Ligand-switchable Substrates for a Ubiquitin-Proteasome System*

Emily L. Egeler‡, Lorenz M. Urner‡, Rishi Rakhit†, Corey W. Liu‡, and Thomas J. Wandless‡

From the ‡Department of Chemical and Systems Biology and §Stanford Magnetic Resonance Laboratory, Stanford University, Stanford, California 94305

Cellular maintenance of protein homeostasis is essential for normal cellular function. The ubiquitin-proteasome system (UPS) plays a central role in processing cellular proteins destined for degradation, but little is currently known about how misfolded cytosolic proteins are recognized by protein quality control machinery and targeted to the UPS for degradation in mammalian cells. Destabilizing domains (DDs) are small protein domains that are unstable and degraded in the absence of ligand, but whose stability is rescued by binding to a high affinity cell-permeable ligand. In the work presented here, we investigate the biophysical properties and cellular fates of a panel of FKBP12 mutants displaying a range of stabilities when expressed in mammalian cells. Our findings correlate observed cellular instability to both the propensity of the protein domain to unfold in vitro and the extent of ubiquitination of the protein in the non-permissive (ligand-free) state. We propose a model in which removal of stabilizing ligand causes the DD to unfold and be rapidly ubiquitinated by the UPS for degradation at the proteasome. The conditional nature of DD stability allows a rapid and non-perturbing switch from stable protein to unstable UPS substrate unlike other methods currently used to interrogate protein stability control, providing tunable control of degradation rates.

With recent advances in genome sequencing, biologists now have a much clearer picture of the primary structures of predicted and known proteins for many organisms. Methods to query protein function, however, have lagged behind this sequencing revolution. We have developed several general methods to conditionally control protein stability in cells using small molecules (1, 2, 49). Destabilizing domains (DDs) are small protein domains, which, when fused to a protein of interest, promote degradation of the entire fusion protein in the non-permissive (i.e. ligand-absent) state (see Fig. 1A). The instability conferred by the DD can be rescued by addition of a cell-permeable high affinity small molecule. In the permissive state (ligand-present), the fusion protein is stable, and the protein of interest can accumulate to functional levels and exert its biological effect. The amount of stabilizing ligand can be varied or washed out, making this a tunable and reversible system.

Previous work by Banaszynski et al. (1) showed that blocking the proteasome prevents degradation of a FKBP-derived DD, and similar behavior is observed with E. coli dihydrofolate reductase-derived DDs (2). The proteasome is a complex multijubiquitin protein responsible for degradation of most regulated proteins found in the cytoplasm and nucleus. Unstructured or oxidized proteins can be degraded directly by the proteasome (3, 4), and a few proteins are delivered to the proteasome via adapter proteins (5, 6). Most proteins, however, are targeted for proteasomal degradation through covalent modification with the small protein ubiquitin (7). Once a protein is monoubiquitinated, usually on a lysine side chain or the N terminus, the ubiquitin cascade can extend this tag to form polyubiquitin chains through conjugation to one of seven internal lysines in ubiquitin, with chains of four ubiquitin moieties being sufficient for proteasome targeting (8, 9).

Post-translational modification with ubiquitin plays a role in a variety of cellular pathways, but one of the best studied is the ubiquitin-proteasome system (UPS) (10, 11). The UPS network of proteins works in concert to tag substrates with polyubiquitin chains, which, in turn, target substrates to the proteasome for degradation. In addition to targeting regulated proteins in response to cellular signals such as cell cycle progression or nutrient availability, the UPS also modifies misfolded proteins (12). Despite a large body of work into proteasomal recognition of polyubiquitin chains, there remain many unanswered questions about substrate selection within the UPS. Few E3s have known substrate requirements (13).

Misfolded proteins can arise from gene mutation, errors in nascent protein translation, folding, localization, or oxidative damage to existing proteins. The protein quality control (PQC) machinery must detect and remove misfolded proteins to maintain cellular homeostasis. Separate PQC branches have been identified in the endoplasmic reticulum (14), in the nucleus (15), at the plasma membrane (16), and in response to cellular stresses such as heat shock (17, 18). However, little is known about the cytosolic PQC pathway in mammalian cells and how it passes substrates to the UPS for degradation, despite identification of a proteins like CHIP (carboxyl terminus of Hsc70-interacting protein), which are capable of binding chap-
erones and UPS machinery (19–21). A better understanding of the mechanisms by which proteins recognize PQC substrates and connect to the UPS would be a significant advance in our understanding of protein homeostasis.

