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An unstructured initiation site is required for efficient proteasome-mediated degradation

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The proteasome is the main ATP-dependent protease in eukaryotic cells and controls the concentration of many regulatory proteins in the cytosol and nucleus. Proteins are targeted to the proteasome by the covalent attachment of polyubiquitin chains. The ubiquitin modification serves as the proteasome recognition element but by itself is not sufficient for efficient degradation of folded proteins. We report that proteolysis of tightly folded proteins is accelerated greatly when an unstructured region is attached to the substrate. The unstructured region serves as the initiation site for degradation and is hydrolyzed first, after which the rest of the protein is digested sequentially. These results identify the initiation site as a novel component of the targeting signal, which is required to engage the proteasome unfolding machinery efficiently. The proteasome degrades a substrate by first binding to its ubiquitin modification and then initiating unfolding at an unstructured region.

Energy-dependent proteolysis is central to the regulation of many cellular processes, including the cell cycle, signal transduction and the immune response[1]. In eukaryotes, this activity is carried out mostly by a large multi-component protease called the 26S proteasome[1]. The proteasome is composed of two subcomplexes, the 20S core particle and the 19S regulatory particle[2].

The 20S core particle consists of two sets of seven different α and β subunits, which assemble as four heptameric rings. The rings are stacked on top of each other to form a hollow cylindrical structure[3]. Proteolytic sites are located on β subunits in the middle of the complex and, by themselves, show little specificity for their substrates. Selectivity in degradation is achieved by sequestering the proteolytic sites within a central chamber inside the core particle and tightly controlling access. Substrates have to reach the proteolytic chamber through a gated channel that runs along the long axis of the core particle[4]. Several constrictions along the length of the channel prevent folded proteins from entering the proteolytic chamber. Therefore, folded proteins must be at least partially unfolded during degradation[5,6].

The 19S regulatory particle is a multi-subunit complex composed of ~18 polypeptides, 6 of which are ATPases. It associates with the small ends of the core particle at the entrance to the degradation channel. The regulatory particle is involved in substrate recognition, unfolding and translocation into the core particle[7–9], and it controls the gating to the degradation channel[4].

The mechanism by which the proteasome unfolds its substrates is not fully understood. The protease degrades substrates by sequentially unraveling them from their degradation tag, and in doing so it destabilizes folded proteins by disrupting the local structure first encountered by the protease[10,11]. In a plausible mechanism, the proteasome may trap local unfolding fluctuations within the substrate in an ATP-dependent manner, which would lead to the cooperative collapse of the remaining protein structure[10]. ATP is also required for protein translocation, raising the possibility that unfolding and translocation are coupled. In this model, the proteasome may exert a force to pull the bound substrate into the degradation channel, mechanically causing the folded domain to unravel.

Most protein substrates are specifically targeted to the proteasome through the covalent attachment of polyubiquitin chains. Ubiquitination is catalyzed by the successive action of three enzymes or enzyme complexes[12,13]. Ubiquitin-activating enzymes (E1) form a thiol linkage with ubiquitin and transfer ubiquitin to conjugating enzymes (E2). The E2 enzymes then collaborate with substrate-specific ubiquitin protein ligases (E3) to transfer the ubiquitin to the substrate protein. The polyubiquitin chain mediates the productive binding of the substrate to the proteasome[7,14]. During degradation, the ubiquitin moieties are removed by deubiquitinating activities while the substrate remains bound to the proteasome[14,15]. Although ubiquitination of the substrate leads to recognition by the proteasome, it does not ensure rapid degradation of all proteins[14,16]. We have investigated the mechanism by which ubiquitinated substrates are unfolded and degraded by the proteasome. We find that a tightly folded protein is degraded only very slowly even when ubiquitinated unless it contains an unstructured region. This unstructured region, when present, serves as the degradation initiation site and leads to the sequential degradation of the substrate. We conclude that efficient degradation of folded proteins requires two steps: binding to the proteasome through the polyubiquitin tag and, subsequently, engaging of the proteasomal

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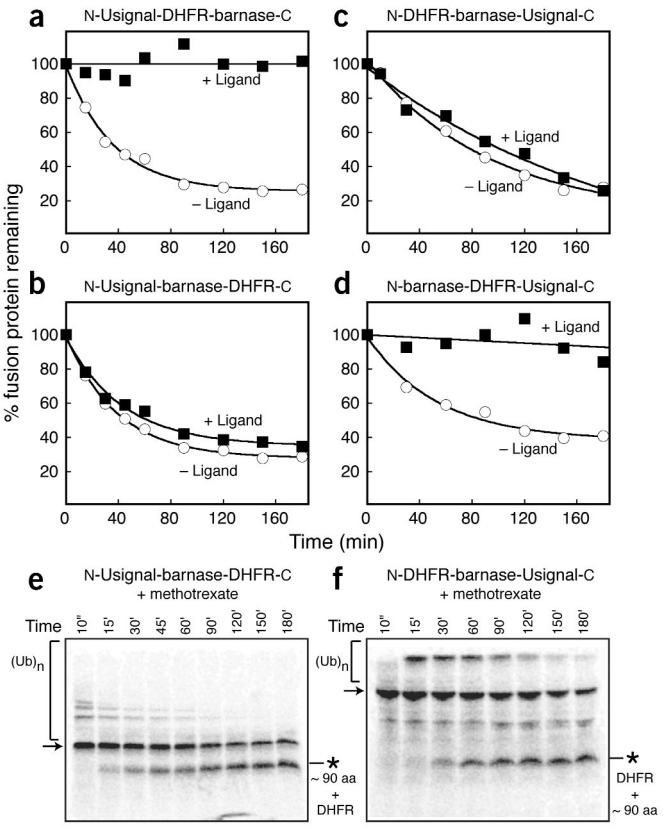

