The proteasome plays a central role in the degradation of regulatory and misfolded proteins. Current models suggest that substrates access the internal catalytic sites by processively threading their termini through the gated substrate channel. Here, we found that latent (closed) and activated (open) proteasomes degraded two natively disordered substrates at internal peptide bonds even when they lacked accessible termini, suggesting that these substrates themselves promoted gating of the proteasome. This endoproteolysis provides a molecular mechanism for regulated release of transcription factors as a means of accessing internal folding defects of misfolded multidomain proteins.

To determine whether proteasome-mediated degradation is initiated from an internal site of a protein, we used two physiological substrates: the cyclin-dependent kinase (CDK) inhibitor p21\(^{\text{wt}}\) (p21) and \(\alpha\)-synuclein (\(\alpha\)-syn). Both proteins are “natively disordered” (10) and are efficiently degraded by the proteasome in the absence of polyubiquitin modification (11, 12). We compared the degradation of these unstable substrates by the latent 20S proteasome (13) with that of the activated, assembled 26S particle (14). The 26S particle, the accepted physiological form of the proteasome, is composed of the 20S core particle and the PA700 (19S) regulatory cap (14). PA700 contains polyubiquitin (15, 16) and misfolded protein (17, 18) binding sites, isopeptidase activity (19, 20), and adenosine triphosphatase subunits presumably involved in unfolding substrates (21). Both the 20S and 26S proteasomes efficiently degraded p21 and \(\alpha\)-syn (Fig. 1A) (22). In contrast, the hyperstable green fluorescent protein (GFP) (melting temperature, \(T_m = 65^\circ C\) (23)) was not degraded by either 20S or 26S (Fig. 1B). The disordered substrates were efficiently degraded by the latent 20S proteasome despite its inability to hydrolyze short peptide substrates (Fig. 1C). Thus, unfolded proteins could open the “gate” that controls access to the otherwise occluded catalytic sites, thereby initiating a process similar to that employed by the proteasome regulators PA700 (24) and PA28 (25).

When fusions between the unfolded substrates and the stable GFP were assessed, both the latent 20S and the active 26S proteasomes efficiently degraded the p21 and \(\alpha\)-syn domains but not the GFP domain of each fusion protein (Fig. 2, A and B), suggesting that additional factors were required for efficient unfolding of this stable domain. We also examined whether either the 20S or the 26S proteasome exhibited a directional preference by comparing the degradation of fusion proteins that contained GFP at either the NH\(_2\)- or COOH-terminus (Fig. 2C). The relative activities of the 20S and 26S proteasomes were equivalent toward the p21 fusions. For the \(\alpha\)-syn fusions, the 20S proteasome degraded the substrates faster, although the 26S reaction did go to completion (inset, Fig. 2B). Apparently, the shorter \(\alpha\)-syn, when blocked with GFP, was less accessible to the longer channel of the 26S proteasome. In this regard, the 26S and 26S proteasomes exhibited similar activity toward unprotected \(\alpha\)-syn (Fig. 1A) and circular \(\alpha\)-syn whose termini were blocked by a peptide bond. For all substrates, degradation was effectively inhibited by MG132 (Fig. 2C), a potent proteasome inhibitor. Finally, the proteasome degraded the substrates blocked at either terminus at equivalent rates (Fig. 2C), indicating that the reported preference for COOH- to NH\(_2\)-terminal degradation (4) is not a general feature of all substrates.

To assess the ability of the proteasome to cleave these substrates at internal sites, we protected both termini of p21 and \(\alpha\)-syn by sandwiching them between nondegradable GFP domains (Fig. 3). As assessed by immunoblottting, GFP-p21-GFP and GFP-\(\alpha\)-syn–GFP proteins were degraded by the 20S and 26S proteasomes, and degradation was inhibited by MG132. The rate of degradation of bilaterally protected substrates was slower than the degradation of p21 alone, \(\alpha\)-syn alone (Fig. 1A), and substrates protected at a single terminus (Fig. 2C), indicating that the endoproteolytic cleavage was less efficient than degradation from the termini. Again, neither GFP-blocking domain
was degraded to any measurable extent in the sandwich proteins, as shown by the stable fluorescence (Fig. 3). Thus, free termini are not required for either the 26S or 20S proteasome to efficiently degrade either p21 or α-syn, suggesting that degradation can be initiated at an internal endoproteolytic site.