Our original screens identified dozens of FKBP mutants that display ligand-dependent stability when expressed in mammalian cells (1, 49). In the work presented here, we examine a subset of these FKBP-derived DDs to study the biophysical behavior of these purified proteins in vitro as well as the intracellular stability of the same DDs when expressed in mammalian cells. The cell-free experiments reveal that the Shield-1 ligand stabilizes the DD protein fold, whereas the DDs appear to be misfolded or rapidly sampling unfolded states in the absence of ligand. When the DDs are expressed in mammalian cells, our studies demonstrate that the withdrawal of ligand causes the DDs to be modified by the UPS before degradation at the proteasome. In the absence of Shield-1, DD recognition by the UPS is rapid, with significant ubiquitination observed within minutes after ligand removal. Recognition is also context-dependent, as DDs stabilized by ligand are not ubiquitinated. Biophysical measures of protein instability for purified proteins correlate well with the extent of ubiquitination and observed cellular stability of DDs expressed in mammalian cells. Identification of the FKBP-derived DDs as substrates of the UPS elucidates both the pathway for DD degradation and suggests that DDs would make excellent model substrates to further probe the molecular mechanisms responsible for recognizing and degrading misfolded cellular proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents**—MG132 powder was purchased from Cayman Chemical and stored as a 10 mM stock in ethanol at −20 °C. Ammonium chloride (15N, 99%) and deuterium oxide (99%) were purchased from Cambridge Isotope Laboratories; isopropyl-β-d-thiogalactoside and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Gold Biotechnology; chloroquine, hexadimethrine bromide (polybrene), and N-ethylmalimide were purchased from Sigma. The antibodies used in this study were anti-GFP (IL-8, Clontech) and anti-α-tubulin (Sigma). Ubiquitin-associated domain (UBA) resin and purified Usp2cc were kindly provided by the R. R. Kopito laboratory (Stanford University).

**Plasmids**—FKBP-derived DD mutants carrying the F36V mutation alone or in combination with L106P, L106A, or V4A/17V were cloned into pBMN vectors as N-terminal fusions to either YFP or Superfolder GFP (sfGFP, Sandia BioTech). For expression in bacteria, DD mutants and wild-type FKBP were cloned into pET15b protein expression constructs were co-transformed with the chaperone plasmid pG-Tf2 (Takara) into BL21(DE3) cells. Cells were grown in LB with 20 ng/ml tetracycline at 37 °C, cultured in growth medium 48 h to allow viral integration, and then assayed for fluorescence or drug selected with blasticidin (10 μg/ml, Invitrogen) or puromycin (2.5 μg/ml, Invitrogen). For Shield-1 washout experiments, adherent cells had Shield-1 medium replaced with growth medium “conditioned” with 5 μM bacterially expressed F36V protein. Cells were either incubated with conditioned medium continuously or for 5 min at 37 °C before replacement with regular growth medium. For suspension cells, cells were harvested by centrifugation at 1,000 rpm for 5 min between each change of medium.

**Bacterial Protein Expression**—For expression of F36V and wild-type FKBP, pET15b protein expression constructs were transformed into BL21(DE3) cells. Cells were grown in Luria-Bertani medium (LB (22)) at 37 °C to an optical density at 600 nm of 0.5–0.7. Protein expression was induced with 2 mM isopropyl-β-d-thiogalactoside at 37 °C for 5 h, after which cells were collected by centrifugation at 5,000 rpm at 4 °C for 20 min. For expression of mutants L106P, L106A, and V4A/17V, pET15b protein expression constructs were co-transformed with the chaperone plasmid pG-Tf2 (Takara) into BL21(DE3) cells. Cells were grown in LB with 20 ng/ml tetracycline at 37 °C to an optical density at 600 nm of 0.5–0.7. Protein expression was induced with 2 mM isopropyl-β-d-thiogalactoside at 37 °C for 5 h, after which cells were collected by centrifugation at 5,000 rpm at 4 °C for 20 min. For NMR experiments, M9 minimal media (22) containing 1 g/liter 15NH4Cl was substituted for LB to make uniformly 15N-labeled protein.

**Protein Purification**—Hexahistidine-tagged protein was purified on PerfectPro nickel-nitritrotriacetic acid-agarose resin (5 PRIME) according to manufacturer’s instructions. For conditioned media, purification was halted here, and protein was dialyzed into PBS with 4% glycerol. For NMR and urea experiments, protein was incubated with MHTΔ238 tobacco etch virus protease (23) in cleavage buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, pH 8) at 30 °C for 4 h or overnight at a ratio of 1:10 mg tobacco etch virus:mg of substrate. The reaction was incubated with regenerated nickel-nitritrotriacetic acid resin, this time collecting the flow-through and first wash.

**NMR Spectroscopy**—15N-labeled protein was dialyzed in Slide-A-lyzer cassettes (3,500 MWKO, Pierce) into NMR buffer (50 mM KH2PO4, 100 mM NaCl, 0.5 mM EDTA, 1 mM TCEP, 2% glycerol) and supplemented to 10% deuterium oxide. Wild-type FKBP was prepared in NMR buffer pH = 5.0, 5.6, 6.2, 6.8, and 7.4. FKBP-derived DDs were prepared in NMR buffer, pH 6.8, as this was the compromise between physiological conditions and hydrogen exchange rates. All NMR experiments were acquired at the Stanford Magnetic Resonance Laboratory. [1H,15N]-HSQC spectra were acquired at 27 °C on Varian Inova 600 and 800 MHz spectrometers running VNMR v6.1C and VNMRI 2.1B, respectively, both equipped with 5-mm inverse detect, triple-resonance 1H[13C,15N] pulse-field gradient probes. The majority of HSQC spectra were acquired on the 600 instrument and used 8395 Hz (1H) and 2431 Hz (15N) spectral widths, centered at 120 ppm in the 15N dimension. 1024 data points in t2, 64 complex t1 points, and a 1-s pre-scan (d1) delay. Spectra were 10 min to 10 h long (4 to 256 transients per
t1 increment) depending on sample protein concentrations. Two HSQC spectra were acquired on the 800 instrument and used 9612 Hz (1H) and 3242 Hz (15N) spectral widths, centered at 120 ppm in the 15N dimension, 1024 data points in t2, 32 complex t1 points, and 1-s pre-scan (d1) delay. Spectra were 2.5–5-h-long (128 to 256 transients per t1 increment), again depending on sample protein concentrations. All HSQC data were processed on the spectrometers and then analyzed with SPARKY (24).

