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Proteasomal Degradation from Internal Sites Favors Partial Proteolysis *via* Remote Domain Stabilization

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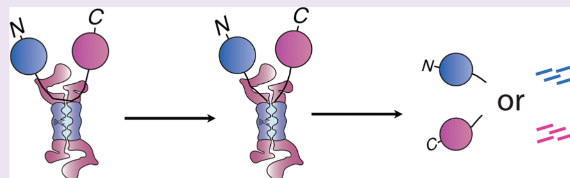
Supporting Information

ABSTRACT: The ubiquitin-proteasome system controls the concentrations of hundreds of regulatory proteins and removes misfolded and damaged proteins in eukaryotic cells. The proteasome recognizes ubiquitinated proteins and then engages its substrates at unstructured initiation regions. After initiation, it proceeds along the polypeptide chain, unraveling folded domains sequentially and degrading the protein completely. *In vivo* the proteasome can, and likely often does, initiate degradation at internal sites within its substrates, but it is not known how this affects the outcome of the degradation reaction. Here we find that domains flanking the initiation region can protect each other against degradation without interacting directly. The magnitude of this effect is related to the stability of both domains and can be tuned from complete degradation to complete protection of one domain. Partial proteasomal degradation has been observed in the cell in three signaling pathways and is associated with internal initiation. Thus, the basic biochemical mechanism of remote stabilization of protein domains is important in proteasome biology.



The ubiquitin-proteasome system (UPS) is the major cytosolic and nuclear mediator of protein turnover in eukaryotes. It controls many cellular processes through targeted degradation of transcription factors and other regulatory proteins and degrades misfolded proteins as part of the cell's stress response.[1−3]

A two-part degron targets proteins to the proteasome for efficient degradation.[4−6] The first part is a proteasome-binding tag, typically a polyubiquitin chain that is added to one or more lysines within the substrate through the action of E1, E2, and E3 enzymes. The second part is an unstructured initiation region, which functions best when it is close in space to a polyubiquitin chain, as displacing it by ~40 Å largely inhibits degradation of model substrates.[7] The ubiquitin tag is recognized by receptors in the proteasome's 19S regulatory cap, and the initiation region is likely engaged by ATPase motors at the base of the cap.[1,8] The motors pull at the initiation region, which leads to the sequential unfolding and translocation of the substrate to the protease active sites buried within the 20S proteasome core particle. Thus, degradation typically begins at the initiation region and proceeds linearly along the polypeptide chain.[6] The proteasome denatures any folded domains as it encounters them so that the end product is the complete degradation of the substrate into small peptides.[9] This processivity prevents the formation of fragments that could have undesired biological activities.

The proteasome can also initiate degradation at internal unstructured loops within larger proteins,[6,10−15] and this appears to be common in cells. However, we do not know how the location of the initiation region affects the rest of the degradation reaction and its end products. The proteasome can degrade circular and disulfide-linked proteins, indicating that the channel that leads to the degradation chamber can accommodate more than one polypeptide chain at once,[10,16] but crystal structures of the proteasome core particles show that the channel will be a tight fit for two chains.[17] This tight fit or the load put on the proteasome by the simultaneous presence of two polypeptide chains and their folded domains could reduce the effectiveness of the unfolding and degradation machinery and thus the proteasome's processivity.

In a few cases, proteins are degraded incompletely by the proteasome in a process referred to as proteasomal processing. The known physiological examples of processing are the p105 and p100 precursors of the p50 and p52 subunits of the mammalian transcription factor NFκB, which functions in immune and inflammatory responses, the yeast Spt23 and Mga2 transcription factors, which are distantly related to NFκB and regulate unsaturated fatty acid biosynthesis, and the *Drosophila* transcription factor Cubitus interruptus (Ci) and its vertebrate homologues Gli2 and Gli3, which function in Hedgehog signaling.[18−25] Partial degradation of these proteins releases fragments with new biological activities. In the case of p105, p100, Spt23, and Mga2, an inert precursor protein is converted into an active transcription factor, whereas in the case of Ci and the Gli proteins, a transcriptional activator is converted into a competitive repressor of transcription. Thus, proteasomal processing represents an additional layer of post-translational regulation that can be used to control biological activity and cellular fate. However, the biochemical mechanism of this processing reaction is only poorly understood, and this is a major stumbling block to the discovery of other examples.