To formally test this possibility, we produced circular α-syn and GFP-p21 proteins that lacked NH₂- and COOH-termini with the use of a protein splicing technique (26, 27) (Fig. 4A). Purified α-syn formed two products after the cyclization process (Fig. 4B). The predominant form, band b, was converted to band a after digestion with thrombin, indicating that band b was the circular α-syn. Furthermore, band b produced no signal from NH₂-terminal amino acid sequence analysis, consistent with it being the circular variant, whereas the NH₂-terminal sequence Cys-Arg-Gly-Asp-Val-Phe-Met for band a indicated that it is the linear α-syn (Fig. 4B). Similarly, band c was circular GFP-p21 and band d was linear GFP-p21 (Fig. 4B). The circular protein composed 80 to 95% of each preparation as determined by Coomassie-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Like linear α-syn, the cyclized α-syn protein showed a random coil conformation (28). Circular α-syn protein migrated faster than linear α-syn on SDS-PAGE, as typically observed (27). In contrast, the linear GFP-p21 protein migrated faster than the circular variant, as previously observed for variants of the maltose-binding protein (27).

Purified 26S and 20S proteasomes effec-

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**Fig. 1.** Proteasomal degradation of natively disordered substrates. (A) For p21 degradation, 400 nM p21 was incubated with 5 nM latent 20S or active 26S proteasome at 37°C in buffer A [20 mM Tris-HCl, pH = 7.1; 200 mM NaCl; 10 mM MgCl₂; 0.25 mM adenosine triphosphate (ATP); and 1 mM dithiothreitol (DTT)]. For α-syn degradation, 500 nM α-syn was incubated with 10 nM latent 20S or active 26S proteasome at 37°C in buffer B [20 mM Tris-HCl, pH = 7.1; 200 mM NaCl; 10 mM MgCl₂; 0.25 mM ATP; and 1 mM DTT]. The degradation time course was monitored by immunoblotting with monoclonal antibodies to p21 or α-syn. 6XHis indicates the hexameric histidine metal affinity tag. 30 + MG132 represents the thirty minute time point in the presence of the proteasome inhibitor. (B) 100 nM GFP was incubated with 20 nM 20S or 26S proteasome at 37°C in buffer B. The stability of GFP was assessed by continuously monitoring fluorescence (λex/em = 395/508 nm; +205, thin trace; +265, thick trace) and by immunoblotting (inset) with an antibody to GFP at the beginning (lane 1) and on completion of fluorescence monitoring (+205, lane 2; +265, lane 3). (C) The latency of the 20S proteasome (black bars) was assessed by comparing its multiple catalytic activities to the activated 26S proteasome (hatched bars) with the use of fluorogenic peptide substrates Z-Leu-Leu-Glu-βNA (LLE, caspase-like activity), Z-Val-Val-Arg-AMC (VVR, trypsin-like activity), and suc-Leu-Leu-Val-Tyr-AMC (LLVY, chymotrypsin-like activity) (14), where Z is benzoyloxycarbonyl, βNA is 2-naphthylamine, AMC is 7-amino-4-methylcoumarin, and suc is succinyl.