**Urea Denaturation**—Protein was purified further by size exclusion chromatography in SEC buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM TCEP, pH 7.5) on a HiPrep 26/60 Sephacryl S-100 HR column (GE Healthcare) using an Akta Explorer FPLC system. Fractions were analyzed by SDS-PAGE, and fractions containing protein were pooled and concentrated (10,000 MWCO, Amicon). For each FKBP mutant, 2 μM protein was incubated with 1 mM DTT and 10 μM Shield-1 or an equal volume of ethanol at 4 °C overnight. Gravimetrically measured 10.22 m urea stock was added to a final range from 0 to 6M for FKBP mutants, or 0 to 7M urea for wild-type FKBP12, with each series containing 40 samples buffered to 50 mM Tris-HCl, pH 7.5, and incubated at room temperature overnight. A SPEX 1680 Double Spectrometer (Horiba Scientific) was used with a band pass of 10 nm, an excitation wavelength of 290 nm, and a recorded emission range of 300–380 nm. Fraction unfolded was calculated according to Equation 1,

\[
\text{Fraction unfolded} = \frac{F_N - \text{ratio}(320/356)}{F_N - F_U} \quad (\text{Eq. 1})
\]

where ratio(320/356) is the ratio of emission values at 320 nm (native) and 356 nm (unfolded), and \(F_N\) and \(F_U\) are the values for the completely native and unfolded fractions in the pre- and postdenaturational regions, respectively.

\[
F_N = \alpha_N + \beta_N \cdot [\text{urea}] \quad (\text{Eq. 2})
\]

\[
F_U = \alpha_U + \beta_U \cdot [\text{urea}] \quad (\text{Eq. 3})
\]

Further description of equations for fitting data can be found in Ref. 25.

**Immunoblotting**—Cell pellets were washed with PBS and lysed on ice with cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.5) containing protease inhibitor (Sigma) and 10 mM N-ethylmalimide. After incubating on ice 30 min, lysates were cleared by centrifugation at 13,300 rpm at 4 °C for 20 min. Proteins were quantified by Bradford protein assay (Bio-Rad) and resolved using SDS-PAGE.

**UBA Affinity Chromatography**—UBA resin was washed in 2 bed volumes of binding buffer (10 mM HEPES, 20 mM imidazole, 150 mM NaCl, 0.5% Triton X-100, pH 7.4) and centrifuged at 4000 rpm for 1 min. UBA resin was resuspended in binding buffer to make a 50% slurry. One milligram of whole cell lysate was diluted with binding buffer to a final volume of 150 μl and incubated with 50 μl of 50% UBA slurry with end-over-end mixing at 4 °C overnight. Resin was washed twice with 1 ml of binding buffer and once with PBS. Protein was eluted off the resin by boiling in 2× SDS loading buffer at 100 °C for 10 min. Supernatant was resolved by SDS-PAGE. For Usp2cc control experiments, 1 mg of lysate was treated with 20 mM DTT at 4 °C for 5 min to neutralize N-ethylmalimide in the lysis buffer. Lysate was incubated with 5 μg of purified Usp2cc at 37 °C for 1 h before proceeding with addition of UBA resin.

**RESULTS**

**FKBP Mutants Display Variety of Stabilities in Cells**—Previous work used FACS based flow cytometry screens to isolate mutants of FKBP that display ligand-dependent stability when expressed in mammalian cells (1, 49). All mutants responded to addition of Shield-1 with increased stability, and we were interested in investigating how protein structural changes correlated with the observed function in cells. For the work presented here, we used four FKBP mutants that exhibit different levels of stability when expressed in cells in the absence of ligand (i.e. basal stability) (Fig. 1B). Shield-1 possesses a synthetic “bump” to prevent its binding to the endogenous wild-type FKBP12. All of the FKBP mutants described herein encode the cavity-forming F36V mutation that accommodates this engineered bump. Thus, Shield-1 binds ~1000-fold more tightly to proteins possessing the F36V mutation (26). Parental F36V is the most stable mutant, showing significant expression over background in the absence of Shield-1 and a modest rise in levels, as expected, upon addition of ligand. The L106P mutant

---

5 T. D. Goddard and D. G. Keller, SPARKY 3, University of California, San Francisco, CA.
is the least stable DD, although the V4A/I7V mutant also shows very low basal levels in the absence of stabilizing ligand. The L106A mutant displays an intermediate phenotype with basal levels significantly above background, but it remains robustly responsive to the addition of Shield-1. Although it is a poor candidate to use as a DD to regulate protein of interest levels, we used the L106A mutant to allow us to see trends in biophysical measurements and recognition by UPS machinery.

