[51] Ubiquitin Fusion Technique and Related Methods

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Abstract

The ubiquitin fusion technique, developed in 1986, is still the method of choice for producing a desired N-terminal residue in a protein of interest *in vivo*. This technique is also used as a tool for protein expression. Over the past two decades, several otherwise unrelated methods were invented that have in common the use of ubiquitin fusions as a component of design. I describe the original ubiquitin fusion technique, its current applications, and other methods that use the properties of ubiquitin fusions.

Introduction

The ubiquitin (Ub) fusion technique was invented through experiments in which a segment of DNA encoding the 76-residue Ub was joined, in-frame, to DNA encoding *Escherichia coli* β-galactosidase (βgal) (Bachmair *et al*., 1986; Varshavsky, 1996b, 2000). When the resulting protein fusion was expressed in the yeast *Saccharomyces cerevisiae* and detected by radiolabeling and immunoprecipitation with anti-βgal antibody, only the moiety of βgal was observed, even if pulse-labeling was close to the time (1–2 min) required for translation of the Ub-βgal's open reading frame (ORF). It was found that the Ub moiety of the fusion was rapidly cleaved off after the last residue of Ub (Fig. 1) (Bachmair *et al*., 1986). The proteases involved are called deubiquitylating enzymes (DUBs) (Amerik and Hochstrasser, 2004; Baker, 1996; Gilchrist *et al*., 1997; Hemelaar *et al*., 2004; Pickart and Cohen, 2004; Verma *et al*., 2002; Wilkinson, 2000; Wilkinson and Hochstrasser, 1998). A mammalian genome encodes at least 80 distinct DUBs that are specific for the Ub moiety. The *in vivo* cleavage of a Ub fusion at the Ub-polypeptide junction is largely cotranslational (Johnsson and Varshavsky, 1994b; Turner and Varshavsky, 2000).

A note on terminology: ubiquitin whose C-terminal (Gly-76) carboxyl group is covalently linked to another compound is called the *ubiquityl* moiety, with derivative terms *ubiquitylation* and *ubiquitylated*. The acronym Ub refers to both free ubiquitin and the ubiquityl moiety. This nomenclature (Varshavsky, 1997; Webb, 1992), which brings Ub-related terms in line with standard chemical terminology, has been adopted by most Ub researchers. Shorthand for ''degradation signal'' is ''degron''

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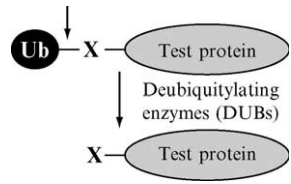

FIG. 1. The ubiquitin fusion technique. Linear fusions of Ub to other proteins are cleaved after the last residue of Ub by deubiquitylating enzymes (DUBs) (see the main text) (Bachmair *et al.*, 1986; Varshavsky, 1996b).

(Dohmen *et al.*, 1994; Gardner and Hampton, 1999; Varshavsky, 1991). Through the use of prefixes, subscripts, or superscripts, this acronym can be employed to denote, in a uniform and succinct way, different types of degradation signals. For example, ''N-degron'' denotes one class of degradation signals recognized by the N-end–rule pathway, specifically those in which an essential determinant is a substrate's destabilizing N-terminal residue (Bachmair and Varshavsky, 1989; Rao *et al.*, 2001; Suzuki and Varshavsky, 1999; Varshavsky, 1996b).

One physiological function of DUB-mediated cleavage reactions (Fig. 1) is the excision of Ub from its natural DNA-encoded precursors, either linear poly-Ub (Finley *et al.*, 1987) or Ub fusions to specific ribosomal proteins (Finley *et al.*, 1989; Redman and Rechsteiner, 1989). Many DUBs that process linear Ub fusions can also cleave Ub off its branched, posttranslationally formed conjugates, in which Ub is conjugated either to itself, as in a branched poly-Ub chain, or to other proteins. A Ub–protein conjugate usually is composed of a single poly-Ub chain covalently linked to an internal Lys residue of a substrate protein. The ubiquitylated substrate is recognized (in part through its poly-Ub chain) and processively degraded by the 26S proteasome, an ATP-dependent multisubunit protease (Baumeister *et al.*, 1998; Rechsteiner and Hill, 2005). For reviews of the Ub system, see Fang and Weissman (2004); Hershko *et al.* (2000); Hicke and Dunn (2003); Petroski and Deshaies (2005); and Pickart (2004).

The finding of a rapid *in vivo* deubiquitylation of Ub fusions (Fig. 1) led to the discovery of N-end rule, a relation between the *in vivo* half-life of a protein and the identity of its N-terminal residue (Fig. 2) (Bachmair *et al.*, 1986). First, it was shown that the cleavage of a Ub-X-polypeptide after the last residue of Ub takes place regardless of the identity of a junctional residue X, proline (Pro) being the single exception. By allowing a bypass of ''normal'' N-terminal processing of a newly formed protein, this finding yielded an *in vivo* method for placing different residues at the N-termini of

Residue X	Half-life of X-βgal	
	E. coli	*S. cerevisiae*
Arg	2 min	2 min
Lys	2 min	3 min
Phe	2 min	3 min
Leu	2 min	3 min
Trp	2 min	3 min
Tyr	2 min	10 min
His	>10 h	3 min
Ile	>10 h	30 min
Asp	>10 h	3 min
Glu	>10 h	30 min
Asn	>10 h	3 min
Gln	>10 h	10 min
Cys	>10 h	>30 h
Ala	>10 h	>30 h
Ser	>10 h	>30 h
Thr	>10 h	>30 h
Gly	>10 h	>30 h
Val	>10 h	>30 h
Pro	>10 h	>30 h
Met	>10 h	>30 h