Figure 1 Sequential degradation of two-domain proteins by the proteasome in two directions. (**a**–**d**) Proteasome degradation of N-DHFR-barnase-C fusion proteins (**a**,**c**) or N-barnase-DHFR-C fusion proteins (**b**,**d**) targeted to degradation from the N terminus (**a**,**b**) or from the C terminus (**c**,**d**) at 30 °C. The graphs plot the amount of undegraded fusion protein, including its ubiquitinated forms, as a percentage of the total amount of substrate present at the start of the experiment. All four proteins were degraded by the proteasome (**a**–**d**, ○). Stabilizing the DHFR domain with 320 μM methotrexate (+ Ligand, ■) prevented the degradation of fusion protein only when the domain was located immediately adjacent to the N-terminal ubiquitination signal (Usignal) (**a**) or C-terminal Usignal (**d**). In **d**, quantification includes both full-length protein and fusion protein with cleaved Usignal. When the barnase domain was inserted between the DHFR domain and the Usignal, methotrexate stabilization of DHFR did not prevent the degradation of the full-length protein (**b**,**c**). Instead, the protein was progressively converted to a smaller end product. (**e**,**f**) Proteasome-dependent degradation of N-Usignal-barnase-DHFR-C (**e**) and N-DHFR-barnase-Usignal-C (**f**) in the presence of 320 μM methotrexate is shown as autoradiograms of the SDS-PAGE gels. The arrow indicates the full-length protein, and a square bracket marks its ubiquitinated forms. Asterisk, partially degraded end product corresponding to the size of DHFR plus an additional ~90 amino acids.

unfolding activity through an unstructured region in the substrate protein, which then serves as the initiation site.

RESULTS

Bidirectional proteasome degradation

ATP-dependent proteases degrade their substrates by processively unraveling them from the substrates' degradation signal[10]. In a multidomain protein, the domain adjacent to the degradation signal is degraded first, followed by domains that are downstream. The functional analogs of the proteasome in bacteria seem to be able to degrade substrates in either the N- to C-terminal[10,17,18] or the C- to N-terminal direction[19,20], depending on the position of the degradation signal. Here we present evidence that the proteasome can do so also.

To investigate the direction of proteasomal degradation, we fused ubiquitination signals to the N or C termini of two-domain model proteins. The ubiquitination signals were derived from the well-characterized N-end rule tag[21] for targeting from the N terminus or from the ubiquitination domain of the NFκB precursor protein p105 (ref. 22) for targeting from the C terminus. The two-domain proteins consisted of *Escherichia coli* dihydrofolate reductase (DHFR) and the ribonuclease barnase fused in two orientations to yield N-DHFR-barnase-C or N-barnase-DHFR-C. The resulting proteasome substrates were degraded efficiently (**Fig. 1a–d**).

Degradation of some proteins is inhibited when they are stabilized against unfolding[6,10]. For example, a DHFR domain becomes degradation-resistant when stabilized against unfolding by the tightly binding ligand methotrexate[6]. In a multidomain protein, a stable domain also protects the downstream part of the precursor from proteolysis[10]. In a substrate in which an N-terminal ubiquitination domain is followed by DHFR and then barnase (N-Usignal-DHFR-barnase-C), stabilizing the DHFR domain prevented the degradation of both DHFR and barnase and led to the protection of full-length substrate (**Fig. 1a**). In contrast, when the order of DHFR and barnase in the substrate was reversed so that DHFR was located at the C-terminal end of the construct (N-Usignal-barnase-DHFR-C), stabilizing DHFR did not protect the barnase domain located between the ubiquitination signal and DHFR (**Fig. 1b**). Degradation of this protein in the presence of methotrexate led to the accumulation of a partially degraded fragment (**Fig. 1e**). Thus, we conclude that these fusion proteins were degraded sequentially from the N-terminal ubiquitination signal toward the C terminus, which is consistent with our previously published results[10].

Attaching the ubiquitination signal at the C terminus of the fusion protein yielded corresponding results, indicating that degradation proceeded in the opposite direction, from the C terminus toward the N terminus. For the construct in which barnase at the N terminus is followed by DHFR and then the ubiquitination signal at the C terminus, stabilizing DHFR protected barnase from proteolysis by the proteasome. Degradation led to the accumulation of a barnase-DHFR fusion fragment, expected to be generated by the clipping of the C-terminal ubiquitination signal (**Fig. 1d**, data not shown). In contrast, stabilizing the DHFR domain at the N terminus of a substrate in which DHFR is followed by barnase and then the ubiquitination domain did not protect the barnase domain from degradation (**Fig. 1c**) but led to the accumulation of a much smaller partially degraded fragment (**Fig. 1f**). Independent of the direction of degradation, the partially degraded substrate fragments seemed to consist of DHFR and ~90 amino acids of barnase, as judged by their mobility in SDS-PAGE[10] (**Fig. 1e**,**f** and data not shown).

Separating ubiquitination and initiation sites

The susceptibility of a protein to degradation by ATP-dependent proteases is determined by the stability of the protein's local structure adjacent to the degradation signal[10,11]. Proteins are readily unfolded and degraded when the degradation tag leads into a surface α-helix or an irregular loop, even when the proteins are greatly stabilized. Proteins are much more difficult to unravel when the degradation tag is followed by a buried β-strand[10].