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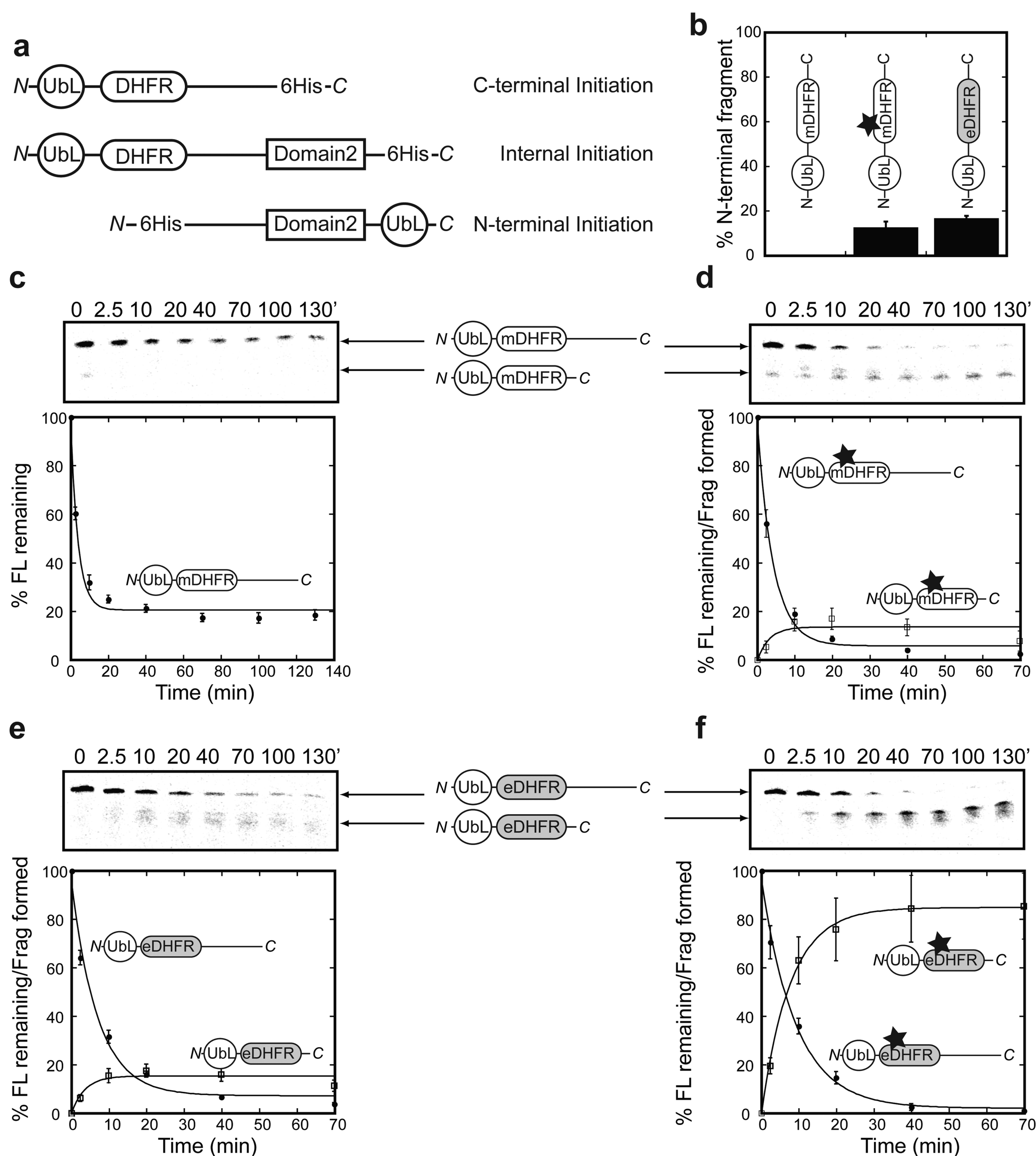

Figure 1. Model substrates for internal initiation. (a) Schematic depiction of degradation substrates, which contain a UbL domain for targeting to the proteasome and either an internal initiation region flanked by two folded domains or lacking a domain on one side of the initiation region. (b) DHFR fragment formation (percent of degraded protein that is converted to fragment) for substrates lacking a C-terminal flanking domain. Star indicates MTX bound to DHFR. (c−f) Representative degradation assays and quantification for UbL-mDHFR— (c), UbL-mDHFR·MTX (d), UbL-eDHFR (e), and UbL-eDHFR·MTX (f). Full-length protein is indicated by filled circles, fragment by open squares. The 26S proteasomal degradation assays were conducted using 20 nM purified proteasome in excess of trace radiolabeled substrate at 30 °C. Error bars represent the SEM of 4 experiments.

Internal initiation may play an important role in processing. For Spt23 and Mga2, degradation must begin internally because both ends of the polypeptide chain are blocked: the N-terminus by the tightly folded IPT domain that is released by the processing reaction and the C-terminus by a membrane anchor.[11,22,23] The ubiquitination sites responsible for processing of p105, Ci, and Gli3 are in the middle of the full-length polypeptide chain,[26−28] and thus the proteasome likely initiates their degradation internally as well. Ci also contains degrons near its termini, but these do not lead to processing.[29−31]

Here we determine whether internal initiation is directly related to processing by following how the structure of model substrate proteins affects proteasome processivity in a purified degradation system. We discover that domains flanking the initiation region stabilize each other without interacting directly. This remote stabilization decreases processivity and can tune

proteasomal degradation from complete proteolysis to almost quantitative fragment formation.