FIG. 2. The N-end rule of the yeast *S. cerevisiae*. Specific residues at the N-terminus of a test protein such as βgal are produced using the Ub fusion technique (Fig. 1 and the main text). The *in vivo* half-lives of the corresponding X-βgal proteins are indicated on the right. Stabilizing N-terminal residues (Met, Gly, Ala, Ser, Thr, Cys, Val, and Pro) are not recognized by UBR1, the E3 Ub ligase of the N-end–rule pathway. The N-end rule of mammalian cells is similar but contains fewer stabilizing residues (Kwon *et al.*, 2002, 2003).

otherwise identical proteins. It was found that the *in vivo* half-lives of resulting test proteins were strongly dependent on the identities of their N-terminal residues, a relation referred to as the N-end rule (Fig. 2) (Bachmair *et al.*, 1986). The underlying, universally present N-end rule pathway has a variety of functions; their list continues to expand (Du *et al.*, 2002; Kwon *et al.*, 2002, 2003; Rao *et al.*, 2001; Turner *et al.*, 2000; Varshavsky, 1996b, 2003; Yin *et al.*, 2004).

The Ub fusion technique (Figs. 1 and 2) remains the method of choice for producing, *in vivo*, a desired N-terminal residue in a protein of interest. The requirement for a "technique" to do so stems from a constraint imposed by the genetic code. All nascent proteins bear N-terminal Met (formyl-Met in prokaryotes). The known methionine aminopeptidases (MetAPs) that remove N-terminal Met would do so if, and only if, a

residue at position 2, to be made N-terminal after cleavage, is small enough (Bradshaw *et al*., 1998; Varshavsky, 1996b). Specifically, MetAPs do not remove N-terminal Met if it is followed by any of the 12 destabilizing residues in the yeast-type N-end rule (Fig. 2). The exception, in metazoans, is Cys, whose side chain is small enough to allow cleavage of the Met-Cys bond by MetAPs. N-terminal Cys is a destabilizing residue in mammals and (apparently) other multicellular eukaryotes, but a stabilizing residue in fungi such as *S. cerevisiae* (Gonda *et al*., 1989; Kwon *et al*., 2002). (All destabilizing residues, including Cys, can be made N-terminal through cleavages by other intracellular proteases, such as separases, caspases, and calpains, which act, in this capacity, as upstream components of the N-end rule pathway.) The Ub-specific DUB proteases are free of constraints imposed by the preceding property of MetAPs, except when the residue X of a Ub-X polypeptide is Pro, in which case the cleavage still takes place but at a much lower rate (Bachmair *et al*., 1986; Johnson *et al*., 1992, 1995). However, there also exists a DUB that can efficiently cleave at the Ub-Pro junction (Gilchrist *et al*., 1997).

Ub fusions can be deubiquitylated *in vitro* as well (Baker, 1996; Catanzariti *et al*., 2004; Gonda *et al*., 1989). High activity and specificity of DUBs make them reagents of choice for applications that involve, for example, the removal of affinity tags from overexpressed and purified proteins. A particularly efficacious version of the Ub fusion technique for high-level production and easy purification of recombinant proteins expressed in *E. coli* was described by R. Baker and colleagues (Fig. 3) (Baker *et al*., 2005; Catanzariti *et al*., 2004).

Yet another advantage of the Ub fusion technique stems from the finding that expression of a protein as a Ub fusion can dramatically augment protein's yield (Baker *et al*., 1994; Butt *et al*., 1989; Ecker *et al*., 1989; Mak *et al*., 1989). The yield-enhancement effect of Ub was observed with short peptides as well (Pilon *et al*., 1997; Yoo *et al*., 1989). This and other applications of Ub fusions are described in the following, with references to original articles and specific constructs.

Production and Uses of N-Degrons

An N-degron is composed of a protein's destabilizing N-terminal residue and an internal Lys residue (Bachmair and Varshavsky, 1989; Hill *et al*., 1993; Suzuki and Varshavsky, 1999; Varshavsky, 1996b). The lysine determinant is the site of formation of a substrate-linked poly-Ub chain (Chau *et al*., 1989; Pickart and Fushman, 2004). One way to produce an N-degron in a protein of interest is to express the protein as a Ub fusion whose junctional residue, which becomes N-terminal on removal of the Ub

