[21] Fusion-Based Strategies to Identify Genes Involved in Degradation of a Specific Substrate

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Abstract

Fusion proteins have been used in many instances to allow genetic screening for genes required for the degradation of a specific substrate. This straightforward, yet powerful, approach can be applied in many circumstances to facilitate gene characterization and discovery. Some general principles are discussed and then several successful uses of these tactics are described in detail.

General Principles of Using Fusion Reporters to Discover Degradation Genes

Because protein degradation is highly processive and evolved to destroy a wide variety of proteins, the addition of fusion partners will often allow normal degradation of the resulting protein. A fusion gene added to a degradation substrate can render an otherwise tedious or infeasible genetic screen facile and practical. In this chapter, some general principles for using this technique will be discussed, and then several detailed examples will be described. The examples are all from studies using yeast. It is hoped that the combination of general concepts and detailed examples will allow the largest number of applications.

Reporter Design: Pathway Fidelity and Degrons

There can be many ways for a protein in a particular compartment to undergo ubiquitin-mediated degradation, and altering a protein by fusion addition could send it down a distinct pathway from the one designated for study. It is important to confirm that the engineered reporter fusion undergoes degradation by the pathway of interest. If some mutants deficient in the degradation pathway already exist, testing the behavior of the reporter protein in those mutants will confirm the fidelity of the fusion. If no mutants are available, characterization of the fusion's fidelity by other means can be useful. In studying the degradation of yeast Hmg2p isozyme of HMG-CoA reductase (HMGR), a GFP fusion reporter was tested both for stabilization by mutants and normal physiological regulation of HMGR stability.

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Sometimes loss of degradation pathway information can be desirable, if the resulting fusion simplifies a genetic analysis. This is the case for the ground-breaking studies of α2 repressor protein degradation (Matα2) in yeast. This very rapidly degraded protein undergoes ubiquitination by two distinct pathways, one using the E2s Ubc6/Ubc7 and the E3 ligase Doa10p, and the other by Ubc4/Ubc5 and a still-unknown ligase (Chen *et al*., 1993). Entry into these two degradation pathways is mediated by separate portions of the protein, called ''degrons,'' with the N-terminal 67 amino acids, called ''deg1,'' mediating only the Ubc6/7 branch (Hochstrasser and Varshavsky, 1990). The genetic analysis of Matα2 degradation was facilitated by the use of only the deg1 portion of the entire Matα2 protein fused to reporters, because this tactic effectively isolated that single branch of degradation for study.

The degron concept is useful in the design of reporter fusions. A degron is defined as a discrete, transferable region of a protein that is necessary and sufficient for the ubiquitin-mediated degradation of the protein in which it resides (e.g., Dohmen *et al*. [1994]). deg1 above is an example of this idea. However, discrete degrons do not always mediate selective protein degradation. There are also cases in which the information for pathway entry involves a large portion of a protein, including sequence or structural elements that are far removed from each other. The entire multi-spanning N-terminal domain (approximately half the protein) of Hmg2p is required for its regulated degradation by the HRD pathway (Gardner and Hampton, 1999; see later). Degradation reporters for Hmg2p must include this complete N-terminal domain (Cronin and Hampton, 1999). When a protein of interest has other activities, it is also useful to know whether these must be included in the reporter. For example, Hmg2p has a discrete C-terminal catalytic domain that is responsible for an essential step of the sterol pathway but not required for regulated degradation. Thus, another decision that goes into making reporters for Hmg2p is whether to leave the C-terminal catalytic region intact or to replace the C-terminal catalytic region with the reporter. In general, an understanding of the protein regions that are and are not needed for physiologically relevant degradation is an important aid in designing the most effective reporter fusions.