The susceptibility of a protein to degradation by the proteasome can also depend on the direction of degradation if the N- and C-terminal parts of the substrate protein form different local structures. For example, in a circular permutant of DHFR, cpLys38, the N terminus

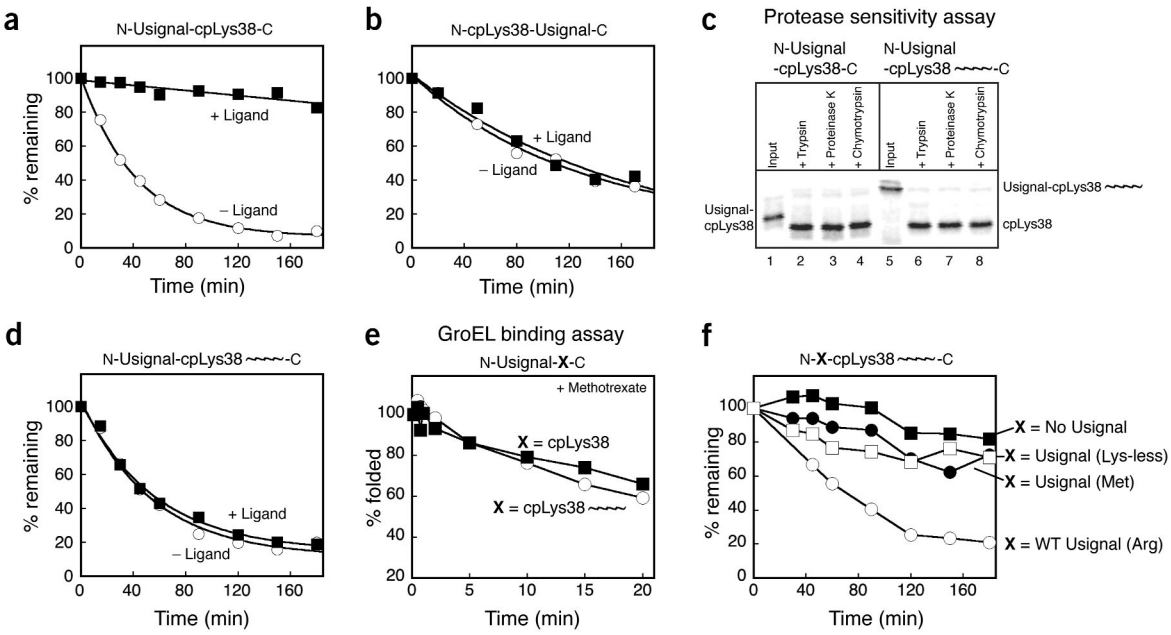

Figure 2 Proteasome degradation is accelerated by an unstructured region in the substrate protein. (**a**,**b**) N-terminally tagged (**a**) and C-terminally tagged (**b**) DHFR mutant cpLys38 were incubated in a proteasome degradation assay at 25 °C in the absence of ligand (○). Stabilization of cpLys38 with 320 μM methotrexate (+ Ligand, ■) prevented its degradation only when targeted from the N-terminal tag (**a**), and the C-terminally tagged protein was degraded rapidly (**b**). (**c**) The N-terminal ubiquitination signal in N-Usignal-cpLys38 (lanes 1–4) as well as the C-terminal region in N-Usignal-cpLys38~~~~C (lanes 5–8) appear disordered and are sensitive to digestion by nonspecific proteases. Protease sensitivity was assayed by incubating the protein with the indicated protease at 4 °C for 1 min. (**d**) N-terminally tagged cpLys38 containing a C-terminal unstructured region was degraded efficiently by the proteasome in the absence (○) or presence of 320 μM methotrexate (■). Thus, the addition of a C-terminal unstructured region significantly accelerated the degradation of N-terminally tagged cpLys38 and completely reversed its methotrexate-induced degradation block. (**e**) An unstructured region does not affect spontaneous unfolding of cpLys38. The rate of spontaneous unfolding of N-Usignal-cpLys38-C (■) and N-Usignal-cpLys38~~~~C (○) was followed in the presence of 100 μM methotrexate as measured by GroEL binding assay at 15 °C. (**f**) Degradation of cpLys38 containing a C-terminal unstructured region depends on ubiquitination at Lys15 and Lys17. N-Usignal-cpLys38~~~~C (○), N-cpLys38~~~~C (■), N-Usignal(Met)-cpLys38~~~~C (●) in which the N-end residue has been mutated from arginine to methionine, and N-Usignal(Lys-less)-cpLys38~~~~C (□) in which Lys15 and Lys17 in the N-end rule tag were mutated to arginine, were degraded by the proteasome at 25 °C in the presence of 320 μM methotrexate.

leads into a buried β-sheet whereas the C terminus leads into a surface α-helix[10]. This protein was degraded efficiently when the ubiquitination signal was fused to its N terminus (N-Usignal-cpLys38-C, **Fig. 2a**) or its C terminus (N-cpLys38-Usignal-C, **Fig. 2b**). Degradation from the N terminus was completely inhibited when the protein was stabilized against global unfolding by methotrexate (**Fig. 2a**), whereas degradation from the C-terminal ubiquitination signal was unaffected by methotrexate binding (**Fig. 2b**). Thus, the fate of cpLys38 depends on the site at which the proteasome initiates degradation.

This conclusion raises the question whether the site of ubiquitination is always the site at which proteasome degradation initiates. The 19S regulatory particle can bind to proteins that lack a ubiquitin modification if these proteins are unfolded or misfolded[9,23], and degradation signals targeting proteins to prokaryotic ATP-dependent proteases are unstructured peptide sequences[24]. Therefore, we speculated that an unstructured region in a proteasome substrate could serve as the degradation initiation site. To test this hypothesis, we attached a lysine-less tail to the C terminus of a protein consisting of an N-terminal ubiquitination signal followed by cpLys38 (yielding N-Usignal-cpLys38~~~~C). Notably, this protein was degraded efficiently even when stabilized by methotrexate (compare **Fig. 2a** to **Fig. 2d**). The C-terminal tail seems to be disordered in the fusion protein, as it was highly sensitive to trypsin, proteinase K and chymotrypsin digestion (**Fig. 2c**) and showed a characteristically increased hydrodynamic radius compared with a natively folded protein as measured by gel filtration experiments[25] (data not shown). The unstructured tail did not simply destabilize cpLys38 against spontaneous global unfolding. The stabilities of cpLys38 with and without the unstructured region are similar as assessed by a GroEL binding assay[26] (**Fig. 2e**). Also, addition of the unstructured region did not change the position of the ubiquitination site. Ubiquitination of the substrate with the C-terminal unstructured region still occurred at the N-terminal ubiquitination signal. The protein was stabilized when the N-end rule tag was inactivated by the mutation of the N-end residue from arginine to methionine[27], by mutating the ubiquitin acceptor lysines, Lys15 and Lys17, in the linker region to arginine[27], or by deleting the N-terminal ubiquitination site entirely (**Fig. 2f**).