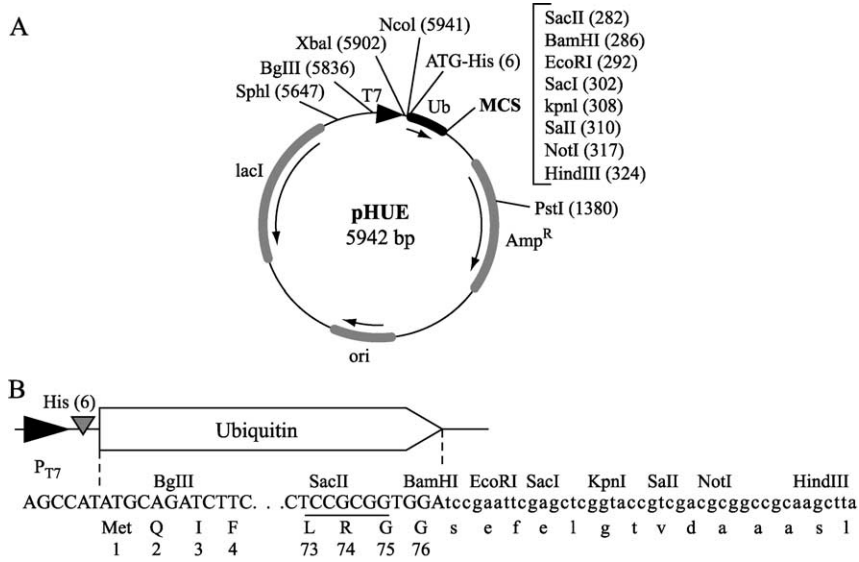

FIG. 3. The ubiquitin fusion-based expression-purification technique of R. Baker and colleagues (Catanzariti *et al*., 2004). (A) Plasmid map of pHUE, a histidine-tagged ubiquitin expression vector. It shows the Ub-coding region (black box), the T7 polymerase promoter (black triangle), and other relevant regions (shaded boxes). Arrows indicate the direction of transcription. Restriction enzyme recognition sites within the multiple cloning site (MCS) are listed, and other useful recognition sites are also shown, all of them unique, except *Bgl*II; locations are cited relative to the start codon upstream of the His-tag, ATG = 1. *(His)6*, polyhistidine tag; *Amp*^{r}, β-lactamase gene; *ori*, colE1 origin of replication; *lacI*, Lac repressor gene. (B) DNA and encoded protein sequence of the 5′ and 3′ ends of the Ub-coding region, showing the engineered *Sac*II site (underlined) within Leu-73, Arg-74, and Gly-75, and the 3′ polylinker. Restriction sites and encoded amino acid residues are shown above and under the DNA sequence, respectively. A Ub fusion expressed using pHUE can be deubiquitylated by the histidine-tagged DUB enzyme USP2 (Baker *et al*., 2005; Catanzariti *et al*., 2004).

moiety, is destabilizing (Fig. 2). An appropriately positioned internal Lys residue (or residues) is the second essential determinant of N-degron. Many natural proteins lack such ''targetable'' lysines, and therefore would remain long-lived even if their N-terminal residue were replaced by a destabilizing one. One way to bypass this difficulty is to link a protein of interest to a portable N-degron that contains both a destabilizing N-terminal residue (produced through a Ub fusion) and a requisite Lys residue(s). The earliest portable N-degron of this kind is still among the most efficacious known (Fig. 4B) (Bachmair and Varshavsky, 1989) and can be improved through insertion of additional Lys residues (Suzuki and Varshavsky, 1999). A screen in the sequence space of two amino acids,

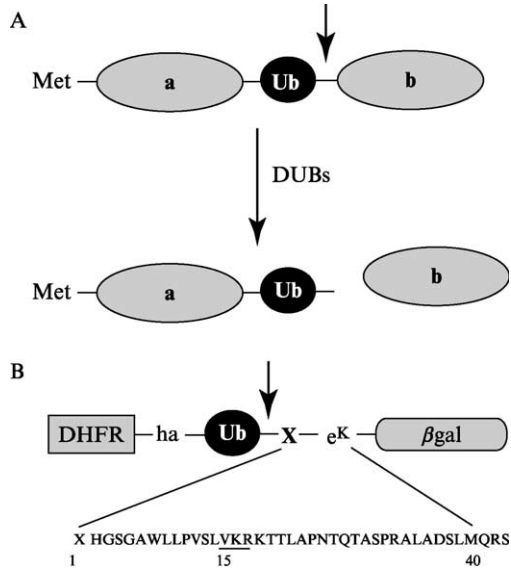

FIG. 4. The ubiquitin-protein-reference (UPR) technique. (A) A tripartite fusion containing **a** the reference protein moiety whose C-terminus is linked, by means of a spacer peptide, to the Ub moiety. The C-terminus of Ub is linked to **b,** a protein of interest (Lévy *et al*., 1996; Suzuki and Varshavsky, 1999). *In vivo*, this tripartite fusion is cotranslationally cleaved (Turner and Varshavsky, 2000) by deubiquitylating enzymes (DUBs) at the Ub-**b** junction, yielding equimolar amounts of the unmodified protein **b** and **a**-Ub, the reference protein **a** bearing a C-terminal Ub moiety. If **a**-Ub is long-lived, determining the ratio of **a**-Ub to **b** as a function of time or at steady-state yields, respectively, the *in vivo* decay curve or the relative metabolic stability of protein **b**. (B) Example of a specific UPR-type Ub fusion (Suzuki and Varshavsky, 1999). This fusion contains the following elements: DHFRha, a mouse dihydrofolate reductase (DHFR) moiety extended at the C-terminus by a sequence containing the hemagglutinin-derived ha epitope; the Ub moiety (more specifically, the Ub^{R48} moiety bearing the Lys → Arg alteration at position 48); a 40-residue, *E. coli* Lac repressor–derived sequence, termed e^K (extension [*e*] containing lysines [*K*]) and shown below in single-letter abbreviations for amino acids; a variable residue X between Ub and e^K; the *E. coli* βgal moiety lacking the first 24 residues of wild-type βgal. A short arrow in (A) and (B) indicates the site of *in vivo* cleavage by DUBs.

Lys and Asn, has shown that certain sequences containing exclusively lysines and asparagines can also function *in vivo* as strong N-degrons (Suzuki and Varshavsky, 1999). At least some natural N-degrons, such as the one in a separase-produced fragment of SCC1, a subunit of *S. cerevisiae* cohesin, are highly efficacious as well (Rao *et al*., 2001) and are also likely to be portable. The modularity and portability of N-degrons

make possible a variety of applications whose common feature is the conferring of a constitutive or conditional metabolic instability on a protein of interest.