**In Vitro Folding Studies of Purified FKBP-derived Mutants**

To look at the effect of Shield-1 binding on the structure of different DD mutants, we bacterially expressed all DD mutants and wild-type FKBP12 with a cleavable affinity tag in 15N-labeled medium. The fully assigned two-dimensional HSQC NMR spectrum has been published for wild-type FKBP12, which facilitated mapping chemical shifts of residues across the DDs (27). Rather than seeing strongly resolved above noise and well dispersed peaks reminiscent of a stably folded protein, the spectrum for L106P in the absence of Shield-1 resembles an unfolded or aggregated protein (Fig. 2A). Spectra display weak signal-to-noise ratios relative to other samples of similar concentration, and cross-peaks are not well dispersed. Spectra of the V4A/I7V mutant show better signal-to-noise levels, and resonances are reasonably well dispersed with one area of clustered cross-peaks (Fig. 2B). Spectra for F36V and L106A mutants both provide resonances with strong signal-to-noise ratios and well resolved cross-peaks in both dimensions, similar to the pattern observed for the well folded wild-type FKBP12 (Fig. 2, C and D; supplemental Fig. S3B). Incubating each protein sample with one molar equivalent of Shield-1 alters the spectra of all mutants so that the spectra converge to produce similar patterns of cross-peaks representative of the folded DD-Shield-1 complex (Fig. 2; supplemental Fig. S3A). Thus, each DD mutant, although providing HSQC spectra characteristic of various states of folding, is fully competent to bind Shield-1. In this manner, Shield-1 may act as a chemical chaperone that captures the stable, folded conformation of each DD mutant as the ligand-free proteins sample poorly folded or unfolded states in a dynamic equilibrium.

To support the NMR analyses, we used tryptophan fluorescence to monitor protein unfolding in the absence or presence of Shield-1 over a range of urea concentrations. FKBP12 has a single tryptophan (Trp-59), which can be used to spectrophotometrically report on the hydrophobic (folded) or hydrophilic (unfolded) state of the protein. Wild-type FKBP12 and a number of its mutants have been shown to unfold according to a two-state model wherein molecules unfold without intermediate folding states (25, 28, 29). For these experiments, we subjected bacterially expressed protein to an additional purification step using size exclusion chromatography to remove any low concentration impurities. Protein samples were incubated with either excess Shield-1 or vehicle overnight before samples were exposed to various concentrations of urea, also overnight.

Without ligand, the L106P mutant is almost entirely unfolded; even in the absence of urea (Fig. 3A). The V4A/I7V mutant also shows a significant fraction of unfolded protein in the absence of urea, but it appears to be slightly more stable.
**Ligand-switchable Substrates for UPS**

### Figure 3: Shield-1 stabilizes DD domains against denaturation.

A, purified protein of mutants L106P (orange), V4A/I7V (blue), L106A (green), and F36V (purple) were incubated with vehicle before exposure to varying concentrations of urea. Fraction of unfolded protein is calculated from the ratio of tryptophan fluorescence at 320 nm/356 nm (as described under "Experimental Procedures"). B, comparison of fraction of protein unfolded for the mutants when incubated with vehicle (dashed lines) or excess Shield-1 (solid lines) before addition of urea. Only best fit curves are displayed.

**FIGURE 3. Shield-1 stabilizes DD domains against denaturation.** A, purified protein of mutants L106P (orange), V4A/I7V (blue), L106A (green), and F36V (purple) were incubated with vehicle before exposure to varying concentrations of urea. Fraction of unfolded protein is calculated from the ratio of tryptophan fluorescence at 320 nm/356 nm (as described under "Experimental Procedures"). B, comparison of fraction of protein unfolded for the mutants when incubated with vehicle (dashed lines) or excess Shield-1 (solid lines) before addition of urea. Only best fit curves are displayed.

than the L106P mutant. The L106A mutant appears folded in the absence of urea but unfolds with the addition of low levels of urea; and the F36V mutant, the most stable domain when expressed in cells, requires the highest concentration of urea to unfold. Incubation with Shield-1 prior to exposure to urea significantly stabilizes the proteins and increases the concentration of urea necessary to unfold all mutants (Fig. 3B). All curves show a sharp transition between folded and unfolded states, indicating a lack of folding intermediates and allowing the data to be fit with a sigmoidal curve according to a two-state unfolding model. Separately, samples were diluted from high urea concentrations to a low concentration of urea to verify unfolded protein was able to refold (data not shown).

These measurements support the more qualitative NMR study findings that very unstable DD mutants are partially to fully unfolded in the absence of ligand and that stabilization with Shield-1 causes the protein domains to adopt more stable folds. These biophysical experiments correlate well with the behavior of the respective DD mutants when expressed in cells (Fig. 1B). The mutants that are stable or only weakly destabilizing when expressed in cells appear relatively well folded in vitro. Likewise, mutants that strongly confer instability to fusion proteins when expressed in cells appear to be unfolded or in dynamic equilibrium between folded and unfolded states when characterized in vitro.

