A Directed Approach for Engineering Conditional Protein Stability Using Biologically Silent Small Molecules

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The ability to regulate the function of specific proteins using cell-permeable molecules can be a powerful method for interrogating biological systems. To bring this type of “chemical genetic” control to a wide range of proteins, we recently developed an experimental system in which the stability of a small protein domain expressed in mammalian cells depends on the presence of a high affinity ligand. This ligand-dependent stability is conferred to any fused partner protein. The FK506- and rapamycin-binding protein (FKBP12) has been the subject of extensive biophysical analyses, including both kinetic and thermodynamic studies of the wild-type protein as well as dozens of mutants. The goal of this study was to determine if the thermodynamic stabilities (ΔΔG_{un}) of various amino acid substitutions within a given protein are predictive for engineering additional ligand-dependent destabilizing domains. We used FKBP12 as a model system and found that in vitro thermodynamic stability correlates weakly with intracellular degradation rates of the mutants and that the ability of a given mutation to destabilize the protein is context-dependent. We evaluated several new FKBP12 ligands for their ability to stabilize these mutants and found that a cell-permeable molecule called Shield-1 is the most effective stabilizing ligand. We then performed an unbiased microarray analysis of NIH3T3 cells treated with various concentrations of Shield-1. These studies show that Shield-1 does not elicit appreciable cellular responses.

Cell-permeable small molecules have long been powerful tools for interrogating biology (1). They can be used to conditionally probe biological processes, often with high temporal resolution (2–4). Interest in using perturbants of this type has grown significantly in the past decade, but one of the pressing questions remains. How can one discover a cell-permeable perturbant for any protein of interest? Nature was the source for many of the early examples, especially when the biologically relevant target of the perturbant was easily discerned. To accelerate the discovery process, many investigators are screening large libraries of small molecules either against purified proteins or against living cells using high content imaging and phenotype-based scoring to evaluate library members (5). Irrespective of the discovery process used to identify the perturbant, the question of specificity remains critical for research biology (6). When a particular reagent is used to conditionally perturb a biological process, how confident can one be that the resulting phenotype can be ascribed to the putative molecular target of the perturbant?

We have chosen an alternate strategy to provide small molecule control of any protein of interest (7). Our approach relies upon one well characterized protein-ligand interaction that can be used to regulate many different proteins of interest. We have engineered small protein domains called destabilizing domains (DDs)2 that are constitutively degraded when expressed in mammalian cells. This instability is conferred to any other protein fused to the DD. A high affinity ligand binds to the DD and prevents it from being degraded. This ligand-mediated stability allows the fused protein to perform its cellular function, provided that it is fully functional in the context of a fusion protein. Specificity is provided by the genetic fusion of the DD to the protein of interest, and speed, reversibility, and tunability are provided by the stabilizing ligand.

One of the challenges to this approach is engineering the desired DDs. What is the most efficient procedure to take an otherwise stable protein-ligand pair and selectively perturb the protein structure to the point of instability in the absence of a ligand and stability in the ligand-bound state? In one approach, one can assume nothing and use an unbiased screening strategy to identify mutants of the protein that endow the structure with the desired ligand-dependent stability (7). Alternatively, one can rely upon thermodynamic analyses of the protein, if available, to rationally choose mutations that might confer ligand-dependent stability. Another challenge for this strategy is the desire to use stabilizing ligands that do not perturb the cellular environment by binding to off-target proteins. In this study, we evaluate the ability of directed amino acid substitutions to confer ligand-dependent stability to a small protein domain. We also investigate the specificity of ligands that stabilize these domains. This comparison of two possible discovery processes (unbiased screening versus biophysics-guided engineering) will probably inform future attempts to develop new destabilizing domains.