N-Degron and Reporter Proteins

A change in the physiological state of a cell that is preceded or followed by the induction or repression of specific genes can be monitored through the use of promoter fusions to a variety of protein reporters, such as, for example, βgal, β-glucuronidase, luciferase, and green fluorescent protein (GFP). A long-lived reporter is useful for detecting the induction of genes but is less suitable for monitoring either a rapid repression or a temporal pattern that involves an up- or down-regulation of a gene product of interest. A short-lived reporter is required in such settings. N-degron–containing X-βgal proteins of the original N-end rule study (Fig. 2) (Bachmair *et al.*, 1986) were the first such reporters. Over the past two decades, other reporters, including the ones mentioned previously, were metabolically destabilized by extending them with either a portable N-degron or a ''nonremovable'' Ub moiety (Dantuma *et al.*, 2000; Deichsel *et al.*, 1999; Paz *et al.*, 1999; Worley *et al.*, 1998). The latter is targeted by a distinct Ub-dependent proteolytic pathway called the UFD pathway (Ub-fusion-degradation) (Bachmair *et al.*, 1986; Johnson *et al.*, 1992, 1995; Koegl *et al.*, 1999). Metabolically unstable reporters are particularly useful in settings in which the reporter's concentration must reflect a recent level of gene activity. Portable N-degrons were also used to destabilize specific protein antigens, thereby enhancing presentation of their peptides to the immune system (Tobery and Siliciano, 1999; Townsend *et al.*, 1988).

N-Degron and Conditional Mutants

Conditional mutants based on N-degrons are described in detail in Chapter 52. A frequent problem with conditional phenotypes is their leakiness (i.e., unacceptably high residual activity of either a temperature-sensitive (*ts*) protein at nonpermissive temperature or a gene of interest in the ''off'' state of its promoter). Another problem is ''phenotypic lag,'' which often occurs between the imposition of nonpermissive conditions and the emergence of a relevant null phenotype. Phenotypic lag tends to be longer with proteins that are required in catalytic rather than stoichiometric amounts.

In one application of Ub fusions and the N-end–rule pathway to the problem of phenotypic lag, a constitutive N-degron (produced as a Ub fusion) was linked to a protein expressed from an inducible promoter (Park

et al., 1992). This otherwise useful method is constrained by the necessity of using a heterologous promoter and by a constitutively short half-life of a target protein, whose levels may, therefore, be suboptimal under permissive conditions. An alternative approach is to link an N-degron to a normally long-lived protein in a strain where the N-end–rule pathway can be induced or repressed. Such strains have been constructed with *S. cerevisiae* (Ghislain *et al.*, 1996; Moqtaderi *et al.*, 1996) but can also be designed for other organisms, including mammalian cells. The metabolic stabilities, and hence also the levels of N-degron-bearing proteins in a cell with an inducible N-end rule pathway, are either normal or very low, depending on whether UBR1, the pathway's E3 Ub ligase, is absent or present (Ghislain *et al.*, 1996; Moqtaderi *et al.*, 1996). These conditional mutants can be constructed with any cytosolic or nuclear protein whose function tolerates an N-terminal extension.

Yet another design is a portable N-degron that is inactive at low (permissive) temperature but becomes active at high (nonpermissive) temperature. Such an N-degron was constructed, using the Ub fusion technique, in the context of a specific *ts* allele of the 20-kDa mouse dihydrofolate reductase (DHFR) bearing N-terminal Arg, a strongly destabilizing residue (Dohmen and Varshavsky, 2005; Dohmen *et al.*, 1994). Linking this DHFR-based, heat-activated N-degron to proteins of interest yielded a new class of *ts* mutants, called *td* (temperature-activated degron). The *td* method does not require an often unsuccessful search for a nonleaky *ts* mutation in a gene of interest. If a protein can tolerate N-terminal extensions, the corresponding *td* fusion is likely to be functionally unperturbed at permissive temperature. (By contrast, a low activity of a *ts* protein at permissive temperature is a frequent problem with conventional *ts* mutants.) The *td* method eliminates or reduces phenotypic lag, because the activation of N-degron results in rapid disappearance of a *td* protein. Yet another advantage of the *td* technique is the possibility of using two sets of conditions: a *td* protein-expressing strain at permissive versus nonpermissive temperature or, alternately, the same strain versus a congenic strain lacking the N-end–rule pathway, with both strains at nonpermissive temperature (Dohmen *et al.*, 1994). This powerful internal control, provided in the *td* technique by two alternative sets of permissive/nonpermissive conditions, is unavailable with conventional *ts* mutants. Since 1994, a number of laboratories described successful uses of the *td* technique to construct *ts* (*td*) alleles of specific proteins (Amon, 1997; Aparicio, 2003; Caponigro and Parker, 1995; Hardy, 1996; Kanemaki *et al.*, 2003; Kesti *et al.*, 2004; Labib *et al.*, 2000; Valasek *et al.*, 2003; Wang *et al.*, 2004; Wolf *et al.*, 1998).

N-Degron and Conditional Toxins

A major limitation of current pharmacological strategies stems from the absence of drugs that are specific, in a predetermined manner, for two or more independent molecular targets. For reasons discussed elsewhere (Varshavsky, 1995, 1998), it is desirable to have a therapeutic agent that possesses a multitarget, combinatorial selectivity, which requires the presence of two or more predetermined targets in a cell and simultaneously the *absence* of one or more targets for the drug to exert its effect. Note that simply combining two or more ''conventional'' drugs against different targets in a multidrug regimen will not yield a multitarget selectivity, because two drugs together would perturb not only cells containing both targets but also cells containing either one of the targets.