Mode and Stability of Expression

Most genetic screens for alterations in degradation score alterations in protein level. For the simple case of a protein that is synthesized at a rate that is independent of the protein's levels, alterations in the protein degradation rate will cause the same proportional change in the steady-state

level. Thus, the difference between the mutant and wild-type steady-state concentration of a reporter will often be proportional to the change in degradation rates for the two cases. If variability in the expression of the reporter is on the order of the difference caused by the desired mutations, there will be more difficulty in finding the desired mutants among false positives that arise because of expression level variation. For example, in yeast, expression plasmids can be either of one of two types of autonomously replicating plasmids, called ARS/CEN or 2 micron (YCp or YEp, respectively), or they can be integrating plasmids (YIp) that must be incorporated into the yeast genome to be replicated (Botstein and Fink, 1988). Autonomously replicating plasmids can vary in copy number over severalfold, whereas an integrating plasmid has a copy number that is preserved because of its presence as a true part of the chromosomal genome. Although our experience is in yeast, the principle of limiting changes in expression that could enhance false positives is applicable to any biological system. In mammalian cell lines, transfected reporters are often present in multiple arrays. Alterations in expression because of changes in reporter gene copy number, unequal recombination, or gene amplification can result in inheritable alterations in steady-state level that are not due to changes in degradation. In general, it is desirable to use the most stable source of expression possible, especially if the difference between the stable and degraded phenotypes is in the range of only fourfold to fivefold.

Another choice in the design of reporter fusions is the choice of promoter to drive the expression of the heterologous gene. Constitutive promoters that are not subject to regulation by signaling pathways limit the number of undesired ways that the steady-state levels can be affected in a screen. In truth, no promoter is completely free from communication with the cell, but those that drive always-needed housekeeping genes can limit the number of spurious phenotypes in a genetic analysis. We have had much success with the yeast GAPDH promoter (TDH3) (Schena *et al.*, 1991). The strength of the promoter used to drive the reporter fusion can also affect some studies, because there are degradation pathways that can be overwhelmed by overly strong production of a pathway substrate (e.g., HMG-CoA reductase [HMGR] in mammals; Sever *et al.* [2003]). Again, the best strategy is to test the behavior of an engineered reporter plasmid before applying it to full-blown screen.

Phenotypes of Degradation

When using a fusion protein to evaluate degradation, the degradation phenotype must be amenable with the throughput demanded by the genetics. Either the steady-state level of the protein is used as a gauge, or the

degradation of the fusion is directly determined. Direct assays of degradation are more difficult to use in screening, but such approaches have been employed successfully. This method of scoring includes subjecting samples from a master plate to conditions in which protein synthesis is curtailed and subsequent analysis of the levels of the degradation substrate under study after a degradation period (e.g., Knop *et al.* [1996]).

Once a screening phenotype has been decided, it is a good idea to test the screen for its ability to detect desired mutants. This is best done by using previously available mutants with the new assay/screen. With the ubiquitin proteasome pathway, one can often use more downstream mutants, such as one of many proteasome alleles (e.g., cim3–1, Hiller *et al.* [1996]; hrd2-1/rpn1, Hampton *et al.* [1996a]), which are still viable but cause a general stabilization of many ubiquitinated substrates. Alternately, proteasome inhibitors such as lactacystin or MG132 can be used to test the involvement of the pathway. One can also use mutant versions of the reporter protein that are resistant to the degradation pathway under study. For example, sometimes changing key amino acids can stabilize a protein under study (Gardner and Hampton, 1999). Creating the analogous fusion reporter with one or several stabilizing mutations provided a reliable alternative to test the feasibility of a screening strategy. We have used such ''in cis phenocopies'' in a variety of approaches, although these tools are luxuries of previous detailed analyses of a substrate under study. Alternately, if a reporter undergoes physiological regulation of its degradation, the stabilizing conditions can be used to determine whether a screen can locate degradation-deficient candidates. For example, plating yeast cells expressing the regulated HMGR reporter Hmg2p-GFP on a small dose of lovastatin causes physiological stabilization that can be scored by an optical colony screen for GFP (see later) (Cronin *et al.*, 2000).

Eliminating Fusion Reporter Mutants in a Screen

Sometimes mutations in the reporter protein that arise in the screen can phenocopy the desired mutants. When possible, the best strategy is to test for plasmid independence of the degradation phenotype. In yeast screens, each candidate mutant is cured of the original reporter-expressing plasmid, retransformed with a fresh sample of the same plasmid, and then scored for the desired degradation phenotype. In cases in which the plasmid cannot be removed easily, such as in larger eukaryotic cell lines, or when the reporter gene is replacing the endogenous allele without flanking homologous DNA, the independence of the degradation phenotype can be rescored by adding a different reporter of the same protein that can be detected while being coexpressed with the original reporter. Alternately

when feasible, the native protein from which the reporter is derived can be evaluated if it is also coexpressed in the candidate mutants, using a method that allows unambiguous detection of this second substrate.

