Protocol

Regulating Protein Stability in Mammalian Cells Using Small Molecules
Emily L. Hagan, Laura A. Banaszynski, Ling-chun Chen, Lystranne A. Maynard-Smith, and Thomas J. Wandless

Department of Chemical and Systems Biology, Stanford University, Stanford, CA 94305, USA

Corresponding author (wandless@stanford.edu)

INTRODUCTION

Regulating protein stability using small molecules provides a rapid, reversible, and tunable method to study a protein of interest’s (POI) role in cells. We recently designed a small protein domain based on the 12-kDa FKBP (FK506 binding protein) that can be fused at either the carboxyl or amino terminus of a protein of interest. This destabilization domain (DD) confers instability to fusion protein partners. The method described here explains how to use a DD fusion to control the biological activity of a POI. In the absence of a small molecule ligand, the DD is unstable and directs the fusion protein for degradation. Addition of the ligand stabilizes the DD, allowing the fusion protein to accumulate in cells and the POI to exert its biological effect. The ligand is specific for the DD and has no detectable off-target effects. By utilizing the specificity of genetic fusion and the speed of small molecule binding, this technique provides an alternative to RNA interference to study a POI’s role in cells.

RELATED INFORMATION

A schematic representation of the theory underlying this procedure is presented in Figure 1. This protocol can be used in conjunction with A General Method for Conditional Regulation of Protein Stability in Living Animals (Sellmyer et al. 2009). A number of commercially available systems (ProteoTuner, Clontech) include the Shield-1 ligand and DD vectors in plasmid, retroviral, and lentiviral formats, and allow for selection by antibiotic resistance and/or fluorescence. Shield-1 can also be purchased through Cheminpharma.

Figure 1. Genetic fusion of the DD to a POI confers instability to the entire fusion product, resulting in protein degradation. Addition of a small molecule ligand (e.g., Shield-1) rescues the fusion and allows the POI to exert its normal biological effect.
MATERIALS

Reagents

Antibiotic (e.g., G418, puromycin) (optional; see Step 13.i)

Cell line (e.g., NIH/3T3 or other cell line for infection; ATCC)

Ethanol

Hexadimethrine bromide (Polybrene, 4 mg/mL in PBS; Sigma)

Lipofectamine 2000 (Invitrogen)

Medium for 3T3 cells

Medium for Phoenix cells

Opti-MEM I reduced-serum medium (GIBCO)

Phoenix ecotropic packaging cell line (Orbigen)

An amphotropic packaging cell line is also available for infecting nonmurine cells.

Phosphate-buffered saline (1X; PBS)

Plasmid, retroviral (e.g., pBMN)

Plasmids, FKBP-12 mutants (F36V and F36V L106P or F36V E31G R71G K105E)

QIAprep Spin Miniprep kit (QIAGEN)

Shield-1 (1 mM, prepared in 100% ethanol; Clontech or Cheminpharma)

Equipment

Biosafety cabinet, Class II

Dishes, tissue culture, 10-cm (Falcon)

Flow cytometer (optional; see Steps 13.ii and 16)

Incubator, humidified, equilibrated with 5% CO₂, preset to 37°C

Protein blotting equipment (optional; see Step 16)

Syringe filters, nylon, sterile, 25-mm, 0.45-μm pore size (Fisher)
METHOD

Construct Cloning

This technology works with transient transfections of viral and nonviral plasmids but requires careful titration of DNA into cells to achieve the proper tunability. Therefore, stable integration of the construct is recommended for cleaner expression of fusion and maintenance of construct.