A strategy for designing protein-based reagents that are sensitive to the presence or absence of more than one target at the same time was proposed a decade ago (Varshavsky, 1995). One key feature of such reagents is their ability to use codominance, the property characteristic of many signals in proteins, including degrons and nuclear localization signals (NLSs). Codominance, in this context, refers to the ability of two or more signals in the same molecule to function independently and not to interfere with each other. The critical feature of a degron-based multitarget reagent is that its intrinsic toxicity (or another intended property) is the same in all cells, whereas its *in vivo* half-life, and, consequently, its steady-state level and overall toxicity, depends on the cell's protein composition, specifically on the presence of multiple ''target'' proteins that have been chosen to define the profile of a cell to be eliminated or otherwise modified (Varshavsky, 1995). A related but different design involves a toxic protein made short-lived (and therefore relatively nontoxic) by the presence of a degradation signal such as N-degron, produced using the Ub fusion technique. For example, if a cleavage site for a specific viral or nonviral protease is placed between the fusion's toxic moiety and the N-degron, the fusion would be cleaved only in cell containing the relevant protease. As a result, the toxic moiety of the fusion would become long-lived, and, therefore, more toxic, only in a target cell population, for example, virus-infected cells (Falnes and Olsnes, 1998; Falnes *et al*., 1999; Varshavsky, 1996a). Analogous Ub fusion–based approaches can use a different degron, for example, the one recognized by the UFD (Ub-fusion-degradation) pathway (Tcherniuk *et al*., 2004). The interference/codominance (IC) concept and the ideas about protein-size multitarget reagents have been extended to small (<1 kDa) multitarget compounds (Varshavsky, 1998).

Overexpression of Proteins as Ubiquitin Fusions

A major application of the Ub fusion technique is its use to augment the yields of recombinant proteins (Baker *et al*., 1994; Butt *et al*., 1989; Ecker *et al*., 1989; Mak *et al*., 1989; Pilon *et al*., 1997). See Fig. 3 for a particularly effective version of the Ub fusion technique for high-level expression and purification of recombinant proteins expressed in *E. coli*, by R. Baker and colleagues (Baker *et al*., 2005; Catanzariti *et al*., 2004).

The yield-enhancing effect of Ub was observed not only with eukaryotic cells (where the Ub moiety is present in a nascent fusion but not in its mature counterpart) but also in prokaryotes, which lack the Ub system, including DUBs, and, therefore, retain the Ub moiety in a translated fusion. (*E. coli* transformed with a plasmid expressing the *S. cerevisiae* DUB UBP1 acquires the ability to deubiquitylate Ub fusions [Tobias *et al*., 1991].) The effect of N-terminal Ub moiety of a fusion on the fusion's yield is likely to stem from rapid folding of the nascent Ub moiety, whose presence at the N-terminus of an emerging protein may thereby either partially protect, in ways that are not understood, a still unfolded part of the fusion from proteolytic attack, facilitate its folding, or both. The Ub-mediated increase in total yield is often accompanied by an increase in solubility of overexpressed protein. In this regard, the effect of Ub is analogous to that of several other proteins, such as thioredoxin and maltose-binding protein (MBP) (Kapust and Waugh, 1999). When these moieties are cotranslationally linked to a protein of interest, they often increase its yield and solubility. A model of the underlying mechanism suggested for MBP (Kapust and Waugh, 1999) may also be relevant to the effect of Ub moiety. Specifically, a partially unfolded nascent protein is presumed to weakly interact with the nearby (upstream) MBP moiety, thereby transiently precluding intermolecular self-interactions that can result in irreversible aggregation before the protein has had the time to attain its mature conformation.

The first engineered Ub fusions used pUB23-X, a family of high-copy plasmids that expressed Ub-X-βgal proteins containing different junctional residues (X) in *S. cerevisiae* from a galactose-inducible, glucose-repressible promoter (Bachmair and Varshavsky, 1989; Bachmair *et al*., 1986). Subsequent designs facilitated the construction of ORFs encoding Ub-X-polypeptide fusions by introducing a *Sac*II (*Sst*II) site within the codons for the last three residues of Ub moiety (Baker *et al*., 1994). In this cloning scheme, an ORF of interest is amplified using PCR and a primer in which the 5′ extension encodes the last three residues of Ub. Another cloning route uses double-stranded oligonucleotides with *Sac*II cohesive ends that are used to join DNA fragments (Baker *et al*., 1994). The expression of a

resulting Ub-X-polypeptide fusion in a eukaryotic cell (or in a prokaryotic cell that contains the *S. cerevisiae* UBP1 DUB) yields an X-polypeptide bearing a predetermined N-terminal residue X (Figs. 1 and 2).

In their natural milieu, proteins of biotechnological or pharmacological interest are often products of secretory pathways and, therefore, are cleaved by signal peptidase on their entrance into the endoplasmic reticulum (ER). This cleavage frequently yields destabilizing residues at the N-termini of these proteins. When the same proteins are overexpressed in the cytosol of a heterologous bacterial or eukaryotic host, their N-terminal Met tends to be retained, because MAPs cannot cleave off N-terminal Met if it is followed by a destabilizing residue (see earlier). It is in these, quite frequent, cases that the expression of a protein as a Ub-X-protein fusion can attain two aims at once: producing a protein of interest bearing the desired N-terminal residue (Fig. 2) and also, quite often, increasing protein's yield compared with an otherwise identical expression of Ub-lacking protein (Baker, 1996).