A variation of this idea is to use two distinct reporter proteins co-expressed in the parent strain or line. The screen would entail scoring separate phenotypes for each reporter; candidates will show both mutant phenotypes. The likelihood of a *cis* mutation in each independently expressed reporter protein causing the desired phenotype is much lower, thus biasing the screen toward the desired genomic mutations. Use of this approach is detailed later in the COD screen, but the idea is generally applicable if independent reporters can be developed.

Sources of Mutants

Along with the classical (and still very useful) randomly mutagenized cells, complete collections of viable null mutations are now available for *Saccharomyces cerevisiae* and undoubtedly other organism collections are on the way. Screening fusions for degradation phenotypes with these organized collections is a nice complement to traditional screening for mutants and has the further advantage of immediate identification of involved genes. The principal hurdle in this sort of analysis is to generate a strain or line of the null array with the desired reporter. In yeast, some creative applications of robotic mating and mating-type–specific selection have been applied to automate this task (Tong *et al*., 2001).

Directed Genetic Screens

Available DNA genomic libraries, cDNA expression libraries, or more recently siRNA libraries for use in *C. elegans* (Kamath *et al*., 2003), *Drosophila* S2 cells (Kiger *et al*., 2003), or mammals (Paddison *et al*., 2004), can also by analyzed with these approaches. In the case of protein-encoding DNA, candidates would be proteins that alter the degradation pathway by being overabundant, either as the native protein or some alteration or truncation that exists in the library. In the case of siRNA-expressing libraries, the phenotypically interesting candidate is presumed to encode a gene required for the wild-type phenotype, such that lowering its levels by siRNA will cause the alteration in degradation that is being screened for. One example of this approach, the high copy screen with a yeast 2micron library, is given in detail, but the principle is the same for many variations of this idea. In all cases, the fusion reporter-expressing wild-type strain is produced by transforming the reporter plasmid into the desired cell line or organism, and the resulting reporter strain is screened with the library to evaluate degradation effects.

Detailed Examples of Screens and Selections

The following examples are from the Hochstrasser laboratory (Yale University, New Haven, CT) or our own work. They are all yeast screens; together they include most of the ideas stated in the preceding general considerations. Standard yeast techniques can be found in a previous volume of this series (Guthrie *et al.*, 2004) or other sources (Burke *et al.*, 2000), including the many-linked *Saccharomyces* Genome Database (www.yeastgenome.org).

The DOA Pathway: Screens and Selections with Reporter Enzymes

The rapidly degraded yeast alpha two repressor (Matα2) is involved in the control of mating-type specific genes in yeast. A yeast genetic analysis of Matα2 degradation was launched early in the studies of this substrate; the resulting genes are collectively known as DOA (*D*egradation *O*f *A*lpha2) (Hochstrasser and Varshavsky, 1990). Degradation of this protein is complex, being mediated by separate regions at the N- and C-terminus. The N-terminal degron is known as deg1 (aa 1–67). deg1 is necessary and sufficient for Ubc6/Ubc7-dependent degradation mediated by the Doa10p ubiquitin ligase (Chen *et al.*, 1993; Swanson *et al.*, 2001). As mentioned previously, use of only deg1 as the fusion partner isolates only this branch of Matα2 degradation for analysis.

The First DOA Screen: deg1-lacZ

The reporter fusion called deg1-lacZ has the first 67 amino acids of α2 fused to the entire β-galactosidase protein and is expressed by inclusion of the natural Matα2 promoter in the plasmid. The fusion has enzyme activity and a very short half-life imparted by the presence of the deg1 degron. The yeast vector used was the low-copy ARS/CEN plasmid YCp50, which is selected in yeast with the *URA3* gene on the vector. Although this plasmid can have between one and four stable copies in yeast, this variability did not have an impact on the success of the screen. The wild-type parent strain included a *ura3-52* mutation, allowing for continued selection for the deg1-lacZ reporter plasmid by growth on uracil-minus medium. The strain also was of the alpha mating type to allow expression of the reporter fusion from the Matα2 promoter included in the expression plasmid. This parental reporter strain harbored very low levels of β-galactosidase because of the continued deg1-dependent degradation of the reporter plasmid.