**Degradation in Cells Is Primarily Proteasome-dependent**—Preliminary evidence showed that degradation of the L106P mutant is hindered by inhibition of the proteasome with MG132 or lactacystin (1). To pursue this matter further, we tested whether other protein degradation pathways could also function as routes for DD degradation. To study the effect of blocking the proteasomal or lysosomal pathways on the DD mutants, NIH 3T3 cell lines stably expressing DD-YFP-HA fusions were pretreated with 1 μM Shield-1 for 24 h to allow fusion proteins to reach maximum stabilization levels. We then removed the Shield-1-containing medium and replaced it with medium conditioned with bacterially expressed purified F36V to quickly and efficiently remove ligand from the cells (supplemental Fig. S5). After using this conditioned medium to wash out Shield-1, cells were incubated with 10 μM MG132, 100 μM chloroquine, or vehicle in the presence or absence of Shield-1. After 6 h, cells were collected, and lysates were resolved by SDS-PAGE and blotted for expression of YFP. Proteasome inhibitors, but not lysosome inhibitors, attenuate the degradation of DDS when Shield-1 is withdrawn (Fig. A4). This finding suggests that the proteasome is the primary site of DD degradation, which is consistent with the view that the proteasome is the destination for most regulated protein turnover, whereas the lysosome is mainly responsible for bulk turnover of cytosol, organelles, and protein aggregates (17, 30).

**FKBP-derived Destabilizing Domains Are Rapidly Ubiquitinated in Absence of Shield-1**—To test whether the DDs are targeted to the proteasome via the UPS pathway, we utilized the UBA protein domain from human ubiquitin 2 conjugated to resin to probe for ubiquitin modifications (31). This UBA resin has affinity for polyubiquitin chains and no observed chain linkage preference. NIH 3T3 cell lines stably expressing the various DD-YFP-HA constructs were treated with Shield-1 for 24 h followed by ligand washout and incubation for 4.5 h in one of three conditions: no ligand and proteasome inhibited with MG132; no ligand and no proteasome inhibition; and Shield-1 ligand present and proteasome inhibited. Equal amounts of lysate for each condition were affinity purified using UBA resin, and the bound fractions were resolved by SDS-PAGE. The DD fusion proteins were probed using antibodies against YFP (Fig. 4B) and HA (data not shown).

When Shield-1 is absent and the proteasome is inhibited, ubiquitin chains are detected at higher molecular weight ladders for L106P, V4A/I7V, and L106A. Relative amounts of ubiquitin chains are inversely proportional to the basal stability of the DD mutant observed in cells, with the less stable L106P and V4A/I7V mutants showing more ubiquitination than the L106A or parental F36V mutants. Ubiquitin chains are not detected after Shield-1 removal when the proteasome is active, indicating that polyubiquitinated proteins are degradation competent. Lastly, ubiquitin chains are not detected when the DD is stabilized by Shield-1, despite inhibition of the protea-
some, showing that ubiquitination is not an artifact of blocking the proteasome. Taking these findings together, we conclude that modification with ubiquitin chains represents processing of the DD proteins for proteasomal degradation when ligand is absent, possibly because the DD is unfolded as evidenced by the experiments with purified protein.

As a control for interaction with the resin, the UBA affinity purification was repeated with lysate that was first treated with Usp2cc, the catalytic core of a deubiquitinating enzyme that removes all ubiquitin chains from proteins (32). After Usp2cc treatment of lysates from cells in which Shield-1 was removed and the proteasome inhibited, high molecular weight species are not captured by the UBA resin (Fig. 4C). Some residual signal may indicate that unstable unmodified DD-YFP proteins (~40 kDa) have affinity for the resin itself.

To investigate the timing of DD ubiquitination, we switched to HeLa S3 cells that could be grown in suspension, which would allow us to rapidly collect samples following Shield-1 washout. This experiment used sfGFP instead of YFP fused to the C terminus of the L106P mutant. Two spinner flasks of L106P-sfGFP cells were adapted to growth in suspension and treated with 1 μM Shield-1 for 24 h. After Shield-1 washout using conditioned medium, cells were cultured in the presence or absence of the proteasome inhibitor bortezomib, and samples were collected at 0, 5 min, 15 min, 30 min, 1.5 h, 2.5 h, 4.5 h, and 6 h. Lysates were affinity purified using UBA resin as described for the prior experiment.

FIGURE 4. DD fusion proteins are ubiquitinated and degraded by the proteasome in the non-permissive state. A, NIH 3T3 cell lines stably expressing DD-YFP-HA were treated with 1 μM Shield-1 (S1) for 24 h, and then ligand was washed out, and cells were incubated for 4.5 h in the absence or presence of 1 μM Shield-1, 10 μM MG132, and/or 100 μM chloroquine (CQ). Whole cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody that recognizes YFP. Tubulin was used as a loading control. B, affinity purification with UBA resin. Same cell treatment as in A, except chloroquine was omitted. Equal amounts of whole cell lysate were incubated with UBA resin overnight. The bound fraction was eluted, resolved by SDS-PAGE, and immunoblotted with anti-GFP antibody that recognizes YFP.

C, Shield-1 + MG132 lysates from B were treated with Usp2cc before UBA purification. D, after 24 h with 1 μM Shield-1 stabilization, L106P-sfGFP Hela S3 cells were collected at various time points after ligand washout, either in the presence or absence of 2 μM bortezomib. Equal amounts of whole cell lysate were incubated with UBA resin overnight. Bound fraction was eluted and immunoblotted with anti-GFP antibody. Tubulin from the input is a loading control.

