activity cells are observed), a subpopulation or clones of inducible cells can be isolated that have low background

activity. However, steps must be taken to ensure that selection for low β-gal activity does not also select for cells that cannot be induced (see below). Alternatively, the constructs should be reintroduced into cells, taking steps to reduce copy number and expression as described above.

Poor Induction of β-gal Activity Is Observed

When induction fails to occur, flow cytometry is again useful to characterize the population. Low induction observed in a mass assay may be due to only a small part of the population responding. The FACS can be used to select for cells that have a high induced β-gal activity, but this criterion alone may result in cells that have constitutive β-gal activity. Ideally, cells will be sorted sequentially for a subpopulation or clones that have low β-gal activity in the absence of inducer, and higher β-gal activity in the presence of inducer (Blakely et al. 2000). Although a FACS simplifies this procedure, such selection is also possible simply by plating a large number of clones and then screening the clones for inducible β-gal activity.

Constitutive β-gal Activity Is Observed with Membrane Proteins

In our experience, constitutive β-gal activity is more likely to occur when the chimeric proteins are both membrane proteins. We suspect that this is because the membrane proteins, limited to the two-dimensional space of the membrane, have a higher effective concentration of complementation partners at a given level of expression than cytoplasmic proteins. Fortunately, membrane proteins offer an opportunity for another level of selection, because antibodies to the extracellular domain of the membrane protein of interest or to an extracellular domain tag can be used to select for chimera expression in live cells using the FACS (Blakely et al. 2000). Thus, instead of selecting only for low β-gal expression in the absence of inducer (ligand), which may also select for cells that fail to express the chimera, these cells can be simultaneously selected for low β-gal expression using fluorescein fluorescence and modest expression of the chimera using an antibody to the protein of interest and a secondary antibody that fluoresces at a different wavelength. We have also observed that high uninduced β-gal activity can be a problem when using a full-length receptor with a large cytoplasmic domain. In some cases, truncating the receptor so that the β-gal mutant is closer to the transmembrane domain increases the fold induction of β-gal activity primarily by reducing the background level of uninduced β-gal activity. In the case of some receptors, such as the EGF receptor, this has the added benefit of blocking ligand-induced internalization of the receptor (Blakely et al. 2000).

Continued Lack of β-gal Activity after Troubleshooting

In some cases, a lack of β-gal activity or constitutive β-gal activity may be difficult to correct. Some interactions will inevitably be inhibited by the attachment of the interacting proteins to the β-gal deletion mutant. In other cases, the interaction may occur, but the β-gal mutants may not be oriented in a manner that permits complementation. Increasing the distance between the interacting protein and the β-gal mutant using peptide linkers may help. For cytoplasmic proteins, moving the β-gal mutant from one end of the protein to the other may also improve complementation. Although a large majority of the interactions that we have tested work with this system, there is no definite way of predicting which ones will work well. However, given two proteins known to interact, it should be possible to generate a range of chimeric proteins in vitro and select those that display the best characteristics.

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