■ RESULTS AND DISCUSSION

Model Substrates To Study Internal Initiation. We examined how the processivity of the proteasome is affected when it initiates degradation in the middle of a polypeptide chain by following the proteolysis of a series of model substrates by purified proteasome. The substrates were designed to allow the proteasome to initiate at a free end (*i.e.*, the N- or C-terminus) or to force it to initiate at an internal linker region flanked by two folded domains (Figure 1a). Each substrate had a proteasome-binding tag derived from the ubiquitin-like domain (UbL) of Rad23.[32,33] We used a UbL domain instead of a polyubiquitin chain because the UbL is better defined, as it consists of a single moiety whose placement within the substrate we can control. For the flanking domains, we used a range of small proteins with well-defined structures. At the N-terminus, we used dihydrofolate reductase (DHFR) either from mouse (mDHFR) or *E. coli* (eDHFR) and at the C-terminus we used *B. amyloliquefaciens* barstar or barnase, or the immunoglobulin domain 27 of human titin.[34−36] The initiation region was derived from the mitochondrial targeting sequence of *S. cerevisiae* cytochrome b_2, which has been used extensively in *in vitro* degradation experiments[6,7,15] (Figure 1a). Substrates were transcribed and translated *in vitro* and then purified by affinity chromatography.

Blocking the C-Terminus of an Initiation Region with a Folded Domain Stabilizes the N-Terminal Domain against Degradation. A substrate consisting of an N-terminal UbL domain followed by mDHFR and an ∼200 amino acid initiation region (UbL-mDHFR—, where — signifies the initiation region) was degraded efficiently by purified yeast proteasome under single-turnover conditions without the formation of any stable fragments (Figure 1b,c). Stabilizing mDHFR by adding the tight-binding ligand methotrexate (MTX)[37] or replacing mDHFR with the more stable eDHFR[38] led to the formation of a small amount of fragment corresponding to the UbL and DHFR domains with a short tail (UbL-DHFR) upon degradation of the full-length protein (Figure 1b,d). About 85% of the times that the proteasome encountered DHFR, it unfolded the domain and proceeded to digest the substrate completely, while the remaining 15% of the times the DHFR domain escaped from the proteasome to be released as a fragment (Figure 1b,e). Further stabilizing eDHFR with MTX led to essentially quantitative release of a DHFR fragment[9,37] (Figure 1f). Degradation was by the proteasome because the reaction was blocked by the proteasome inhibitor MG132 (Supplementary Figure 1).

Next, we blocked the free C-terminus of the initiation region by attaching a barnase domain to create the protein UbL-eDHFR—barnase and thus forced the proteasome to initiate degradation between the eDHFR and barnase domains. The full-length protein was degraded efficiently and partially converted into fragments (see below). The proteasome initiated degradation at the internal linker because shortening the linker to ∼20 amino acids prevented degradation (Supplementary Figure 2), whereas increasing or decreasing the linker by 40 amino acids had little effect on the rates or extent of the reaction (Supplementary Figure 3), as expected for internal initiation from earlier studies.[15]

Forcing the proteasome to initiate degradation at the internal linker caused it to stop prematurely at the DHFR domain approximately twice as frequently as before (Figure 2a). Thus, a domain flanking the initiation region at one end can stabilize a domain at the other end. The simplest explanation for this observation would be if the domains stabilized each other by a direct interaction. However, this mechanism is unlikely because stabilization was not specific to barnase and attaching the titin Ig domain to the C-terminus of the initiation region instead of barnase had a similar effect. Thus, the stabilization must occur by a different mechanism.