The N-terminal and C-terminal regions of wild-type DHFR both form buried β-strands. This protein is efficiently degraded when targeted to the proteasome by an N-terminal degradation tag, and methotrexate stabilization prevents its degradation[6] (data not shown). Attaching an unstructured region to the C terminus did not release the methotrexate-induced block to degradation but instead led to clipping of the protein (**Fig. 3a**,**b**,**d**). To determine the site of the clipping, we attached a His_6 sequence to the C terminus of the protein as an epitope tag. An immunoblot against the C-terminal tag showed that the protein is clipped at the C terminus (**Fig. 3c**,**d**). Together, these results suggest that degradation of methotrexate-stabilized N-Usignal-cpLys38~~~~C and N-Usignal-DHFR~~~~C initiates at the C-terminal unstructured region and not at the ubiquitination site.

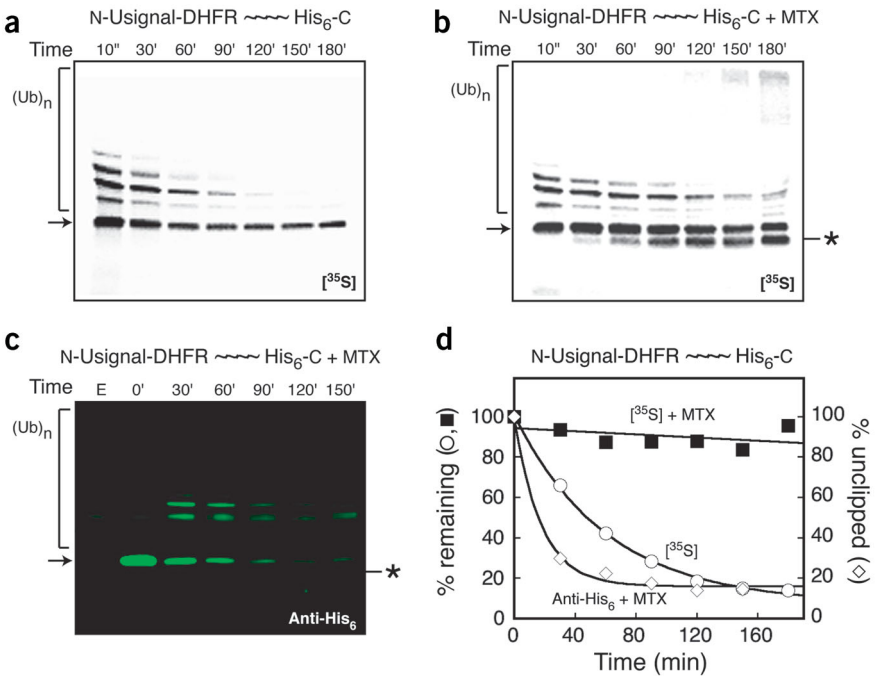

Figure 3 Proteasome degradation initiates at an unstructured region in the substrate protein. (**a**,**b**) Proteasome degradation of N-Usignal-DHFR~~~~His_6-C at 25 °C in the absence (**a**) or presence (**b**) of 320 μM methotrexate (MTX) is shown as autoradiograms of SDS-PAGE gels separating the reaction products. Each lane represents the amount of protein remaining at the indicated time points. Arrow, bands representing full-length substrate protein; square bracket, ubiquitinated species. Stabilization of DHFR with MTX prevented the complete degradation of the full-length protein and led to the clipping of the substrate. The asterisk in **b** indicates the truncated degradation end product that forms when DHFR is stabilized with 320 μM MTX. (**c**) Western blot, using antibody to His-tag, of the degradation of N-Usignal-DHFR~~~~His_6-C at 25 °C in the presence of 320 μM MTX. Lane E contains reticulocyte lysate as background control. The degradation end product is not recognized by antibodies specific to His-tag, demonstrating that the full-length protein was truncated at its C terminus. The asterisk in **c** indicates the expected position of the degradation end product as seen by autoradiography in **b**. (**d**) Quantification of autoradiograms from **a** (○) and **b** (■) and western blot from **c** (◇). In the presence of MTX, N-Usignal-DHFR~~~~His_6-C is rapidly clipped at the C-terminal unstructured region without the degradation of the DHFR domain. Undegraded (○, ■) or unclipped (◇) substrate, including ubiquitinated forms, at each time point are plotted as a percentage of the total substrate present at the beginning of the time course.