There are numerous examples of Ub-mediated increases in the yield and solubility of overexpressed proteins. For instance, a conventional heterologous expression of the *Streptomyces* tyrosinase in *E. coli* yielded inactive enzyme, whereas expression of tyrosinase as a Ub fusion resulted in an abundant and active enzyme (Han *et al*., 1994). Another example was a high yield, in *E. coli*, of the soluble human collagenase catalytic domain as a Ub fusion (Gehring *et al*., 1995). In contrast, the expression of the same protein in the absence of N-terminal Ub moiety resulted in low yield and insoluble product. A 60-fold increase in the yield of the human pi class glutathione transferase GSTP1 was observed on the addition of a Ub-coding sequence to the *GSTP1* ORF (Baker *et al*., 1994). A strong increase of protein yield in *E. coli* was reported using a combination of the T7 RNA polymerase promoter system and Ub fusions (Koken *et al*., 1993). Several other examples of the Ub fusion approach to protein overexpression (Coggan *et al*., 1995; Mak *et al*., 1989; Rian *et al*., 1993; Sabin *et al*., 1989) are described in an earlier review (Baker, 1996). More recently, Vierstra and colleagues applied the Ub fusion technique to augment protein expression in transgenic plants (Hondred *et al*., 1999).

Ubiquitin-Assisted Analysis of Protein Translocation across Membranes

A method, developed in 1994 and called UTA (ubiquitin translocation assay), uses Ub as an *in vivo* kinetic probe in the context of signal sequence-bearing Ub fusions (Johnsson and Varshavsky, 1994b). After emerging from ribosomes in the cytosol, a protein may remain in the

cytosol or may be transferred to compartments separated from the cytosolic space by membranes. With a few exceptions, noncytosolic proteins begin journeys to their respective compartments by crossing membranes that enclose intracellular organelles such as the ER and mitochondria in eukaryotes or the periplasmic space in bacteria. Amino acid sequences that enable a protein to cross the membrane of a compartment are often located at the protein's N-terminus. These ''signal'' sequences (Walter *et al.*, 1984) are targeted by translocation pathways specific for each compartment. The translocation of a protein across a compartment's membrane can start before the protein's synthesis is completed, resulting in docking of the still translating ribosome at the transmembrane channel. The UTA technique takes advantage of rapid (largely cotranslational) cleavage of a Ub fusion to examine temporal aspects of protein transport across the ER membrane in living cells (Johnsson and Varshavsky, 1994b). Specifically, if a Ub fusion that has been engineered to bear an N-terminal signal sequence (SS) upstream of the Ub moiety is cleaved in the cytosol by DUBs, the fusion's reporter moiety would fail to be translocated into the ER. Conversely, if a nascent SS mediates the docking of a translating ribosome at the transmembrane channel rapidly enough, or if the fusion's Ub moiety is located sufficiently far downstream of SS, then by the time the Ub moiety emerges from the ribosome, the latter is already docked, and the nascent Ub moiety enters the ER before it can fold and/or be targeted by DUBs. Thus, the cleavage at the Ub moiety of an SS-bearing Ub fusion in the cytosol can serve as an *in vivo* kinetic marker and a tool for analyzing targeting in protein translocation (Johnsson and Varshavsky, 1994b). The temporal sensitivity of the UTA technique stems from rapid folding of the nascent Ub moiety that precludes its translocation and makes it a substrate of DUBs in the cytosol shortly after the emergence of the fusion's Ub moiety from the ribosome.

Split-Ubiquitin Technique for Detection of Protein–Protein Interactions *In Vivo*

Another Ub-based method, termed the split-protein sensor (SPS), makes it possible to detect and monitor a protein-protein interaction as a function of time, at the natural sites of this interaction in a living cell (Johnsson and Varshavsky, 1994a). These capabilities of the split-Ub technique distinguish it from the two-hybrid assay (Phizicky and Fields, 1995). The key idea of the split-Ub technique was applied by other groups to design a variety of split-protein assays, termed PCA (protein complementation assays), that use ''split'' versions of other proteins, such as dihydrofolate reductase (DHFR), β-lactamase, and green fluorescent pro-

tein (GFP) (Cabantous *et al*., 2005; Galarneau *et al*., 2002; Pelletier *et al*., 1998; Remy and Michnick, 1999; Zhang *et al*., 2004).

The split-Ub technique is based on the following observations: when a C-terminal fragment of the 76-residue Ub (C_{ub}) was expressed as a fusion to a reporter protein, the fusion was cleaved by DUBs only if an N-terminal fragment of Ub (N_{ub}) was also expressed in the same cell. This reconstitution of native Ub from its fragments, detectable by the *in vivo* cleavage assay, was not observed with a mutationally altered N_{ub}. However, if C_{ub} and the altered N_{ub} were each linked to polypeptides that interact *in vivo*, the cleavage of the fusion containing C_{ub} was restored, yielding a generally applicable assay for kinetic and equilibrium aspects of *in vivo* protein interactions (Johnsson and Varshavsky, 1994a).