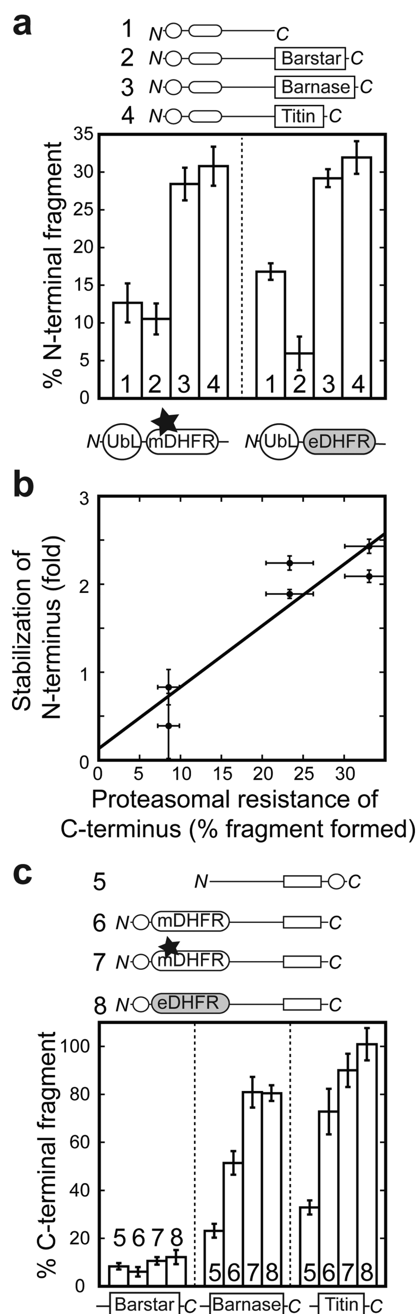

Figure 2. Internal initiation increases proteasomal processing. (a) DHFR fragment formation for substrates lacking or containing different C-terminal flanking domains. (b) Correlation between relative DHFR fragment formation (from A) for internal initiation constructs and amount of C-terminal fragment formed in constructs lacking DHFR. R^2 = 0.86. (c) C-Terminal domain fragment formation for substrates lacking or containing an N-terminal flanking DHFR domain. Error bars represent the SEM of 4−9 experiments.

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Not all C-terminal domains can protect the N-terminus, and placing barstar at the end of the constructs did not stabilize the N-terminal DHFR domain (Figure 2a). Barstar is substantially less stable than either barnase or the titin Ig domain, both thermodynamically and kinetically.[34−36] Furthermore, barstar's halftime for spontaneous unfolding ($t_{1/2}$ ~10 s[34]) is much shorter than the halftime of degradation ($t_{1/2}$ ~7 min), such that the proteasome is able to capture spontaneously unfolded barstar rather than having to actively induce unfolding. As a result, barstar is much more easily degraded by the proteasome than barnase or titin. Indeed, the proteasome digested barstar by itself almost completely, whereas barnase or titin can escape degradation some 30% of the time (e.g., compare —barstar-UbL and —barnase-UbL in Figure 2b,c). The relationship held quantitatively, and the ability of a C-terminal domain to protect the N-terminal domain from degradation depended on its own stability (Figure 2b).

Stabilizing Effect of Flanking Domains Is Reciprocal. If a C-terminal flanking domain can protect the N-terminal domain from proteasomal degradation, the reverse might also be the case. To test this prediction, we manipulated the N-terminal domain and examined the extent to which the C-terminal domain escaped degradation. As described above, degradation of the barnase and titin domains from an N-terminal initiation site without a flanking domain led to the formation of ~30% fragment. Fusing mDHFR to the N-terminus more than doubled the formation of C-terminal barnase and titin fragments (Figure 2c). Stabilizing the N-terminal mDHFR with MTX or replacing it with eDHFR further increased the amount of C-terminal fragment, such that almost none of the C-terminal domain was degraded (Figure 2c). The effect did not depend on a specific pair of domains, as the N-terminal DHFR stabilized either the titin Ig domain or barnase. However, domains differed in their ability to be stabilized. Barstar was only slightly protected even by eDHFR, probably because of barstar's intrinsic instability. On the other hand, the combination of the two most stable domains, titin and eDHFR, led to essentially quantitative release of a titin fragment.

Together, these results indicate that the processivity of the proteasome is modulated by the domains flanking the site at which it initiates degradation. Depending on the stability of the domains, the proteasome can function as a highly processive degradation machine or become prone to releasing undegraded fragments.

Reduced Processivity after Internal Initiation Is Conserved. It seemed possible that the effect of flanking domains on proteasome processivity might simply reflect a deficiency of the yeast proteasome in dealing with complex substrates. To test whether the mammalian proteasome can deal with two domains more effectively, we affinity-purified mammalian proteasome[39] and followed the degradation of substrates targeted to the proteasome by the UbL from human Rad23b (Figure 3). Capping the C-terminus of the initiation region of an eDHFR substrate with barnase led to an ~2-fold increase in the amount of DHFR fragment formed, equivalent to the effect seen with the yeast proteasome (Figure 3a). Likewise, an N-terminal eDHFR domain almost doubled the amount of C-terminal barnase fragment formed (Figure 3b). Thus, internal initiation reduces proteasomal processing in both the yeast and mammalian degradation systems.

Sequence of the Initiation Region Can Affect Processing. For two of physiological examples of proteasomal processing, the NFκB precursors and Ci, the amino acid sequence of the region preceding the resistant domain affects the extent of fragment formation.[19,20] In particular, stretches of a strongly biased or simple amino acid composition, a glycine-rich region in the NFκB precursors and an asparagine-rich region in Ci, appear to enhance processing.[19,20] We therefore replaced the initiation region in the model substrates with sequences of equal length from the relevant regions of NFκB and Ci and determined how proteasome processivity was affected. Yeast Spt23 does not contain strongly biased sequences in the relevant regions,[11] and we also inserted the appropriate Spt23 sequences into the model substrates for comparison.