Requirement for unstructured initiation sites

The results described above suggest that degradation can begin at an unstructured region in a substrate that is separated from the ubiquitination site in primary structure. This finding raises the question whether an unstructured region is always required for the efficient degradation of stably folded substrate proteins. A DHFR construct with the N-end rule degradation signal becomes ubiquitinated on Lys15 or Lys17 of the 40-residue tag[27], and mutating the two lysines to arginine seemed to abolish ubiquitination (data not shown). The tag itself is largely unstructured (**Fig. 2c**), and either the first 14 amino acids preceding the lysines or the 22 amino acids following the lysines could serve as the site at which the proteasome engages the substrate protein. Truncating the N-end rule tag by deleting residues 25–40 of the tag considerably stabilized the DHFR construct against proteasome degradation both *in vitro* (compare N-Usignal-DHFR-C and N-UsignalΔ15-DHFR-C, **Fig. 4a**) and *in vivo*[27]. The slower degradation of the truncated substrate, however, was not caused by reduced ubiquitination, and the truncated substrate was ubiquitinated at the same rate and to the same extent as the full-length substrate (**Fig. 4b–d**). Also, degradation of the truncated substrate was completely restored when either of two unrelated unstructured tails lacking any lysine residues were attached at its C terminus (N-UsignalΔ15-DHFR~~~~~-C and N-UsignalΔ15-DHFR-^^^-C) (**Fig. 4a**). The three proteins N-Usignal-DHFR-C, N-UsignalΔ15-DHFR-C and N-UsignalΔ15-DHFR~~~~~-C have similar stabilities against global unfolding as measured by GroEL binding assay[26] (data not shown). Together, these results suggest that the unstructured region in the N-end rule degradation tag is required for efficient degradation by the proteasome.

To design a substrate that lacks an unstructured region completely, we constructed a protein that is targeted to the proteasome by a concatamer of four ubiquitin moieties fused to each other in frame[14,28]. When this tetraubiquitin tag was fused directly to DHFR, the resulting fusion protein (N-Ub_4-DHFR-C) was degraded only inefficiently (**Fig. 4e**). Attaching either of two unstructured extensions to the C terminus of the substrate (N-Ub_4-DHFR~~~~~-C and N-Ub_4-DHFR-^^^-C) led to its rapid degradation (**Fig. 4e**). Unfolding of DHFR was still required, as methotrexate binding prevented the degradation of N-Ub_4-DHFR~~~~~-C (data not shown). The amino acid sequences of the two degradation initiation sites tested here were unrelated. Therefore, it is improbable that degradation initiation requires the presence of a specific sequence motif that is recognized by the proteasome. Additional experiments, in which parts of the initiation sites were deleted, suggested that stretches of as few as 20 amino acids can serve as degradation initiation sites (data not shown). From these results, we conclude that rapid degradation of a folded protein by the proteasome requires the presence of an unstructured region in addition to a ubiquitin modification. Degradation initiates at the unstructured region, and the region presumably serves to engage the unfolding activity of the proteasome.

Internal initiation sites

Next we wanted to determine any restrictions on the location of the unstructured region in the substrate protein and whether free N or C termini were required. We constructed a fusion protein consisting of a short N-terminal ubiquitination signal followed by cpLys38 and then by barnase (N-UsignalΔ15-cpLys38-barnase-C). Stabilizing the cpLys38 domain by methotrexate prevented its N- to C-terminal degradation and hence protected both cpLys38 and barnase from proteolysis (**Fig. 5**). When the unstructured region was placed between the two domains (N-UsignalΔ15-cpLys38~~~~barnase-C), the protein was degraded rapidly even when cpLys38 was stabilized with methotrexate (**Fig. 5**). Presumably, degradation of this precursor initiated at the internal unstructured segment. In agreement with an earlier study[29], this result suggests that a free N or C terminus is not required for proteasome degradation. Attaching an unstructured region to the C terminus of the two-domain protein (N-UsignalΔ15-cpLys38-barnase~~~~~-C) stimulated degradation only mildly. A possible explanation could be that the unstructured region in a substrate

