Making It Easier to Regulate Protein Stability

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The ability to induce degradation of a protein of interest is a powerful experimental tool used to ascertain protein function. Iwamoto et al. (2010) describe a method that allows reversible and dose-dependent modulation of the stability of any target protein.

The best way to determine the function of an uncharacterized protein in a cell is to remove it and see what happens. The most common ways to do this are to disrupt expression of the gene encoding the protein by altering the gene at the DNA level or to manipulate mRNA levels by RNA interference. However, these techniques have the limitation that some proteins persist in the cell for a long time after their synthesis is blocked. Thus, it would be useful to have a way to directly eliminate individual proteins from cells.

Physiologically, proteins are most often removed from the cell by the ubiquitin-proteasome system (UPS). At the center of this system is a large protease complex called the proteasome. Individual substrates are tagged for destruction by a removable protein tag called ubiquitin, which is attached by a cascade of enzymes consisting of an E1 (ubiquitin-activating enzyme), an E2 (ubiquitin-conjugating enzyme), and an E3 (ubiquitin-protein ligase). Several methods have been developed to redirect the UPS to remove desired target proteins artificially. Each approach has its own advantages and disadvantages, but none has yet seen widespread usage. In this issue, Iwamoto et al. (2010) present a new technique that uses a small molecule to specifically modulate the stability of, in principle, any protein of interest. Because this technique has several advantages over the existing methods, it has the potential to become widely employed.

In one class of approaches to degrade a protein of interest, the ubiquitination machinery is redirected to the new target. For example, there is a ubiquitination signal or degron that only becomes active at high temperatures; when this degron is fused to a target protein, degradation can be induced by an increase in temperature (Dohmen et al., 1994). Alternatively, one can retarget ubiquitinating enzymes by fusing them to a domain that specifically recognizes the desired substrate (reviewed in Banaszynski and Wandless (2006)). The target protein then becomes ubiquitinated and is degraded. In a particularly sophisticated version of this method, the ubiquitination machinery is redirected by a small molecule that binds both the protein of interest and an E3 (Sakamoto et al., 2001). The advantages of this are that it does not require any gene modification and it is inducible by a small molecule, which also means that this method could potentially be used therapeutically to remove undesirable proteins.

It is not always easy to find a ligand that binds the target protein. To get around this restriction, another method uses the plant hormone auxin to bring the protein of interest to an E3 complex. Auxin binds both to a plant E3 and to a protein receptor, which can be fused to the target protein (Nishimura et al., 2009); auxin addition then leads to ubiquitination and degradation of the desired substrate. Auxin is not found in mammalian cells and so it does not have off-target effects, but both the auxin-binding E3 component and the modified protein of interest have to be introduced into cells.

A second class of methods works by directly relocating proteins in the cell. In the examples developed to date, this is done through an inducible dimerization system based on the small molecule rapamycin, which causes the two proteins FKBP and mTOR to bind to each other (reviewed in Crabtree and Schreiber (1996)). If the rapamycin-binding domain of mTOR, called FRB, is fused to the target protein and FKBP is attached to a subunit of the proteasome, then addition of rapamycin will cause the target protein to bind to the proteasome and be degraded (Janse et al., 2004). Proteins can also be inactivated by sequestration. When FKBP is fused to a target protein and FRB is attached to a mitochondrial import signal, the addition of rapamycin causes the target protein to be very rapidly localized to mitochondria, which inactivates it (Robinson et al., 2010).

The third and last class of approaches works somewhat differently; here, the target protein is first destabilized so that it is constitutively degraded until it is rescued by drug treatment. This approach requires the protein of interest to be fused to a destabilizing domain (DD), which targets it for degradation. The protein can then be rescued by the addition of a ligand that binds the DD and inactivates it. The system is reversible, and when the ligand is withdrawn, the protein is degraded again. In one implementation of this approach, the DD is a mutant version of the FRB domain. The FRB fusion is stabilized by a rapamycin analog, which induces dimerization with endogenous FKBP. This method can be very effective and has been used to create conditional alleles in mice (Stankunas et al., 2003). However, there are also drawbacks; for example, the stabilized target protein is part of a fairly large complex whose bulk and geometry may affect its function. It is possible to modify the FRB and FKBP domains so that rapamycin addition causes release of the target protein in its native form, without additional domains, but this also means that stabilization can no longer be reversed (Pratt et al., 2007).

In this issue, Iwamoto et al. (2010) describe the development of a new DD with superior properties. This DD is derived from *E. coli* dihydrofolate reductase (ecDHFR) and is stabilized by the DHFR inhibitor trimethoprim (TMP). Iwamoto et al. (2010) isolated the ecDHFR mutants used as DDs in a cell-based

screen that selected domains causing almost complete removal of the fusion protein. TMP stabilizes the DD-target protein fusion in a dose-dependent manner up to 100-fold, which gives the system a substantial dynamic range. Stabilization is reversible and removal of TMP leads to rapid degradation back to background levels. In addition, the ligand TMP works by itself and does not require dimerization with a second protein, similar to an earlier version developed by the same group (Banaszynski et al., 2006). This system appears to be so effective that it can even control the levels of transmembrane proteins, which greatly increases its potential utility and applicability.

The stabilizing ligand used for this system also brings some important practical advantages. TMP is commercially available, inexpensive, and has good pharmacological properties, which makes its usage in in vivo experimental systems easier. TMP has very few off-target effects in mammalian cells because it inhibits ecDHFR much more strongly than the endogenous mammalian DHFR. In fact, it is used as an antibiotic. Additionally, TMP crosses the blood-brain barrier and can therefore be used to modulate the stability of proteins in the central nervous system of live animals. Finally, TMP also crosses the placental barrier, which should permit the study of proteins that are essential in the earliest stages of development. If the offspring contain a gene modified with ecDHFR, TMP could be administered to the mother throughout gestation to ensure the presence of the protein of interest. At the desired times, TMP could then be withdrawn so that the target protein is degraded. This would make it possible to investigate the role of the targeted protein at various stages of development.

Thus, the new ecDHFR-derived DD method represents a substantial step forward for inducible protein degradation systems, and it could become a practical addition to our tools for regulating protein concentrations.

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Creating Designer Laccases

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High redox potential laccases from white-rot fungi are recalcitrant to engineering. Maté et al. (2010) employed directed evolution to improve the activity and expression level of the fungal laccase from basidiomycete PM1, followed by rational design to restore thermostability lost during evolution, resulting in a highly active and stable enzyme.

Highly active and stable enzymes are desirable for industrial biotechnology. Two methods are commonly used to obtain enzymes with such properties: screening microorganisms to identify novel enzymes, and engineering existing enzymes to improve these properties. While traditional strain screening is still widely used, enzyme engineering, in particular directed-enzyme evolution, has increasingly become an alternative to generate enzymes with desired properties. This is particularly true when enzyme properties, such as enzymatic activity and thermal stability, can be analyzed in a high throughput manner to facilitate the identification of superior mutants from fairly large mutant libraries. In a typical directed-evolution experiment, screening for enhanced enzymatic activity is performed concomitantly with screening for improved thermostability to generate mutants that are both more active and more stable. With such a procedure, some mutants with enhanced activity but less stability are obtained, while others with enhanced stability but less activity are also frequently identified. If the additive mutations fail to be combined, either the more active mutant or