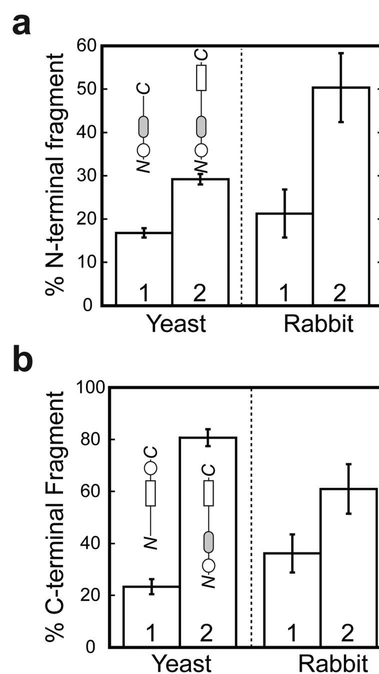

Figure 3. Internal initiation increases processing with both yeast and rabbit proteasome. (a) DHFR fragment formation for a substrate lacking or containing a C-terminal barnase domain, for both yeast and mammalian proteasome. (b) Barnase fragment formation for a substrate lacking or containing an N-terminal DHFR domain, for both yeast and mammalian proteasome. Error bars represent the SEM of 4−9 experiments.

As expected, the NFκB and Ci sequences led to a substantial increase in the amount of N-terminal DHFR fragment formed even in the absence of a C-terminal domain. Intriguingly, the addition of a C-terminal domain did not further enhance the levels of processing. The Spt23 sequence, on the other hand, did not by itself increase fragment formation, and consequently flanking domains still increased processing in these constructs (Figure 4a). The effect of the sequence insertions was symmetrical, and the NFκB and Ci linkers, but not the Spt23 linker, also led to a substantial increase in the amount of C-terminal fragment in the absence of an N-terminal domain (Figure 4b). Addition of an N-terminal domain had little if any effect in substrates with the NFκB and Ci sequences but increased the amount of fragment in substrates with the Spt23 sequence. The effect of the different linker sequences on processing does not merely reflect their inability to support degradation. Although the sequences affect the rates of degradation, these rates do not correlate with the extent of processing (Supplementary Table 1). Thus, simple sequences appear to reduce the processivity of the proteasome enough that the addition of a flanking domain has no further effect on fragment formation.

Physical Model for Remote Stabilization by the Proteasome. The proteasome usually degrades proteins completely to avoid the formation of protein fragments with unwanted activities. Here, we find that this processivity is compromised when the proteasome initiates degradation between folded domains. The stability of one domain not only affects its own degradation but also that of the flanking domain. The effect on proteasome processivity can be tuned by adjusting the stability of the two flanking domains so that degradation can be complete or lead to quantitative formation of protein fragments. The protective effect is not due to a direct or specific interaction between the two flanking domains and occurs with domains that are separated by a long linker. There are precedents for protein stabilization against degradation through the binding of another protein or ligand (*e.g.*, see refs 9, 22, 37, 40, 41), but stabilization without a direct interaction is unexpected.

The simplest physical model for this remote stabilization is that it is mediated by the proteasome itself. The proteasome degrades its substrates sequentially by running along their polypeptide chains.[9] The polypeptide chain is translocated into the degradation channel, and this leads to unraveling of any folded domains that are encountered. Single molecule force measurements find that the bacterial proteasome analogue ClpXP pulls at its substrates generating forces of several piconewtons that mediate protein unfolding.[42,43] The eukaryotic proteasome presumably acts similarly. After initiation at an internal site, the first cleavage creates two protein fragments, and domains flanking the initiation region could stabilize each other through three different mechanisms. First, the proteasome could be optimized to apply force to a single domain at a time such that the two fragments compete for the unfoldase activity, which in turn slows the unfolding and degradation of both substrates. Substrates are pulled into the degradation channel of ATP-dependent proteases by aromatic-paddle containing loops that can grab and translocate substrates in response to ATP binding and hydrolysis.[8,44−46] The substrate tails could compete for access to the translocation paddles, which would slow the unfolding process, for example, by reducing the number of pulls per time period each domain receives until one domain is unfolded. Fewer unfolding attempts would then increase the likelihood that the domain falls off the proteasome and escapes.[47] Second, the presence of two substrates simultaneously could affect the geometry with which the proteasome pulls at them. The proteasome presumably traps local unfolding fluctuations that transiently weaken substrates if they coincide with a motor power-stroke.[42,43] Two substrates at the entrance of the degradation channel at once might interfere sterically with one another and thereby prevent the proteasome from efficiently capturing these fluctuations and converting them into protein unfolding. Third, the presence of two substrates simultaneous could create friction in the channel. The degradation channel allows simultaneous passage of two polypeptide chains[10,16,17] but is only ~13 Å wide at its narrowest point as judged in crystal structures.[17] This size suggests a tight fit for the substrate, possibly creating friction and restricting the movement of the polypeptide chains, depending on the amino acid sequence, packing geometry, and the flexibility of the channel.