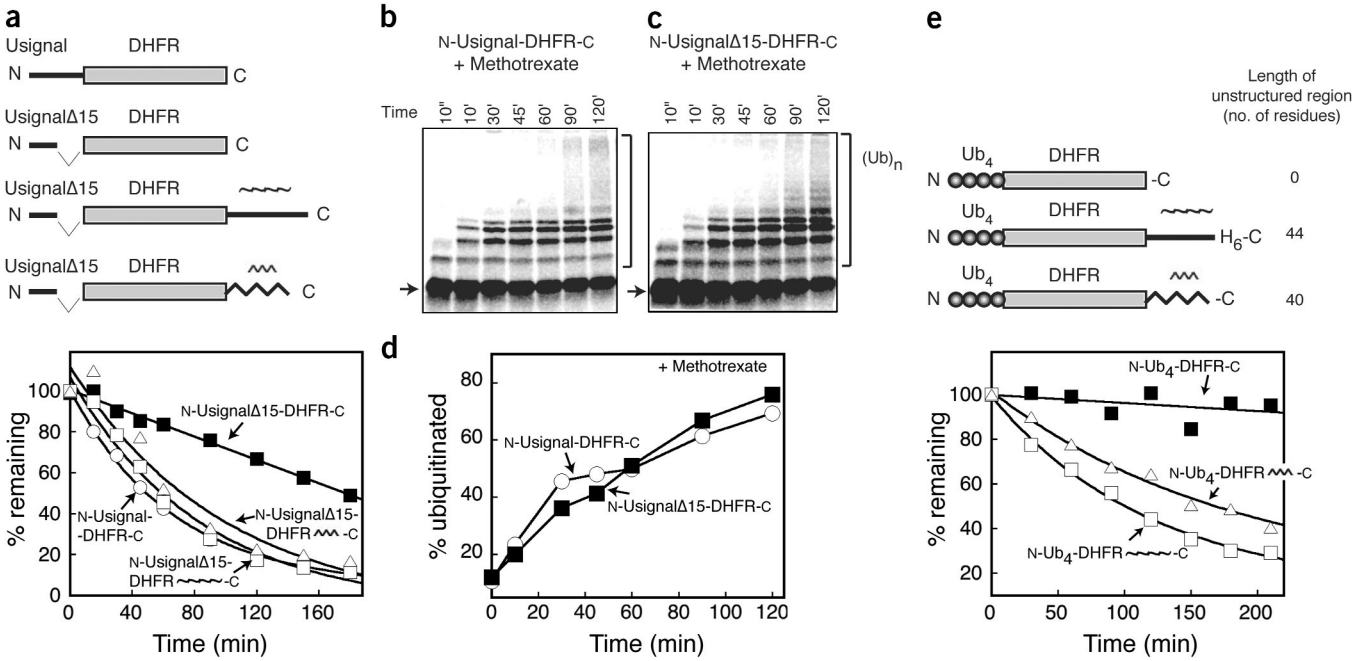

Figure 4 Tightly folded substrates must contain an unstructured region for efficient proteasome degradation. (**a**) Degradation of N-Usignal-DHFR-C requires the unstructured region of the Usignal. Deletion of the portion of unstructured Usignal following the ubiquitin acceptor lysines, N-UsignalΔ15-DHFR-C, substantially stabilized the protein against degradation compared with N-terminally tagged DHFR at 25 °C. This stabilization was overcome when an unstructured region was added to the C terminus of the protein carrying the deletion to create N-UsignalΔ15-DHFR~~~C or N-UsignalΔ15-DHFR-ᔓC. (**b**,**c**) The truncation of the Usignal did not alter the ubiquitination of the substrate. The ubiquitination rate of N-Usignal-DHFR-C (**b**) and N-UsignalΔ15-DHFR-C (**c**) in reticulocyte lysate was measured in a ubiquitination assay in the presence of 320 μM methotrexate. The SDS-PAGE gels resolve the ubiquitinated forms (square bracket) from the nonubiquitinated protein (arrow), and the lanes represent the progress of the ubiquitination reaction. (**d**) Quantification of autoradiograms from **b** and **c**. The amount of ubiquitinated species at the indicated times was quantified and plotted as a percentage of the initial nonubiquitinated protein. (**e**) Degradation of N-Ub_4–tagged DHFR-C by proteasome at 25 °C. N-Ub_4-DHFR-C lacks any unstructured region and was degraded very inefficiently. Degradation was accelerated several-fold when an unstructured region was added to the C terminus of the protein, N-Ub_4-DHFR~~~C and N-Ub_4-DHFR-ᔓC.

protein has to be located near to the ubiquitination site to serve as an efficient initiation site for degradation.

DISCUSSION

The eukaryotic proteasome degrades a wide range of cellular proteins that differ greatly in their structures, stabilities and functions. Most of these proteins are targeted for degradation by ubiquitination, and the ubiquitination sites[30] can be found near the substrates' N termini[31,32], near their C termini[33] or internally[22,34]. We reported previously that the proteasome can degrade substrate proteins sequentially by engaging a substrate near an N-terminal ubiquitination site and running along the polypeptide chain toward the C terminus. Here we show that the proteasome can also degrade substrates in the opposite direction, from the C terminus toward the N terminus. The direction of degradation is not strictly determined by the location of the ubiquitin modification on the substrate, and degradation does not necessarily initiate at the ubiquitination site. For example, proteolysis of a substrate that is ubiquitinated at its N terminus can begin at an unstructured region provided at the C terminus. Degradation then proceeds from the C terminus toward the N-terminal ubiquitination site (**Fig. 2**).

The presence of an unstructured region in a folded proteasome substrate greatly enhances the efficiency with which the protein is degraded (**Fig. 4**). Our results suggest that ubiquitination alone is not sufficient for the efficient degradation of tightly folded proteins. Instead, it seems that the proteasome binds its substrate through the ubiquitin chain and then engages the protein at an unstructured region. This requirement for an unstructured initiation site is somewhat reminiscent of the mechanism by which helicases engage their substrates. Helicases are multidomain ATPases that are also sometimes multimeric and that separate the strands of nucleic acid double helices processively. Helicases require a single-stranded region in their substrates as the initiation site to unwind the double-stranded remainder of the nucleic acid[35]. In at least one helicase, the initiation site can be clearly separated from the substrate recognition site[36]. The mechanism we propose for the proteasome is also reminiscent of the way in which some prokaryotic ATP-dependent proteases engage their substrates through adaptor proteins such as SspB[37] or UmuD[38]. For example, the protease ClpXP binds its substrate UmuD′ indirectly by using UmuD as an adaptor and then engages the substrate directly through a defined initiation site[38]. In other protease substrates, such as SsrA-tagged proteins, the substrate recognition sites and initiation sites seem to be either overlapping or close to each other[38]. The latter scenario is similar to the way precursor proteins engage the mitochondrial unfolding and import machinery, where the recognition and initiation sites in the mitochondrial targeting sequence are not easily separated.

The proteasome can actively unfold proteins[9,10] and degrade them faster than they would unfold spontaneously by trapping local unfolding fluctuations in the substrate[10,11]. We propose that the proteasome is only able to trap these local fluctuations efficiently after it has engaged its substrate fully through an unstructured region, possibly by threading it into the degradation channel. In the absence of an unstructured region, much larger and therefore rarer unfolding

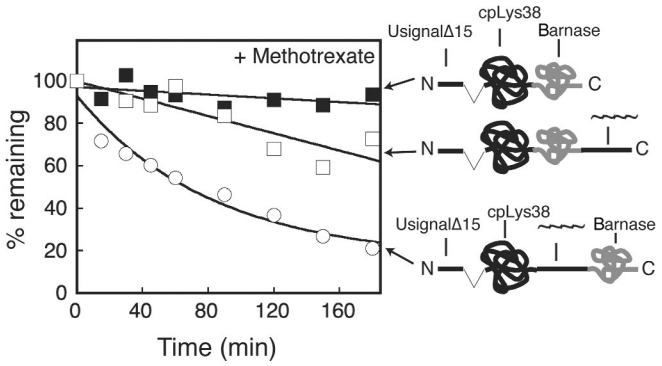

Figure 5 Characterization of degradation initiation sites. Degradation of N-terminally tagged cpLys38-barnase fusion protein by the proteasome at 25 °C in the presence of 320 μM methotrexate. Stabilizing cpLys38 with methotrexate prevents the N- to C-terminal sequential degradation of N-UsignalΔ15-cpLys38-barnase-C (■). Under the same conditions, the fusion protein containing an unstructured degradation initiation site between cpLys38 and barnase domain, N-UsignalΔ15-cpLys38~~~~barnase-C, was degraded efficiently in the presence of methotrexate (○). However, adding the unstructured region to the C terminus of the fusion protein to create N-UsignalΔ15-cpLys38-barnase~~~~C did not accelerate degradation (□).

fluctuations may have to occur in a folded substrate for it to fully engage the proteasome. Thus, the requirement for an unstructured region should become greater when a substrate is more stable against global unfolding.