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**Fig. 2.** Proteasomal degradation of unilaterally protected p21 and α-syn. (A and B) 100 nM GFP-p21 and GFP–α-syn fusion proteins were incubated with 20 nM 20S or 26S proteasome in buffer A (GFP-p21) or buffer B (GFP–α-syn). Reactions were monitored by immunoblotting (inset) and fluorescence monitoring as in Fig. 1. A precursor-product relationship was demonstrated during the time course of degradation with the use of 20S proteasome (right half of each panel). (C) 100 nM substrates with GFP either NH₂-terminal to [as in (A) and (B)] or COOH-terminal to the p21 or α-syn moieties were incubated with 10 nM 20S or 26S proteasome as in (A), and the degradation time course was assessed by immunoblotting. Substrate remaining in the 20S proteasome reactions in the absence (solid lines) or presence (dashed lines) of MG132 was quantitated by densitometry and normalized to the 0 time point.
tively degraded both circular substrates and were inhibited by MG132 (Fig. 4C), again leaving the GFP moiety of the p21-GFP substrate as in Figs. 2 and 3 (29). This endoproteolysis activity cosedimented with the intact proteasome complex on glycerol gradients (Fig. 4D), excluding the possibility that contaminating proteases or fragments of the proteasome were responsible.

These results call into question several fundamental aspects of currently favored models of proteasome function and regulation. First, they demonstrate that the proteasome can catalyze endoproteolytic cleavage of polypeptide bonds (Fig. 4E). These data differ appreciably from models in which proteasome-mediated degradation is restricted to processive hydrolysis of a polypeptide chain from a free NH2- or COOH-terminus (4, 5) and instead support a model in which a disordered polypeptide chain loop can enter the axial channel to permit initial endoproteolytic cleavage. In this regard, crystal structures of the active proteasome with open access gates (24, 30) could easily accommodate a /H9252-hairpin structure. Studies with some disulfide-trapped substrates (31) but not others (32)
suggest that there is space for up to three extended chains within the chamber.

Second, little difference in the rates of endoproteolytic cleavage of these disordered substrates was detected between latent 20S and active 26S proteasomes (Figs. 1 to 4) in which the status of the gate that controls entry to the central axial channel of the proteasome is closed and open respectively (Fig. 1C) (24, 30).

Physiological regulators of the proteasome, such as PA700 (19S cap) and PA28, increase proteasome activity in part by opening this gate, thereby increasing access of substrates to the proteasome’s catalytic centers (24, 25).

The ability of closed, latent 20S proteasome to catalyze cleavage of these natively disordered, physiological substrates suggests they possess certain features that also promote “gating” of the proteasome (Fig. 4E), features that folded proteins lack. This mechanism suggests a potential role for the free 20S proteasome found in the absence of bound regulatory proteins in many cells (33). It is possible that these inherent signals could target substrates directly for 20S proteasomal degradation without the need for polyubiquitin modification.

References and Notes

22. Materials and Methods are available as supporting material on Science Online.
28. The far-ultraviolet circular dichroism spectrum of the mixed population of circular and linear α-syns exhibits a predominant negative absorption at about 198 nm, characteristic of a random coil conformation.
29. GFP fluorescence was stable during the degradation of the linear and circular GFP-p21 substrates, as in Figs. 1 to 3.
34. We thank S. J. Elledge (Baylor College of Medicine) for his generous gift of p21ΔDNA; R. Nussbaum (National Human Genome Research Institute) for α-syn cDNA; C. Wigley, R. Stithman, and S. Mualem for critical suggestions; and members of our laboratories for helpful comments. This work was supported by grants from the Welch Foundation [P.J.T.], Muscular Dystrophy Association [G.N.D.], and NIH [grants DK46818 (G.N.D.) and DK49835 (P.J.T.)].

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Production of α,1,3-Galactosyltransferase–Deficient Pigs

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The enzyme α,1,3-galactosyltransferase (α,1,3GT or GGTA1) synthesizes α,1,3-galactose (α,1,3Gal) epitopes (Galα1,3Galβ1,4GlcNac-R), which are the major xenogenins causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of α,1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the α,1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the α,1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the α,1,3GT protein. Four healthy α,1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the α,1,3GT gene hold significant value, as they would allow production of α,1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.

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