Remote stabilization is surprising but can be modeled quantitatively by a simple kinetic mechanism using chemically reasonable parameters.[48] When the proteasome encounters a folded domain (shown as a blue sphere in Figure 5a), the domain can either be unfolded and degraded or it can be released. The relative rates of unfolding and release will determine how often a domain escapes from the proteasome (fraction escaped = $(k_{\text{release}})/(k_{\text{deg}} + k_{\text{release}})$) (Figure 5a). When the proteasome engages a substrate at a linker between two domains, the first cleavage creates two fragments (blue and pink in Figure 5) associated with the proteasome. Each fragment can then partition between unfolding and rapid degradation or dissociation (Figure 5b). As discussed above, the presence of a second polypeptide will likely slow substrate unfolding (*i.e.*, decrease k_{deg1} in Figure 5b). After one of the domains has been degraded, the other can be digested more rapidly (Figure 5b). The thermodynamic stabilities of mDHFR and eDHFR differ approximately 20-fold.[49,50] Decreasing the degradation rate for the N-terminal domain (k_{degN}) in the model 20-fold increases the amount of the other domain that is released as a fragment by approximately 40%, which is similar to the 60% we observed experimentally (see Supporting Information for details; Supplementary Figure 4).

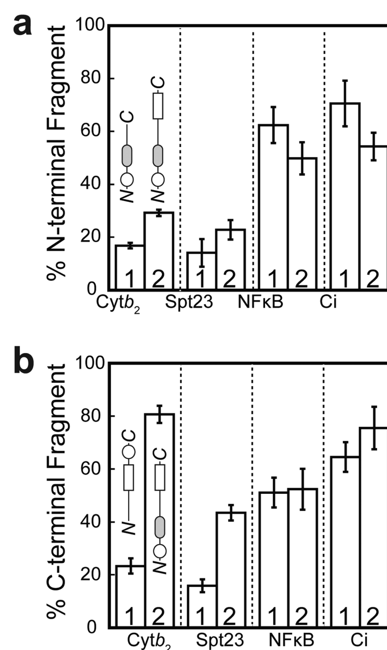

Figure 4. Effects of initiation region sequence on internal initiation. (a) DHFR fragment formation for substrates containing different initiation sequences and lacking or containing a C-terminal barnase domain. (b) Barnase fragment formation for substrates containing different initiation sequences and lacking or containing an N-terminal DHFR domain. Error bars represent the SEM of 4−9 experiments.

Possible *in Vivo* Mechanisms To Determine Whether Fragments Are Degraded or Released. In the physiological examples of protein processing, one-half of the substrate is degraded completely, while the other half persists to elicit a particular biological function. How then does the cell determine which side of the substrate escapes after the initial cleavage? One mechanism could be through the relative stabilities of the flanking domains. The domain that unravels more easily will be degraded first but may still enhance the accumulation of the other half of the protein. For example, mDHFR (in the absence of methotrexate) was not degradation-resistant enough to give a fragment but still enhanced the formation of a C-terminal fragment. In Spt23, the initial cleavage most likely occurs between an N-terminal IPT domain and two C-terminal ankryin repeats, which are followed by a transmembrane domain.[11,22,23]

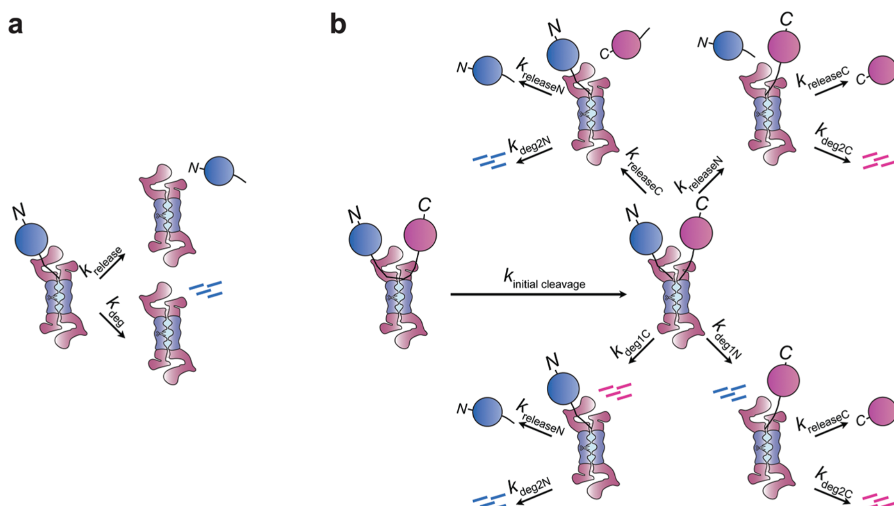

Figure 5. Proposed processing mechanism. (a) Model showing degradation of a substrate from a terminus in which the folded domain can be unfolded and degraded or can be released. (b) Model showing how processing results from internal cleavage followed by slowed unfolding (due to the presence of two domains) in competition with release. Once a domain is unfolded and degraded, the remaining domain can be degraded more quickly.