The requirement of an unfolded region for efficient proteasomal degradation of tightly folded proteins may explain some unexpected experimental observations. For example, a chain of four ubiquitin moieties linked through Lys48 is a sufficient targeting signal for degradation by the proteasome[14]. However, attachment of tetraubiquitin to a ubiquitin-DHFR fusion protein led to notably slow degradation of this protein in an *in vitro* assay[14]. The explanation for this observation could be that the substrate lacked an appropriate unstructured region to serve as the degradation initiation site. In addition, there are several examples of authentic cellular proteins that associate with the proteasome but escape degradation. For example, the cell division cycle regulator Cdc34 and the Hsc70 cochaperone BAG-1 are extensively ubiquitinated and recognized by the proteasome but are not degraded[15,39,40]. Similarly, the adaptor proteins Rad23 and Dsk2 bind both proteasome and substrates but avoid degradation themselves[41–43]. A possible explanation of these observations is that the proteins lack appropriate degradation initiation sites. However, inspection of the domain structure of the proteins in the Pfam database[44] suggests that they contain extensive unstructured regions. Three different explanations are possible. First, the unstructured regions may be located too far away from the ubiquitination sites or proteasome interaction domains to function as effective initiation sites. This situation would be analogous to that observed with the model protein in which the ubiquitination site and unstructured region were separated by two folded domains (N-UsignalΔ15-cpLys38-barnase~~~~C). This protein was degraded only slowly, presumably because of the large physical distance between proteasome binding and degradation initiation sites (**Fig. 5**). A second potential explanation has been proposed in the context of the degradation of the cyclin-dependent kinase inhibitor Sic1 (ref. 16). Sic1 can become ubiquitinated at any of its 20 lysine residues, but only ubiquitination near the N terminus leads to its rapid degradation[16]. It is possible that the proteasome can engage the substrate at several unstructured regions but that the structure of the folded domain of Sic1 is more easily unraveled from the initiation site close to the N-terminal lysines[16], just as the DHFR mutant cpLys38 is more easily unfolded from the C terminus than the N terminus (**Fig. 2**). This scenario could also explain the behavior of the transcription factor inhibitor IκBα. The protein is ubiquitinated on Lys21 and Lys22 of a ~70-residue N-terminal region, but degradation requires the presence of a 40-residue tail at its C terminus[45]. The C-terminal tail is not involved in the ubiquitination step but has a second, unknown function, possibly to serve as a degradation initiation site. Finally, a third possible explanation for the experimental observations listed earlier is that the proteasome may exhibit sequence specificity such that the effectiveness of degradation initiation sites depends on their amino acid composition. For example, the proteasome might be unable to initiate degradation of IκBα at its N terminus because the amino acid sequence does not interact well with the proteasome.

In summary, we demonstrate that polyubiquitination of proteins, though necessary, is not sufficient for rapid degradation of tightly folded proteins. Their efficient degradation requires an additional unstructured region that serves as the initiation site to engage the proteasomal unfolding machinery (**Fig. 6**). Once the substrate has fully engaged the proteasome through its unstructured region, the substrate is unfolded and degraded. In a competing process, the proteasome-associated deubiquitinating enzymes continuously remove the ubiquitin moieties from the bound substrate, releasing the substrate from the complex[46,47]. Therefore, failure to successfully engage the proteasome unfolding machinery would eventually lead to substrate dissociation. Even a substrate that fully engages the proteasome can be released if the degradation is blocked or slowed down considerably by a particularly stable domain. This dissociation would result in a partially degraded substrate and seems to be the mechanism for the processing of some transcription factors, such as NFκB and Spt23p[10]. Our results indicate that differences in substrate structure can have important

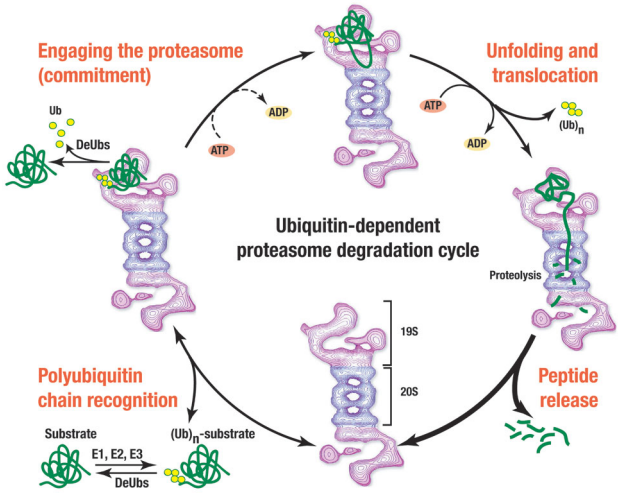

Figure 6 Schematic representation of the proposed proteasome degradation cycle. Polyubiquitinated proteins bind to the proteasome through the ubiquitin chain. Unfolding and degradation, however, ensue only after the substrate has engaged the proteasome through an unstructured domain or the degradation initiation site. Once the substrate is engaged, it is degraded sequentially along the polypeptide chain from the degradation initiation site. The EM reconstruction of the proteasome is reproduced from Larsen and Finley[54].

consequences in determining fate, providing another mechanism by which the proteasome can achieve specificity of degradation.