High molecular weight ubiquitin ladders are detected in both time courses, although the polyubiquitinated species are degraded after ~1.5 h when the proteasome is active (Fig. 4D). Ubiquitin chains are visible within 5 min following ligand washout, suggesting that in the non-permissive state, DDs are rapidly modified by the UPS. We also observe increased affinity over time of L106P-sfGFP for the UBA resin when the proteasome is blocked, presumably because L106P-sfGFP is unfolding and exposing hydrophobic residues that bind nonspecifically to the resin. Treatment of lysate from the 6-h time point of L106P-sfGFP plus bortezomib with Usp2cc prior to incubation with UBA resin reduced this binding, suggesting that ubiquitin may play a role in this process.

FKBP-derived Destabilizing Domains Are Cellular Substrates for UPS—We next designed an experiment to perturb the ubiquitin chain-forming process of the UPS in living cells while monitoring DD stability in individual cells by analytical flow
cytometry. We incorporated a ubiquitin mutant encoding all seven lysines converted to arginine (UbK0) into a retroviral vector that also expressed a red fluorescent protein (HcRed) behind an internal ribosome entry site. Without available lysines to enable further ubiquitin conjugation, UbK0 should act as a chain terminator. If the degradation of DD fusion proteins is ubiquitin-dependent in cells, expressing UbK0 would be expected to slow the degradation of DD fusion proteins by increasing the half-life of species with short, degradation-incompetent ubiquitin chains.

NIH3T3 cell lines stably expressing the various DD-YFP-HA constructs were subjected to a second round of infection with virus encoding either UbK0 and HcRed or an empty multiple cloning site with HcRed behind the internal ribosome entry site. Infections were done in triplicate, and cells were analyzed by flow cytometry 48 h after infection and in the absence of Shield-1. Using HcRed fluorescence as a marker of infection, the median level of DD-YFP fluorescence was compared in cells infected with virus encoding UbK0 versus the control empty vector.

Fluorescence of all DD-YFP-HA fusion proteins increased in the presence of UbK0 compared with the empty vector, with statistically significant increases for the L106P (<0.0001), V4A/I7V (<0.0001), and L106A mutants (<0.001) as calculated from a two-sample *t* test (Fig. 5). The parental F36V fusion protein appeared slightly more fluorescent upon UbK0 infection, but the variation in fluorescence within triplicate infections was too large for a statistically significant difference between UbK0 and empty vector (*p* < 0.1). The fact that infection with the chain-terminating UbK0 stabilizes the unstable DD mutants in the absence of Shield-1 further supports our evidence that in the non-permissive state DD fusion proteins are substrates of the UPS.

Stabilization of FKBP mutants by addition of UbK0 is significantly less than stabilization with saturating amounts of Shield-1. We compared change in median fluorescence of GFPodc, an unstable, ubiquitin-independent proteasome substrate, after infection with UbK0 or empty vector (6). As expected, infection with UbK0 had no effect on degradation of GFPodc (*p* > 0.1). GFPodc control shows that UbK0 is not interfering with ubiquitin-independent proteasome function. The modest effect of UbK0 on DD stability likely results from a weak dominant-negative effect due to a combination of low concentration of UbK0 relative to wild-type ubiquitin and a recent study that reports UbK0 disproportionately incorporates into Lys-63 linkages over Lys-48 (33).

**DISCUSSION**

Our panel of FKBP-derived DD mutants contains members displaying a range in basal stability when expressed in mammalian cells. The findings presented here link this cellular instability to both the propensity of the protein domain to unfold and the extent of ubiquitination in the non-permissive state. We propose a model in which DDs unfold or sample an unfolded state upon removal of Shield-1-stabilizing ligand, are recognized by as yet unknown members of the PQC machinery and are modified by the UPS en route to destruction at the proteasome. The inherent propensity of a given destabilizing domain to unfold dictates the extent of recognition by the PQC, thus more stable, folded mutants such as F36V accumulate to higher levels in cells in the ligand-free state.

Results from the two-dimensional HSQC NMR and urea denaturation show that DDs with low stability in cells are unfolded (or significantly sampling unfolded states) and that Shield-1 binding promotes a more stable structure. The unfolded and folded states are reversible, as purified DD mutants expressed under non-permissive conditions in bacteria are capable of binding Shield-1 and folding into a stable structure. It is interesting to note that the V4A/I7V mutant shows a more stable structure than the L106P mutant in the absence of ligand in urea denaturation and NMR studies, and yet YFP fusions to the two mutants show similar levels of instability in cells by flow cytometry. This could suggest that cells have a stability threshold below which proteins are efficiently targeted for degradation or alternatively that certain structural features of FKBP unfolding are more important than others for PQC recognition.