The ankyrin repeats are likely only marginally stable[51,52] and so may enhance N-terminal fragment formation, while they themselves are completely degraded. Ligand binding or covalent modification could further modulate the stability of domains.

Second, the presence of the ubiquitin modification may play a role in deciding the fates of the fragments. A ubiquitin chain causes a fragment to remain associated with the proteasome so that the degradation machinery could re-engage the fragment, even if the initiation site is temporarily released. In this study, substrates were targeted to the proteasome *via* an N-terminal UbL domain, which makes it more likely that the N-terminal fragment remains bound to the proteasome even if the initiation site is released. Ubiquitin chains are normally removed from substrates in one of the first steps during degradation so they can be recycled.[1] However, it is possible that a ubiquitin tag persists briefly after the initial cleavage step. Also, many substrates contain more than one degron so that fragments can contain additional ubiquitin chains that retarget them for degradation. For example, p105 is ubiquitinated both at an internal site, which leads to processing, and at a C-terminal site, which leads to complete degradation[53] and could also target any postprocessing C-terminal fragment for degradation. Similarly, Ci and Gli proteins contain degrons that lead to complete degradation in addition to the degron that leads to processing.[28] The proteasome also contains at least one subunit (Hul5) with protein ubiquitin ligase activity.[54] Hul5 may ubiquitinate one substrate fragment preferentially, either because of the presence of secondary degrons or simply due to the position of the substrate chain. Indeed, deleting Hul5 reduces the processivity of degradation.[55]

A third possibility is that the sequence of the linker determines which fragment is released. Simple sequences in a substrate may weaken its interaction with the motor proteins,[20] which would either slow its unfolding or accelerate its release (Figure 5a). A model of this type has been discussed for the role of the glycine-rich region in p105 processing.[9,19,20] In our *in vitro* experiments, the simple sequence regions from p105 and Ci led to increased formation of both N- and C-terminal fragments, which seems to argue against this model. However, in these constructs, the simple sequences are in the fragment that contains the UbL domain and therefore remain associated with the proteasome, perhaps enhancing its effect on processivity. In p105 and Ci, the ubiquitin modification targeting the proteins to the proteasome are on the opposite fragment, not the one containing the simple sequences.

Internal Initiation May Be Common in Cells. Internal initiation of proteasomal degradation could be quite common in cells, raising the possibility that remote stabilization reduces the processivity of the proteasome in more cases than would initially be expected. A recent proteomic screen identified the ubiquitin modifications in 220 proteins in mammalian cells,[60] and 30% of the ubiquitination sites were located at least 200 amino acids away from either terminus of the protein (Supplementary Table 2). The proteasome initiates degradation near the ubiquitin modification,[7] which suggests that in many proteins the proteasome initiates degradation internally. Many proteins, especially regulatory proteins and transcription factors, contain long unstructured regions that separate folded domains.[61] In at least ~10% of the proteins identified in the screen, the ubiquitination sites were located between two annotated and presumably folded domains or between a domain and a transmembrane segment. It is possible that these examples of partial degradation have simply been missed. On the other hand, the fact that many proteins contain more than one degron could in turn reduce the number of partially degraded protein fragments that are observed *in vivo*.

In summary, we find that during proteasomal degradation, two domains in a substrate can stabilize each other against degradation without interacting directly with one another, presumably by competing for the proteasome's unfolding activity. We propose that this remote stabilization reduces the processivity of the proteasome and may either allow the formation of biologically active fragments or require mechanisms such as multiple ubiquitination to prevent fragment accumulation in eukaryotic cells.

■ METHODS

Constructs. Constructs encoding substrate proteins were cloned into pGEM-3Zf+ (Promega). The N-terminal UbL domain of yeast Rad23 consisted of amino acids 1−99 of *S. cerevisiae* Rad23, followed by an eight amino acid linker. The C-terminal UbL was a 20 amino acid linker followed by amino acids 1−76 of Rad23. The cytochrome

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b_2 initiation region consisted of the first 91 amino acids of the 95 amino acid yeast cytochrome b_2 mitochondrial targeting presequence followed by a repeat of the final 92 amino acids with lysines changed to arginines or glutamines and methionines changed to alanines. The Spt23 sequence consisted of residues 595−708 and 775−842 of yeast Spt23, the processing region with the ankyrin repeats removed, which has been previously shown to be processed *in vivo*.[11] The NFκB sequence consisted of residues 354−535 of human p105. The Ci sequence consisted of residues 604−786 of *D. melanogaster* Cubitus interruptus. All initiation regions were cloned into *Pst*I and XhoI sites between DHFR and the C-terminal domain. Sequences encoding UbL, *Bacillus amyloliquefaciens* barnase, *B. amyloliquefaciens* barstar, *E. coli* DHFR, mouse DHFR, Spt23, Ci and cytochrome b_2 were synthesized and codon optimized for *E. coli* by GenScript or using gene synthesis oligos designed using DNAWorks.[56] Constructs were assembled using a combination of traditional restriction enzyme cloning and In-Fusion (Clontech).