The enhancement of Ub reconstitution by interacting polypeptides linked to fragments of Ub is caused by a local increase in concentration of one Ub fragment in the vicinity of its ''complementing'' counterpart. This, in turn, increases the probability that the two Ub fragments coalesce to form a quasinative Ub moiety, whose (at least) transient formation results in irreversible cleavage of the fusion by DUBs. This cleavage can be detected readily and can be followed as a function of time or at steady state (Dunnwald *et al*., 1999; Johnsson and Varshavsky, 1994a). Unlike the two-hybrid method, which is based on the apposition of two structurally independent protein domains whose folding and functions do not require direct interactions between the domains, the split-Ub assay and its descendants that use split versions of other proteins involve reconstituting the conformation of a relatively small, single-domain protein. One application of the split-Ub sensor has shown that this assay is capable of detecting transient *in vivo* interactions such as the binding of a signal sequence of a translocated protein to SEC62, a component of the ER channel (Dunnwald *et al*., 1999). Different reporter readouts and selection-based screens have been devised for the split-Ub assay, making it possible to use this method for discovering *in vivo* ligands of a protein of interest, similarly to the main application of the two-hybrid assay (Stagljar *et al*., 1998; Wittke *et al*., 1999). This and other uses of the split-Ub technique are described elsewhere (Dues *et al*., 2001; Eckert and Johnsson, 2003; Johnsson, 2002; Laser *et al*., 2000; Raquet *et al*., 2001; Wittke *et al*., 2000, 2002). As mentioned previously, the invention of the split-Ub method (Johnsson and Varshavsky, 1994a) led to the development of many other split-protein sensors, including those that use split DHFR and split GFP (Cabantous *et al*., 2005; Galarneau *et al*., 2002; Pelletier *et al*., 1998; Remy and Michnick, 1999; Tafelmeyer *et al*., 2004; Zhang *et al*., 2004).

Ubiquitin–Protein-Reference (UPR) Technique

Direct measurements of the *in vivo* degradation of intracellular proteins require a pulse-chase assay. It involves the labeling of nascent proteins for a short time with a radioactive precursor (''pulse''), the termination of labeling through the removal of radiolabel and/or the addition of a translation inhibitor, and the analysis of a labeled protein of interest at various times afterwards (''chase''), using immunoprecipitation and SDS-PAGE, or analogous techniques. Its advantage of being direct notwithstanding, a conventional pulse-chase assay is fraught with sources of error. For example, immunoprecipitation yields may vary from sample to sample, and the volumes of samples loaded on a gel may vary as well. If the labeling for specific chase times is done with separate batches of cells (as is the case, for example, with anchorage-dependent mammalian cell cultures), the efficiency of labeling is yet another unstable parameter of the assay. As a result, pulse-chase data tend to be semiquantitative at best, lacking the means to correct for these errors.

A way to address these problems through an ''internal-reference'' strategy, termed the ubiquitin-protein-reference technique (UPR), was described in 1996 (Lévy *et al*., 1996). This method, an extension of the Ub fusion technique, was applied to pulse-chase assays with mammalian cells and *S. cerevisiae* (Kwon *et al*., 2002; Lévy *et al*., 1999; Rao *et al*., 2001; Suzuki and Varshavsky, 1999; Turner *et al*., 2000), and with *Xenopus* oocytes as well (Sheng *et al*., 2002). The UPR technique can compensate for several sources of data scatter (Fig. 4). This method uses a linear fusion in which Ub is located between a protein of interest and a reference protein moiety (Fig. 4A). The fusion is cotranslationally cleaved by DUBs after the last residue of Ub, producing equimolar amounts of the protein of interest and the reference protein bearing the C-terminal Ub moiety. If both the reference protein and the protein of interest are immunoprecipitated in a pulse-chase assay, the relative amounts of the protein of interest can be normalized against the reference protein in the same sample (Suzuki and Varshavsky, 1999; Turner *et al*., 2000). The UPR technique can thus compensate for the scatter of immunoprecipitation yields, sample volumes, and other sources of sample-to-sample variation. The increased accuracy afforded by UPR underscores insufficiency of the ''half-life'' terminology, because the *in vivo* degradation of many proteins strongly deviates from a first-order kinetics. For a discussion of this issue and specific terminology for describing nonexponential decay, see Lévy *et al*. (1996) and Suzuki and Varshavsky (1999).

A more recent study (Qian *et al*., 2002) with UPR-type constructs and transiently transfected mammalian cells has shown that a fusion containing

a C-terminal Ub moiety (analogous to the DHFR-Ub^{R48} "reference" module in Fig. 4B) can be conjugated to other intracellular proteins, *a la* Ub itself. We also observed such a conjugation in transiently transfected mammalian cells that strongly overexpressed DHFR-Ub^{R48} (derived from DHFR-Ub^{R48}-X-e^{K}-βgal) (Fig. 4B) (Z. Xu and A.V., unpublished data). The extent of this conjugation seemed to be negligible at significantly lower levels of transient expression in mammalian cells (unpublished data) and was not observed at all, thus far, in similar UPR assays with *S. cerevisiae* (Suzuki and Varshavsky, 1999; Turner *et al*., 2000, and data not shown). Thus, some Ub ligases in mammalian cells, but not in yeast, can use as a conjugation substrate (apparently at low efficiency) even a Ub moiety that bears both a protein-size N-terminal extension and the Lys → Arg alteration at position 48. Although the relative efficiency of this "undesirable" conjugation in mammalian cells seems to be low, it is a complication to be addressed. A way to do so would be to identify mutations of C-terminal Ub moiety (Fig. 4B) that render it completely inactive as a substrate for conjugation by Ub ligases without perturbing significantly its recognition by DUBs in the context of a protein fusion. It remains to be seen whether such a "deconvolution," through either targeted or random mutagenesis of Ub, is actually feasible.

Ubiquitin Sandwich Technique

Nascent polypeptides emerging from the ribosome may, in the process of folding, present degradation signals similar to those recognized by the Ub system in misfolded or otherwise damaged proteins. It has been a long-standing question whether a significant fraction of nascent polypeptides is cotranslationally degraded. Determining whether nascent polypeptides are actually degraded *in vivo* has been difficult, because at any given time the nascent chains of a particular protein species are of different sizes and, therefore, would not form a band on electrophoresis in a conventional pulse-chase assay. The Ub sandwich technique, published in 2000, makes it possible to detect cotranslational protein degradation by measuring the steady-state ratio of two reporter proteins whose relative abundance is established cotranslationally (Turner and Varshavsky, 2000).