To find *doa* mutants, colonies deficient for deg1-lacZ degradation were screened for increased levels of β-galactosidase (Hochstrasser and

Varshavsky, 1990). Wild-type cells were mutagenized with ethyl methane sulfonate to ~20% survival. A typical procedure involves growing $\sim 10^8$ cells to stationary phase in YPD medium, washing the cells by centrifugation, and resuspending in 1–2 ml 0.1 *M* sodium phosphate buffer (pH 7). Fifty microliters of EMS is added, and the cells are incubated at 30° for a time calibrated to give the desired level of killing (usually an hour). Cells are resuspended and washed twice with 200 μl 5% sodium thiosulphate ($Na_2S_2O_3$, which neutralizes the EMS) and then stored refrigerated in sodium phosphate buffer. Some investigators let cells divide once or twice in liquid so that mutagenized S/G2/M cells do not give rise to chimeric colonies composed of wt and mutant cells. (*Caution*: EMS is a powerful mutagen; all equipment that contacts EMS should be thiosulfate treated). Mutagenized cells were then plated onto uracil-deficient agar plates containing the chromogenic substrate X-gal, which is hydrolyzed to a blue product by β-galactosidase. The X-gal plates have a final concentration of X-gal of 80 μg/ml, and the medium is phosphate buffered at pH 7.0 as required for the indicator (7 g Na_2HPO_6, 3 gNaH_2PO_4 in 100 ml, pH 7, per final liter of medium). The X-gal is added after the sterilized agar is allowed to cool to ~50° because of its thermolability. Many companies that sell two-hybrid interaction systems (like Clonetech MATCHMAKER) have detailed descriptions of media formulations and use, as do many investigator's, web sites (e.g., Dr. Russel Finely (Wayne St. University, Detroit MI) (http://proteome.wayne.edu/Interactiontrap.html).

Colonies were plated to a density that allows visual scoring, approximately 200–300 per plate, ~40,000 colonies total. After 3–5 days, the plates were evaluated for colonies with elevated blue color. The candidate mutants were then isolated, colony purified on selective medium, and individually tested for a true-breeding phenotype. Next, candidates were subjected to individual pulse-chase analysis to ascertain whether the heightened steady-state levels of β-galactosidase activity were, indeed, due to slow degradation of the reporter. The candidates were then analyzed using yeast classical and molecular genetics.

A DOA Selection: Use of an Enzyme Reporter to Complement an Auxotrophy

When deg1 is fused to the *URA3* gene product Ura3p, the resulting enzyme is rapidly degraded in a *DOA* gene–dependent manner. Consequently, wild-type cells that express this reporter have very low levels of the deg1-Ura3p protein, whereas doa mutants have elevated levels of the fusion gene. The difference in levels of the degraded and stabilized report was sufficient to use complementation of uracil auxotrophy as a doa

phenotype. That, is, wild-type *ura3-52* cells expressing the deg1-Ura3p fusion cannot grow on uracil-deficient medium, whereas doa mutants can (Chen *et al*., 1993). Thus, plating a large number of the parent strain on uracil-deficient medium imposed a selection for doa mutants, allowing a second independent approach for discovering DOA genes. In fact, the selection revealed the ubiquitin ligase, Doa10p, which is responsible for ubiquitination of deg1-bearing proteins (Swanson *et al*., 2001).