Substrates. Radioactive substrates were *in vitro* translated using the RTS 100 *E. coli* HY kit (5 PRIME), supplemented with ^{35}S-methionine. After high-speed ultracentrifugation, substrates were affinity purified using either Talon magnetic beads (Clontech) or S5a agarose (Boston Biochem). Talon purification was done according to manufacturer's protocols, except that all buffers were supplemented with 0.1 mg mL^{-1} BSA, 0.05% (v/v) tween, and 20 mM β-mercaptoethanol. S5a resin was washed with PBS (containing 0.1 mg mL^{-1} BSA, 0.05% (v/v) Tween-20, and 20 mM β-mercaptoethanol), substrates were allowed to bind for 1 h at 4 °C, the beads were washed three times with PBS, and then substrates were eluted with 50 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 0.1 mg mL^{-1} BSA, 0.05% (v/v) Tween-20, and 20 mM β-mercaptoethanol for 30 min at 4 °C. After purification, substrates were typically >95% pure by autoradiography. The domains were fully folded, as chymotrypsin treatment (0.05 mg mL^{-1} for 2.5 min on ice) led to degradation of unstructured regions and quantitative conversion to an N-terminal and a C-terminal fragment (Supplementary Figure 5). Substrates were typically doped with a loading control consisting of a hyperstable mutant of the Villin headpiece domain[57] fused to a hexahistidine tag (not shown in the gels above); this substrate was translated and purified using Talon resin as described above.

Proteasome Purification. Yeast proteasome was purified *via* a FLAG-tag on subunit Rpn11 as described previously[58] with modifications. Briefly, *S. cerevisiae* (strain YYS40) were lysed in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 4 mM ATP, 1 mM DTT, 0.02 mg mL^{-1} creatine kinase (Roche), 20 mM phospho-creatine, 10% (v/v) glycerol. Following clarification, lysate was allowed to bind to Anti-Flag M2 Affinity gel (Sigma), was washed with 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 10% (v/v) glycerol, and was eluted with 100 μg mL^{-1} 3X Flag Peptide (Sigma) in wash buffer. Rabbit proteasome was purified using a GST-UbL column, in a protocol modified from.[39] Briefly, rabbit red blood cells (Lampire Biologicals) were lysed by osmotic shock and then clarified in 25 mM HEPES pH 7.4, 5 mM $MgCl_2$, and 10% (v/v) glycerol (UbL buffer) supplemented with 1 mM ATP, 150 mM NaCl, 1 mM DTT, 0.5 mg mL^{-1} creatine kinase and 10 mM creatine phosphate. The supernatant was mixed with GST-UbL and GSH-Sepharose (GE) and allowed to bind for 2 h at 4 °C, then was poured into a column housing, washed with UbL buffer supplemented with 1 mM ATP, 150 mM NaCl and 1 mM DTT, and eluted with UbL buffer supplemented with 1 mM ATP, 1 mM DTT and ∼1 mg mL^{-1} His_{10}-UIM from human S5a. His_{10}-UIM was then removed with NiNTA-agarose (Qiagen), and proteasome concentration was determined using the Pierce 660 assay (Pierce), using yeast proteasome as a standard. Constructs encoding GST-UbL and His_{10}-UIM were a kind gift from Alfred Goldberg.

Proteasomal Degradation Assay. The 26S proteasomal degradation assays were conducted using 20 nM purified proteasome in excess of trace radiolabeled substrate at 30 °C. Degradation was carried out in buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5% glycerol (v/v), 1 mM ATP, 10 mM creatine phosphate, 0.1 mg mL^{-1} creatine kinase, and 100 μM MTX or 1% (v/v) DMSO as a control. At designated time points, samples were mixed with SDS-PAGE loading buffer to stop proteolysis and then analyzed by SDS-PAGE and electronic autoradiography or exposure to phosphorimager cassettes. Bands were quantified using ImageJ software,[59] and intensities were normalized relative to the loading control and to the number of methionines predicted to be within the full-length or fragment protein (∼80 amino acids beyond the folded domain). This prediction may lead to small errors (<10%) in the extent of fragment formation for the Spt23 and NFκB sequences due to ambiguities as to the number of methionines in the fragment; for the cytochrome b_2 and Ci sequences all potentially ambiguous methionines were replaced with alanine.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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