METHODS

Substrate proteins. Protein substrates were derived either from barnase, a ribonuclease from *Bacillus amyloliquefaciens*[48] or from *E. coli* DHFR[49]. The circular permutant of DHFR cpLys38 was constructed by connecting the N and C termini of *E. coli* DHFR with a linker consisting of six glycine residues and rearranging the gene so that the resulting protein started with what was formerly Lys38 and ended with Asn37 (ref. 50). Ubiquitination at the N terminus of substrate proteins occurred through the N-end rule pathway[51]. In these constructs, an ubiquitin moiety was attached to the N terminus of the substrate protein via a 40-residue linker derived from lac repressor[6]. In reticulocyte lysate, the ubiquitin moiety is rapidly cleaved, and a new N terminus is formed at the amino acid that follows the last residue of the ubiquitin sequence. Depending on the identity of the new N-terminal amino acid, the substrates are then ubiquitinated at two lysine residues in the linker region[27]. The C-terminal ubiquitination signal consisted of amino acids 352–646 of the NFκB precursor protein, p105 (ref. 22). The Ub_4 degradation signal was constructed by connecting four ubiquitins through their N and C termini. The signal was then attached directly to the N terminus of DHFR. The ubiquitin moieties in the concatamer contain G76V mutations to prevent their deconjugation. The unstructured regions were derived from residues 1–95 of cytochrome b_2 (~~~~) or residues 1–40 of lac repressor (^^^). These regions seem to be disordered, as judged by their increased sensitivity to unspecific proteases and by their increased hydrodynamic volume[25]. All proteins were constructed using standard molecular biology techniques in pGEM-3Zf(+) vectors (Promega) and verified by DNA sequencing.

Radioactive proteins were expressed from a T7 promoter by *in vitro* transcription and translation in reticulocyte lysate (Promega), supplemented with [^{35}S]methionine. Arg-Ala dipeptide (12 mM) was included during the translation of N-end rule substrates to prevent their ubiquitination and degradation in the translation mix. Proteins were then partially purified by high-speed centrifugation and ammonium sulfate precipitation as described[52]. N-Usignal-DHFR~~~~His_6-C protein for western blot analysis was expressed from the pGEM-3Zf(+) T7 promoter in *E. coli* strain BL21 (DE3) pLysS and purified from clarified cell lysate by affinity chromatography using a 1-ml Ni^{2+}-NTA agarose column (Qiagen) according to the supplier's protocol.

Proteasome degradation assays. Degradation by the proteasome was followed in rabbit reticulocyte lysate (Green Hectares) that had been ATP-depleted as described[53]. *In vitro*–translated protein was precipitated by ammonium sulfate, resuspended in one-fifth reaction volume of resuspension buffer (25% (v/v) glycerol, 25 mM $MgCl_2$, 0.25 M Tris-HCl, pH 7.4) and diluted five-fold into ATP-depleted reticulocyte lysate supplemented with 1 mM DTT, 5 mM methionine and 25 μM ubiquitin. Methotrexate was included in the reaction wherever mentioned, and the control reactions contained an equal volume of DMSO. The reaction was prewarmed at 25 °C for 3 min, and ubiquitination and degradation were initiated by addition of ATP and an ATP regeneration system (0.5 mM ATP, 10 mM creatine phosphate, 0.1 mg ml^{-1} creatine phosphokinase). Incubation was continued at 25 °C, and at the indicated times equal-volume samples were withdrawn from the reaction mix and added to SDS-PAGE sample buffer to stop the reaction. Samples were resolved by SDS-PAGE, and the radioactive proteins were analyzed and quantified by electronic autoradiography (Instant Imager; Packard). The His_6-tagged proteins were analyzed by immunoblotting using rabbit antibodies to His_6 (Bethyl Labs) and Alexa Fluor 680–labeled secondary antibody specific to rabbit (Molecular Probes) and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

Protease sensitivity assay. ^{35}S-labeled DHFR proteins were incubated at 4 °C in 50 mM Tris-Cl, pH 7.4, containing 100 μM methotrexate, 5 mM $MgCl_2$, 5% (v/v) glycerol and 0.1 mg ml^{-1} trypsin or proteinase K or chymotrypsin. The reaction was stopped at the indicated times with two volumes of 10% (w/v) TCA, and the TCA-insoluble precipitate was solubilized in SDS-PAGE sample buffer. The products were analyzed by denaturing PAGE and electronic autoradiography.

Ubiquitination assays. The condition for the ubiquitination assay of N-Usignal-DHFR-C and N-UsignalΔ15-DHFR-C was similar to the proteasome degradation assay except that 2 μM Ubal (Calbiochem) was included to inhibit the deubiquitinating enzymes. To prevent the degradation of the protein, 320 μM methotrexate was included in the reaction. The ubiquitination rate was measured by quantifying the amount of ubiquitinated protein as a percentage of the total amount of nonubiquitinated substrate at the start of the experiment.

Stability assays. The stability of substrate proteins against global unfolding was assessed using a GroEL binding assay[26]. Substrate proteins were resuspended in GroEL buffer (50 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 10 mM KCl, 100 μM methotrexate, 5 mM $MgCl_2$) and incubated at the indicated temperature. The reaction was started by adding 1 μM $GroEL_{14}$ to the reaction mix. At different time intervals 10-μl samples were withdrawn and added to protease buffer (GroEL buffer supplemented with 0.1 mg ml^{-1} proteinase K, 100 μM methotrexate and 1 mM DTT) kept at 4 °C. After 10 min the reaction was stopped by TCA precipitation, and the samples were analyzed by SDS-PAGE and electronic autoradiography (Instant Imager; Packard).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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