1. Use standard molecular biology techniques to clone a fusion of the DD and the protein of interest into a retroviral plasmid. Use F36V as a stable control, and either L106P or E31G R71G K105E as the unstable DD:

<table>
<thead>
<tr>
<th>FKBP-12 mutation(s)</th>
<th>Stability</th>
<th>Use at</th>
</tr>
</thead>
<tbody>
<tr>
<td>F36V</td>
<td>Stable (Control)</td>
<td>Amino and carboxyl termini of POI</td>
</tr>
<tr>
<td>F36V L106P</td>
<td>Unstable</td>
<td>Amino and carboxyl termini of POI</td>
</tr>
<tr>
<td>F36V E31G R71G K105E</td>
<td>Unstable</td>
<td>Carboxyl terminus of POI</td>
</tr>
</tbody>
</table>

The order of fusion (i.e., amino-/carboxy-terminal DD) and presence or absence of a linker will be dictated by folding constraints of the POI.Appending an epitope tag such as HA or FLAG after the POI is often useful, especially when specific antibodies for the protein of interest are not available. In order to later select for cells containing the fusion, it is best to include a marker for infection (e.g., drug-resistance or a fluorescent protein) behind an internal ribosome entry site.

2. Use the QIAprep Spin Miniprep kit to prepare at least 10 μg DNA of the sequence-verified plasmid.

Viral Particle Packaging

3. On the evening of the first day, plate 2 x 10^6 Phoenix cells into a 10-cm dish for each construct. Culture the cells in medium for Phoenix cells at 37°C. This should be sufficient to produce cells at ~80% confluency at the time of transfection.

4. The following morning, prepare DNA/lipid complexes (10 μg DNA with 25 μL Lipofectamine 2000 in 3 mL Opti-MEM) according to the manufacturer’s instructions.

5. Transfect the DNA/lipid mix into Phoenix cells. Incubate for 4 h at 37°C.

6. After 4 h, replace the Opti-MEM medium with 10 mL medium for Phoenix cells. Return the plates to the incubator.

7. The following evening (i.e., Day 3), plate 1 x 10^6 NIH/3T3 cells per 10-cm plate to be infected. For other cell lines, plate at a cell density such that the cells are ~30%-40% confluent at the time of infection.

8. Forty-eight hours after transfection (i.e., Day 4), harvest the viral particles by filtering the medium
from the Phoenix cells through a 0.45-µm syringe filter.

9. Add polybrene to a final concentration of 4 µg/mL to the viral particle suspension.

10. Proceed immediately to creation of the cell line (Step 11). Alternatively, the viral supernatant can be frozen and stored at -80°C. However, freezing viral supernatants reduces the viral titer by ~50%.

**Cell Line Creation**

11. Aspirate the medium from the NIH/3T3 cells. Add 3 mL of filtered viral supernatant to each dish. The remaining supernatant can be frozen at -80°C for later use.

12. Four hours after infection, replace the viral medium with 10 mL of medium for 3T3 cells.

13. Forty-eight hours after infection, select cells with stable integration of the DD-POI construct: Typically, experiments can be conducted with heterogeneous sorted populations. If clonal populations are desired, screen several clones to account for variation in transgene expression. Comparing clones can be especially useful for POIs with very low functional thresholds, such as transcription factors. See Troubleshooting.

   For drug selection:
   i. Grow cells under selection for at least 7 d.

   For flow cytometric selection using a fluorescent marker:
   ii. Sort a number of cells sufficient for use in downstream experiments.

**Regulating Protein Stability in Mammalian Cells**

14. To test for ligand-dependent control of protein stability, plate equal numbers of DD-POI cell lines in two separate dishes.

15. Add Shield-1 to a final concentration of 1 µM to one plate, and an equal volume of ethanol to the other plate.

16. Twenty-four hours later, assay the protein stability by flow cytometry or protein blotting. A 24-h incubation period and 1 µM Shield-1 is usually sufficient to provide steady-state levels of protein stability. However, these parameters should be optimized empirically depending on the cell type and POI. See Troubleshooting.

17. To more fully characterize the DD-POI fusion, perform a dose-response curve with Shield-1 (1 nM to 3 µM):

   i. Measure the half maximal effective concentration (EC$_{50}$) and maximum stabilization.

   ii. Assay stabilization kinetics with growth and decay time courses after addition and removal of drug, respectively. *Dose cells with Shield-1 simply by adding the drug to the medium. Remove Shield-1 by*
washing the cells three times with fresh medium. For additional information on kinetics experiments, see Banaszynski et al. (2006).

TROUBLESHOOTING

Problem: Low infection is observed when creating cell lines.