Operationally, the Ub sandwich technique (Turner and Varshavsky, 2000) is a three-protein version of the UPR technique described previously. A polypeptide to be examined for cotranslational degradation, termed **B**, is sandwiched between two stable (lacking posttranslational degrons) reporter domains **A** and **C** in a linear fusion protein. The three polypeptides are connected through Ub moieties to yield a fusion protein of the form **A**Ub-**B**Ub-**C**Ub. The independent polypeptides **A**Ub, **B**ub, and **C**Ub that result

from cotranslational cleavages of **A**Ub-**B**Ub-**C**Ub by DUBs are called modules below. The DUB-mediated cleavages establish a kinetic competition between two mutually exclusive events during the synthesis of the **A**Ub-**B**Ub-**C**Ub fusion: cotranslational cleavage at the **B**Ub-**C**Ub junction to release the long-lived **C**Ub module or, alternately, cotranslational degradation of the entire **B**Ub-**C**Ub nascent chain by the 26S proteasome. In the latter case, the processivity of proteasome-mediated degradation results in the destruction of Ub moiety between **B** and **C** *before* it can be recognized by DUBs. The resulting drop in the level of **C**Ub module relative to levels of **A**Ub, referred to as the C/A ratio, reflects the cotranslational degradation of domain **B**. This measurement provides a *minimal* estimate of the total amount of cotranslational degradation, because nonprocessive cotranslational degradation events that do not extend into the **C** domain are not detected. The Ub sandwich method was recently used to demonstrate that more than 50% of nascent protein molecules bearing an N-degron can be degraded cotranslationally in *S. cerevisiae*, never reaching their mature size before their destruction by processive proteolysis (Turner and Varshavsky, 2000). Similar conclusions, through the use of other approaches, were also reached by other groups (Adachi *et al.*, 2004; Reits *et al.*, 2000; Schubert *et al.*, 2000).

If cotranslational protein degradation by the Ub system is found to be extensive for at least some wild-type proteins, it could be accounted for as an evolutionary tradeoff between the necessity of identifying and destroying degron-bearing mature proteins and the mechanistic difficulty of distinguishing between posttranslationally and cotranslationally presented degrons. Cotranslational protein degradation may also represent a previously unrecognized form of protein quality control, which destroys nascent chains that fail to fold correctly. These and other questions about physiological aspects of the cotranslational protein degradation can now be addressed directly in living cells through the Ub sandwich technique.

Concluding Remarks

The Ub fusion technique is made possible by the ability of DUBs to cleave a Ub fusion *in vivo* or *in vitro* after the last residue of Ub irrespective of sequence context downstream from the cleaved peptide bond. Since its development two decades ago, the Ub fusion technique gave rise to a number of applications whose common feature is use of the (largely) cotranslational and highly specific cleavage of a Ub-containing fusion by DUBs. Among these applications are the UPR technique, which increases the accuracy of pulse-chase assays, and the Ub sandwich technique, which makes it possible to determine the extent of cotranslational protein

degradation *in vivo* for any protein of interest. One useful feature of the Ub moiety is its ability, as a part of linear fusions, to increase the yields and solubility of overexpressed proteins or short peptides in either eukaryotic or bacterial hosts. In yet another class of Ub-based applications, the demonstrated coalescence of peptide-size Ub fragments into a quasinative Ub fold yielded the split-Ub assay for detecting protein interactions *in vivo*, an advance that led to the development of several other split-protein assays. Other recent applications include a ubiquitin-based assay for detecting the uptake, by intact cells, of proteins containing ''transduction'' domains that enable the crossing of plasma membrane (Loison *et al*., 2005). Ub fusions continue to be useful in a remarkable variety of ways.

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[52] Heat-Inducible Degron and the Making of Conditional Mutants

By R. JÜRGEN DOHMEN and ALEXANDER VARSHAVSKY

Abstract

Conditional mutants retain the function of a specific gene under one set of conditions, called permissive, and lack that function under a different set of conditions, called nonpermissive; the latter must be still permissive for the wild-type allele of a gene. Such mutants make possible the analysis of physiological changes that follow controlled inactivation of a gene or gene product and can be used to address the function of any gene. Temperature-sensitive (*ts*) mutants, first used in functional studies more than half a century ago, remain a mainstay of genetic analyses. One limitation of the classical *ts* approach is the uncertainty as to whether a given gene can be mutated to yield a *ts* product. Another problem with conventional *ts* mutations is that they are often too leaky to be useful. In 1994, we described a new method, based on a heat-activated degradation signal (degron) that is targeted by the N-end–rule pathway in the yeast *Saccharomyces cerevisiae*. The corresponding mutants were termed *td* (temperature-activated degron) to distinguish them from conventional *ts* mutants. The *td* method requires neither a missense mutation in a gene of interest nor an alteration in its expression patterns. Arg-DHFR^{ts}, a *ts* variant of dihydrofolate reductase–bearing N-terminal Arg residue (a destabilizing residue in the N-end rule) was shown to function as a portable, heat-activated degron, in that Arg-DHFR^{ts} was long-lived at 23° but became short-lived at 37°, owing to activation of its previously cryptic degron. Linking, in a linear fusion, this portable *ts*-degron to a protein of interest results in destruction of the latter at 37°, thereby yielding a *ts* (*td*)

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