When expressed in cells, the unstable DD mutants are tagged with ubiquitin chains as substrates of the UPS in the absence of Shield-1. These ubiquitin chains are degradation competent, as they do not persist when the proteasome is inhibited (Fig. 4B). The extent of DD ubiquitination after Shield-1 removal correlates with the observed basal stability in cells. Importantly, the ubiquitin chains are not artifacts of proteasome inhibition because the presence of Shield-1 prevents polyubiquitination of DD mutants. Many proteins have been shown to exhibit ubiquitin-dependent degradation at the proteasome (9, 12, 15, 34, 35). Few experiments, however, measure how quickly these proteins are recognized by the UPS machinery and targeted for degradation, as most proteins do not possess a conditional switch for stability (i.e. Shield-1). The data presented here suggest that recognition of unstable DDs and their polyubiquitination is very rapid, with degradation at the proteasome occurring more slowly. Further cellular studies of this pool of ubiquitinated but undegraded protein are warranted to determine whether it forms micro-aggregates and what protein interactions are occurring. Rapid ubiquitin chain accumulation seems less pronounced when the proteasome is inhibited. This may be attributed to activation of a stress response or competition with other cellular proteins for recognition by the UPS machinery or...
for ubiquitin itself, as bortezomib causes rapid depletion of free ubiquitin in cells (36).

DD fusion proteins were stabilized by impairment of UPS targeting in living cells upon expression of UbK0. This stabilization occurs without any Shield-1 washout, supporting the model that the UPS is degrading nascent and misfolded DDs. The studies presented here demonstrate relationships in DD mutants between high basal instability in cells and their in vitro propensity to unfold, the extent of ubiquitination after removal of ligand, and sensitivity to a chain-terminating mutant ubiquitin. Taken together, these experiments support a model that in the absence of Shield-1 FKBP-derived DDs are unfolded, rapidly ubiquitinated, and degraded by the proteasome. Further studies are necessary to determine which specific PQC and/or UPS proteins are responsible for recognition and processing of FKBP-derived DDs, although this question may be difficult to answer due to redundancy in substrate specificity of ubiquitin ligases in mammalian cells. It will also be interesting to look more closely at the kinetic behavior of the UPS for fusion proteins restricted to different cellular compartments to query different arms of the PQC machinery.

It is clear that proper maintenance of PQC is important for normal cellular function, with deficits resulting in diseases such as Huntington disease, Alzheimer disease, cancer, and aging (37–39). Different cellular compartments and different cell types display a wide range in PQC response to misfolded proteins (20, 40). Most of what is currently known about the PQC machinery, however, has been elucidated with disease-related protein substrates, whose relative stability is fixed. The conditional nature of DD stability allows a rapid and non-perturbing switch from stable to unstable protein unlike other methods currently used to study protein quality control, affording ligand-dependent control of degradation rates. Future studies with DDs can help probe how the PQC and UPS pathways interconnect, from early PQC recognition events of misfolded protein, to substrate handoff between PQC and UPS protein components, and ultimate regulation by degradation at the proteasome.

Since its development, FKBP12-derived DD technology has been applied in numerous systems to a variety of protein targets (41–47). Recently, an orthogonal system using a different small protein–ligand pair was developed, allowing researchers to control two proteins independently in one cell (2). Early reports suggest that the E. coli dihydrofolate reductase-derived DD system will be equally portable to different organisms and protein targets (48). Continued studies of DD degradation in different living systems and with E. coli dihydrofolate reductase-derived DDs will help identify conserved links between protein quality control pathways and protein degradation pathways, highlighting common mechanisms cells use to maintain protein homeostasis.

Acknowledgments—We acknowledge the Stanford Shared FACS Facility for use of instruments. We also thank Ron Kopito and members of the Kopito Laboratory at Stanford University for valuable reagents and advice. The Stanford Magnetic Resonance Laboratory is supported by the Stanford University School of Medicine.