The DOA uracil auxotrophy selection was performed using wild-type Ura-cells (with the non-reverting *ura3-52* mutation) harboring an ARS/CEN plasmid expressing the deg1-URA3 fusion gene. This expression plasmid was marked with *LEU2*, thus allowing selection for the plasmid that is independent of the uracil auxotrophy needed to distinguish mutant from wild type. Cells were mutagenized with EMS at a level of ~70% killing. A total of 3×10^6 mutagenized cells were plated on 80 uracil-deficient agar plates and incubated at 30°. The first 960 colonies to appear on the selection plates were collected in 96-well dishes and retested for maintained uracil prototrophy. The candidates were tested for complementation group by mating with a number of previously known doa mutants. This is done by replica plating candidate mutants onto a lawn of test nulls of opposite mating time, with auxotrophies such that only the diploids will grow on the replica-plated medium. The resulting diploids were tested for growth on uracil-minus medium by streaking on separate plates. More than 600 of the candidates were alleles of the previously known *DOA2* (*UBC6*), one of the ubiquitin E2s involved in the DOA pathway. More than 300 others were alleles of *DOA10* that encodes the ligase.

In comparing the two DOA isolation strategies, the deg1-lacZ method is a screen, in which every colony is queried for the desired phenotype (blue color in this case), and the deg1-Ura3p is a selection, in which only the mutant candidates were allowed to grow. Both have strengths and weaknesses. Selections allow use of far more individual genomes (in this case more than 10^6 cells), whereas screens typically allow analysis of tens of thousands of colonies. In the DOA uracil auxotrophic selection, the authors isolated two very rare mutants (one each in almost 1000 candidates) that would not have turned up in the lacZ screen. However, screens have the advantage that poorly growing colonies are included in the scoring, whereas in a selection there is a greater chance that these could be missed.

The HRD Pathway: Use of Optical Proteins for Genetics

HMG-CoA reductase (HMGR) is an essential early enzyme of the sterol synthetic pathway. This ER-anchored essential enzyme undergoes regulated, ubiquitin-mediated degradation (Hampton, 2002). In yeast, the

Hmg2p isozyme is subjected to this regulation. When sterol pathway activity is high, the Hmg2p protein is degraded rapidly. When sterol pathway activity is slowed, as when the cells are treated with the HMGR-inhibitor lovastatin, Hmg2p degradation is slowed (Hampton and Rine, 1994). The first yeast genetic analysis of the pathway revealed that this process was ubiquitin-mediated, because the *HRD2* gene encodes a proteasome subunit, and *HRD1* encodes an ER-membrane bound ubiquitin ligase that recognizes Hmg2p and numerous other substrates (Hampton, 2002). The first *HRD* genes were isolated using lovastatin killing to select for cells with elevated levels of Hmg2p because of slow degradation (with some modifications to allow the selection to work with an acceptable background). Subsequently, the availability of GFP allowed construction of an Hmg2p-GFP reporter fusion, in which the catalytic C-terminal domain of Hmg2p was replaced with GFP (Hampton *et al*., 1996b). The resulting protein consisted of the large N-terminal multispanning membrane domain (525 amino acids), which is required for both ER localization and regulated HRD-dependent degradation, followed by the GFP reporter. The cloning and properties of this fusion reporter, and its use in the study of degradation, has been extensively described in an earlier volume of this series (Cronin and Hampton, 1999). In all cases, Hmg2p-GFP is expressed in cells that also have active HMGR, because this is an essential enzyme, and the optical reporter has no catalytic domain. Hmg2p-GFP has been used in a variety of genetic screens, two of which will be described below.

Regulation of the HRD Pathway: Isolation of *COD1* by Two-Protein Screening

Hmg2p and Hmg2p-GFP undergo regulated degradation in yeast. When the sterol pathway is slowed with HMGR inhibitor lovastatin, the signals for degradation decrease, and the Hmg2p-GFP reporter protein is stabilized. Hmg2-GFP was used to find *cod* mutants (*CO*trol of hmgr *D*egradation) that continue to degrade Hmg2p-GFP even when the signals for degradation were low (Cronin *et al*., 2000). Specifically, the desired mutants remain dark when plated on a low dose of lovastatin that normally causes stabilization of the fluorescent reporter and brightening of the cells. Because many uninteresting mutations could make the cells dark (poor expression of Hmg2p-GFP, poor permeability to lovastatin, increased metabolism of the drug, mutations in the *HMG2-GFP* reporter itself), we included another reporter protein, a functional copy of the Hmg2p enzyme with a myc tag (1myc-Hmg2p) expressed from an integrated plasmid at a locus distinct from the optical reporter protein. Although this is not technically a fusion protein, its use as a second reporter in the same cells as a

built-in secondary screen is instructive and can be applied to many other circumstances in which plasmid-based reporters with distinct phenotypes are available.