[Step 13]

Solution:

1. Verify that polybrene was added to the viral supernatant before infection. Polybrene is a cationic lipid that helps diminish the electrostatic repulsion of the viral particle with the cell surface, thus aiding binding.

2. Try concentrating the viral supernatant with a 100,000 MWCO Amicon spin concentrator before adding polybrene. Concentrating the sample from 10 mL to 3 mL increases the viral titer.

3. Cleaning up and concentrating the vector DNA with a Midi- or Maxi-Prep can increase the efficiency of the lipofection (Step 5). Ideally, the DNA concentration should be at least 800 ng/µL.

Problem: No signal for drug-dependent stabilization of the DD-POI fusion is detected by protein blotting.

[Step 16]

Solution: The POI might not tolerate a fusion on that terminus of the protein. Try changing the fusion protein to place the DD at the other terminus of the POI. A linker is not usually necessary, but could help the POI fold properly. Using the F36V domain as a stable control can help identify if the DD-POI fusion is properly expressed.

Problem: The fusion protein is not destabilized.

[Step 16]

Solution: Moving the DD to the other terminus of the POI can change fusion protein stability. However, this technique has not been validated for every single protein; it is possible some proteins are not amenable to the DD system. Note that E31G R71G K105E is a more effective destabilizer than L106P at the C terminus of proteins.

DISCUSSION

Modulating a protein of interest’s stability with a DD provides the user with unparalleled control of protein function. This technique is faster than RNA interference, reversible and tunable (unlike the Cre/lox system), and more specific than most chemical perturbants (Banaszynski et al. 2006; Maynard-Smith et al. 2007). Notably, addition of 1 µM Shield-1 has no detectable off-target effects, as judged by microarray (Maynard-Smith et al. 2007). To date, this system has successfully regulated examples of intracellular, nuclear, and secreted proteins (Banaszynski et al. 2008). However, it is unlikely that this technology will
work for every protein in a cell. The POI might lose its functionality when fused, especially if its active state is part of a higher-order multimer. The DD placement, however, is very flexible. DDs have been shown to work at both termini, as well as in an internal loop of the POI (Chu et al. 2008). It should be noted that a large intracellular pool of unmodified POI can overwhelm the DD-POI effect and obscure a phenotype for ligand-dependent control of protein stability. For this reason, targeting the regulation of monomeric proteins with low endogenous levels or with a dominant phenotype is recommended.

REFERENCES


Recipe

**Medium for 3T3 cells**

Donor bovine serum, heat-inactivated (10%; GIBCO)

Dulbecco’s modified Eagle medium, high-glucose (1X; GIBCO 11965)

Glutamine (2 mM; GIBCO)

Penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively; GIBCO)

Recipe

**Medium for Phoenix cells**

Dulbecco’s modified Eagle medium, high-glucose (1X; GIBCO 11965)

Fetal bovine serum, heat-inactivated (10%; GIBCO)

Glutamine (2 mM; GIBCO)
Penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; GIBCO)

Recipe

Phosphate-buffered saline (PBS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1X solution)</th>
<th>Final concentration (1X)</th>
<th>Amount to add (for 10X stock)</th>
<th>Final concentration (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
<td>137 mM</td>
<td>80 g</td>
<td>1.37 M</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
<td>2.7 mM</td>
<td>2 g</td>
<td>27 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44 g</td>
<td>10 mM</td>
<td>14.4 g</td>
<td>100 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24 g</td>
<td>1.8 mM</td>
<td>2.4 g</td>
<td>18 mM</td>
</tr>
</tbody>
</table>

If necessary, PBS may be supplemented with the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1X solution)</th>
<th>Final concentration (1X)</th>
<th>Amount to add (for 10X stock)</th>
<th>Final concentration (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂•2H₂O</td>
<td>0.133 g</td>
<td>1 mM</td>
<td>1.33 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂•6H₂O</td>
<td>0.10 g</td>
<td>0.5 mM</td>
<td>1.0 g</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

PBS can be made as a 1X solution or as a 10X stock. To prepare 1 L of either 1X or 10X PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.