REFERENCES

1. Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G., and Wandless, T. J. (2006) Cell 126, 995–1004
2. Iwamoto, M., Björklund, T., Lundberg, C., Kirik, D., and Wandless, T. J. (2010) Chem. Biol. 17, 981–988
3. Dunlop, R. A., Brunk, U. T., and Rodgers, K. J. (2009) IUBMB Life 61, 522–527
4. Yano, M., Koumoto, Y., Kanezaki, Y., Wu, X., and Kido, H. (2004) Biomacromolecules 5, 1465–1469
5. Janse, D. M., Crosas, B., Finley, D., and Church, G. M. (2004) J. Biol. Chem. 279, 21415–21420
6. Matsuzawa, S., Cuddy, M., Fukushima, T., and Reed, J. C. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 14982–14987
7. Schrader, E. K., Harstad, K. G., and Matouschek, A. (2009) Nat. Chem. Biol. 5, 815–822
8. Behrends, C., and Harper, J. W. (2011) Nat. Struct. Mol. Biol. 18, 520–528
9. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) EMBO J. 19, 94–102
10. Finley, D. (2009) Annu. Rev. Biochem. 78, 477–513
11. Venancio, T. M., Balaji, S., Iyer, L. M., and Aravind, L. (2009) Genome Biol. 10, R33
12. Kaganovich, D., Kopito, R., and Frydman, J. (2008) Nature 454, 1088–1095
13. Su, L., Lineberry, N., Huh, Y., Soares, L., and Fathman, C. G. (2006) J. Immunol. 177, 7559–7566
14. Hirsch, C., Gauss, R., Horn, S. C., Neuber, O., and Sommer, T. (2009) Nature 458, 453–460
15. Rosenbaum, J. C., Fredrickson, E. K., Oeser, M. L., Garrett-Engele, C. M., Locke, M. N., Richardson, L. A., Nelson, Z. W., Hetrick, E. D., Milac, T. L., Gottschling, D. E., and Gardner, R. G. (2011) Mol. Cell 41, 93–106
16. Okiyoneda, T., Barrière, H., Bagdàny, M., Rabeh, W. M., Du, K., Höhfeld, J., Young, J. C., and Lukacs, G. L. (2010) Science 329, 805–810
17. Bukau, B., Weissman, J., and Horwich, A. (2006) Cell 125, 443–451
18. Wiseman, R. L., Haynes, C. M., and Ron, D. (2010) Cell 140, 590–599.e2
19. Bengtson, M. H., and Joazeiro, C. A. P. (2010) Nature 467, 470–473
20. Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y., and Patterson, C. (1999) Mol. Cell Biol. 19, 4535–4545
21. McDonough, H., and Patterson, C. (2003) Cell Stress Chaperones 8, 303–308
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. A.1–3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
23. Blommel, P. G., and Fox, B. G. (2007) Protein Expr. Purif. 55, 53–68
24. Deleted in proof
25. Main, E. R., Fulton, K. F., and Jackson, S. E. (1998) Biochemistry 37, 6145–6153
26. Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F. J., Gilman, M., and Holt, D. A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 10437–10442
27. Rosen, M. K., Michnick, S. W., Karplus, M., and Schreiber, S. L. (1991) Biochemistry 30, 4774–4789
28. Fulton, K. F., Main, E. R., Daggett, V., and Jackson, S. E. (1999) J. Mol. Biol. 291, 445–461
29. Main, E. R., Fulton, K. F., and Jackson, S. E. (1999) J. Mol. Biol. 291, 429–444
30. Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005) Molecular Cell 17, 351–365
31. Bennett, E. J., Shaler, T. A., Woodman, B., Ryu, K. Y., Zaïtseva, T. S., Becker, C. H., Bates, G. P., Schulman, H., and Kopito, R. R. (2007) Nature 448, 704–708
32. Ryu, K. Y., Baker, R. T., and Kopito, R. R. (2006) Anal. Biochem. 353, 153–155
33. Ziv, I., Mattiuhin, Y., Kirkpatrick, D. S., Erpazazoglou, Z., Leon, S., Pantazopoulou, M., Kim, W., Gygi, S. P., Huguenauer-Tsapis, R., Reis, N., Glickman, M. H., and Kleinfeld, O. (2011) Mol. Cell Proteomics 10, M111.009753
34. Bence, N. F., Bennett, E. J., and Kopito, R. R. (2005) Methods Enzymol. 399, 352–358
Ligand-switchable Substrates for UPS

481–490
35. Ravid, T., and Hochstrasser, M. (2008) Nat. Rev. Mol. Cell Biol. 9, 679–690
36. Xu, Q., Farah, M., Webster, J. M., and Wojcikiewicz, R. J. (2004) Mol. Cancer Ther. 3, 1263–1269
37. Hegde, A. N., and Upadhya, S. C. (2011) Biochim. Biophys. Acta 1809, 128–140
38. Huang, Q., and Figueiredo-Pereira, M. E. (2010) Apoptosis 15, 1292–1311
39. Vernace, V. A., Schmidt-Glenewinkel, T., and Figueiredo-Pereira, M. E. (2007) Aging Cell 6, 599–606
40. Minami, R., Hayakawa, A., Kagawa, H., Yanagi, Y., Yokosawa, H., and Kawahara, H. (2010) J. Cell Biol. 190, 637–650
41. Armstrong, C. M., and Goldberg, D. E. (2007) Nat. Methods 4, 1007–1009
42. Banaszynski, L. A., Sellmyer, M. A., Contag, C. H., Wandless, T. J., and Thorne, S. H. (2008) Nat Med 14, 1123–1127
43. Dolan, B. P., Li, L., Veltri, C. A., Ireland, C. M., Bennink, J. R., and Yewdell, J. W. (2011) J. Immunol. 186, 2065–2072
44. Herm-Götz, A., Agop-Nersesian, C., Münter, S., Grimley, J. S., Wandless, T. J., Frischknecht, F., and Meissner, M. (2007) Nat. Methods 4, 1003–1005
45. Madeira da Silva, L., Owens, K. L., Murta, S. M., and Beverley, S. M. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 7583–7588
46. Perng, Y. C., Qian, Z., Fehr, A. R., Xuan, B., and Yu, D. (2011) J. Virol. 85, 4841–4852
47. Schoeber, J. P., van de Graaf, S. F., Lee, K. P., Wittgen, H. G., Hoenderop, J. G., and Bindels, R. J. (2009) Am. J. Physiol. Renal Physiol 296, F204–211
48. Muralidharan, V., Oksman, A., Iwamoto, M., Wandless, T. J., and Goldberg, D. E. (2011) Proc. Natl. Acad. Sci. U.S.A. 108, 4411–4416
49. Maynard-Smith, L. A., Chen, L. C., Banaszynski, L. A., Ooi, A. G., and Wandless, T. J. (2007) J. Biol. Chem. 282, 24866–24872