We scored for poor regulation of the catalytically active 1myc-Hmg2p by toxicity of lovastatin, seen at much higher doses than those used to test the regulation of Hmg2p-GFP. As the sole active HMGR in the cells, 1myc-Hmg2p activity is essential for cell growth, so at sufficiently high doses of lovastatin, the cells will die. In cells in which lovastatin induces stabilization of Hmg2p, the elevated levels of the Hmg2p blunt the toxicity of the lovastatin, shifting the killing curve of lovastatin to the right compared with strains that cannot slow the degradation of Hmg2p. In other words, more lovastatin is required to kill cells that can stabilize 1myc-Hmg2p (wild-type) than needed to kill cells that cannot stabilize 1myc-Hmg2p (cod mutants). We confirmed this idea by using engineered variants of Hmg2p with sequence changes that removed lovastatin-induced stabilization. The increase in sensitivity that accompanies loss of regulation is about threefold to fourfold, so that 200 μg/ml lovastatin on agar plates will kill the *cod* mutants but not the wild-type cells, thus providing an independent phenotype for poor Hmg2p regulation. Because the dose used to kill the cells is much higher than the concentration that first causes the regulatory response (12.5 μg/ml), the use of low-dose lovastatin to first score the optical phenotype of the coexpressed Hmg2p-GFP does not affect the growth of the cod mutants.

The Two-Gene COD Screen

The wild-type parent strain for the cod screen coexpressed Hmg2p-GFP and 1myc-Hmg2p from separate integrating plasmids with the TDH3 promoter. The 1myc-Hmg2p expression plasmid was integrated at the native locus and was maintained in cells by virtue of its being the only form of the essential HMGR activity in the cells. The Hmg2p-GFP expression vector was integrated at the *ura3-52* locus and was maintained by complementation of uracil prototrophy. We confirmed that each reporter underwent HRD-dependent regulated degradation when the two were coexpressed before launching the screen. This can be important, because there are cases in which degradation pathways can be saturated by an overabundance of a specific substrate.

The COD screen was performed by plating EMS mutagenized cells ($\sim$30% survival; see earlier) onto agar plates supplemented with the appropriate nutrients and 12.5 μg/ml lovastatin to a colony density of approximately 250 colonies per plate. After 2–3 days of growth, the plates were examined for fluorescent colonies, using a plate-based assay developed for

this purpose. This technique has been detailed in a previous volume of this series (Cronin and Hampton, 1999). A narrow bandpass filter with a maximum wavelength of 488 nm was custom-made by Omega Optical, Inc. (Brattleboro, VT; www.omegafilters.com; cost ~$200). The filter was designed to exclude both lower and higher wavelengths that normally come from a bright white light. The filter had the dimensions of a photographic slide (50 mm × 50 mm), so that it could be put into a Kodak slide projector (now readily available in the surplus sites of many universities), providing an intense blue light field for inspection of multiple plates. The blue-illuminated plates are examined through a Kodak No. 12 Wratten filter placed on a pair of laboratory goggles. The colony assay was calibrated both with hrd mutants and with strains expressing variants of Hmg2p-GFP that do not respond to lovastatin to ensure that the screen would distinguish the desired mutants. Once established, this assay is quite facile; 300,000 colonies were examined for lovastatin-induced GFP fluorescence in the cod screen. Colonies with low fluorescence were isolated on no-lovastatin plates. These unresponsive candidates were then tested for growth onto agar plates with 200 μg/ml lovastatin, a dose that will kill hypersensitive cod mutants but not the wild-type parent cells. Candidates that were both unresponsive to lovastatin in the low-dose optical screen and hypersensitive to lovastatin in high-dose toxicity assay were then checked directly for altered regulation of Hmg2p-GFP by *in vivo* flow cytometry and biochemical analysis. Finally, successful candidates were analyzed by classical and molecular genetic means. From this, the *cod1-1* mutant and 39 independent alleles of the same gene, were isolated.

The use of these two independent reporters automatically rules out a large number of distinct false positives. For example, if a candidate were sufficiently impermeable to lovastatin, the optical screen would score as a mutant (unresponsive to lovastatin), but the secondary screen would not show increased sensitivity to the drug at higher doses. Furthermore, the use of two separate Hmg2p-based reporters strongly decreases the isolation of mutations in the Hmg2p-GFP reporter itself, because these would not affect the regulation of the independent 1myc-Hmg2p reporter. With the plethora of reporters available, it is often possible to devise a two-reporter screen that obviates many of the typical concerns of classical genetic screening.

Use of the Optical Screen to Find Genes That Block Hmg2p Degradation

Another powerful approach in many organisms is to test expression libraries for the ability to cause phenotypes of interest. We used a wild-type strain expressing Hmg2p-GFP from the *TDH3* promoter to screen for

yeast genes that disrupt the degradation of Hmg2-GFP at high doses (20–40 copies per cell) (22, 23). The recipient strain expressed Hmg2p-GFP and (from an independent locus) high levels of the soluble catalytic domain of Hmg2p. This source of HMGR activity provided a high level of the cellular degradation signal to ensure rapid degradation of the Hmg2p-GFP and, consequently, low colony fluorescence in the parent strain. The low wild-type fluorescence in the parent strain allowed more sensitive detection of candidates with increased fluorescence because of degradation-inhibiting plasmids.

The source DNA was a publicly available 2 μ yeast genomic library with a *LEU2* prototrophy marker. These plasmids are harbored in yeast cells at a level of 20–40 copies of a single plasmid per cell. The screen is performed on leucine-deficient and uracil-deficient medium to allow continuous selection for the library plasmid and the Hmg2p-GFP reporter plasmid, respectively. Cells were transformed with library DNA and spread on the leucine-deficient agar plates to give 250 colonies per plate (actual number used depends on investigator eyesight). The plates were allowed to grow for 2–3 days and examined for colony fluorescence. Because a typical genomic fragment is represented approximately once every 1000–2000 times in a genomic library, it is good to examine at least 5–10 times this number to improve the possibility of finding a desired clone. Colonies that have elevated fluorescence were isolated, regrown on selective medium, and, if still bright, grown in liquid medium and tested by flow cytometry for direct effects on the degradation rate of the Hmg2p-GFP reporter.

The plasmid DNA was next isolated in bacteria from interesting candidates and purified. Candidate plasmids were analyzed by restriction analysis, and each unique plasmid was tested for phenotypic fidelity by transformation into the parent strain. Successful candidates were analyzed by sequencing, and the candidate coding regions were then individually analyzed by subcloning and retesting in the parent strain. In this way, we isolated both a dominant-negative truncated version of *HRD1* and a yeast homolog of INSIG (Flury *et al.*, 2005), a protein involved in regulated degradation of HMGR in mammals (Sever *et al.*, 2003)

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[22] Bi-substrate Kinetic Analysis of an E3-Ligase–Dependent Ubiquitylation Reaction

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Abstract

Little is known about the kinetic mechanism of E3 ubiquitin ligases. This work describes basic methodology to investigate the kinetic mechanism of E3 ubiquitin ligases. The method used steady state, bi-substrate kinetic analysis of an E3 ligase–catalyzed monoubiquitylation reaction using ubiquitin-conjugated E2 (E2ub) and a mutant IκBα as substrates to evaluate whether the E3-catalyzed ubiquitin transfer from E2ub to protein substrate was sequential, meaning both substrates bound before products leaving, or ping pong, meaning that ubiquitin-conjugated E2 would bind, transfer ubiquitin to the E3, and debind before binding of protein substrate. The method requires the E3 reaction to be rate limiting and at steady state. This was accomplished through optimization of the conditions to ensure that the E3-dependent transfer of ubiquitin from E2ub to substrate was rate limiting. We observed a sequential bi-substrate E3-dependent ubiquitylation reaction on using E2UBCH7 and IκBαSS32/36EE (IκBαee as substrates and a partially purified Jurkat cell lysate as a source for the E3 ligase activity). The sequential bi-substrate kinetic mechanism is consistent with the formation of a ternary complex among E2UBCH7, IκBαSS32/36EE, and E3 before the transfer of ubiquitin from E2UBCH7

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