2 The abbreviations used are: DD, destabilizing domain; FKBP or FKBP12, human 12-kDa FK506- and rapamycin-binding protein; HcRed, red fluorescent protein from Heteractis crispa; SAM, significance analysis of microarrays; SLF, synthetic ligand for FKBP; YFP, yellow fluorescent protein.
EXPERIMENTAL PROCEDURES

FKBP Mutant Generation—The retroviral vector pBMN FKBP-YFP-i-HcRed-tandem was assembled by ligating FKBP mutants into the pBMN YFP HA-i-HcRed-t vector. QuikChange site-directed mutagenesis (Stratagene) was used to incorporate single point mutations into the FKBP-F36V gene. Double and triple mutants were obtained by sequential mutagenesis or PCR amplification. Transfection quality DNA was produced in Escherichia coli.

Tissue Culture and Transfections—NIH3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated donor bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 μg of streptomycin. The FNX ectopic packaging line was cultured with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 μg of streptomycin. The FNX cell line was transfected using standard Lipofectamine 2000 protocols. Viral supernatants were harvested 48 h post-transfection, filtered, and concentrated using an Amicon Ultra centrifugal filter (100-kDa cut-off; Millipore Corp.). NIH3T3 cells were incubated with the concentrated viral supernatant supplemented with 4 μg/ml Polybrene for 4 h at 37 °C. The viral supernatant was then removed, and fresh medium was added to the cells. Cells were cultured for 24 h to allow for viral integration.

Flow Cytometry—Transduced NIH3T3 cells were plated at 1 × 10^5 cells/well of a 12-well plate and treated as described 24 h prior to analysis. Cells were removed from wells using trypsin-EDTA (Invitrogen) and quenched with 1 ml of growth medium. The medium was removed, and cells were resuspended in 300 μl of PBS. Cells were analyzed at the Stanford Shared FACS Facility with 10,000 events represented.

Antibodies—Immunoblotting was performed using the following antibodies: FKBP (mouse, 554091; BD Biosciences) and YFP, Aequorea victoria (JL-8; Clontech).

Microarray Analysis—NIH3T3 cells were cultured in medium containing vehicle or 1 μM, 100 nM, or 10 nM Shield-1 for 24 h. Total RNA was extracted using the RNeasy kit (Qiagen), labeled, and hybridized to the mouse exonic evidence-based oligonucleotide arrays. The hybridizations were performed by the Stanford Functional Genome Facility. After overnight hybridization at 65 °C, each array was washed and scanned using the GenePix 4000A microarray scanner (Axon Instruments). SAM was used to identify genes whose expression had significantly changed after treatment with Shield-1 (19). A complete description of the experiment including data filtering and analysis is included in the supplemental material.

FKBP Ligands—Reagents are available upon request. Shield-1 will be distributed to all qualified investigators.

RESULTS

Human FKBP12 (hereafter called FKBP) has been the subject of numerous kinetic and thermodynamic analyses of protein folding and stability (8–13). In addition to the wild-type protein, the relative contributions of dozens of individual amino acid mutations to the stability of FKBP have been analyzed in vitro (8). These biophysical studies provide a rich source of data with which to guide the “rational design” strategy for engineer-
fluorescence polarization-based competition binding assay revealed that the affinities of the four ligands for the F36V protein were experimentally indistinguishable (supplemental Fig. 1) (18). Fluorescence polarization was also used to measure the affinities of Shield-1 for the F36V parent protein as well as for the three mutants (supplemental Figs. 2 and 3). The dissociation constants of Shield-1 for the four proteins varied between 2 and 4 nM, suggesting that the different potencies of stabilization observed for the analogs are the result of differences in cellular permeability rather than differences in affinity for FKBP.

We next tested all three FKBP mutants using Shield-1 as the stabilizing ligand. With the FKBP mutants fused to the N terminus of YFP, a 1 μM dose of Shield-1 fully stabilized YFP expression levels (Fig. 3A). A triple mutant incorporating all three destabilizing mutations showed very low expression with no detectable Shield-1 dependence (Fig. 3A). The FKBP mutants are not as destabilizing when they are fused to the C terminus of YFP (Fig. 3B). Shield-1 stabilizes the C-terminal fusions, although the expression levels do not quite reach those of the parent F36V protein. Treatment of the F36V parent fusion proteins with Shield-1 causes a modest rise in expression levels, suggesting that the binding of the ligand to the FKBP domain reduces the rate of degradation. When fused to the C terminus of YFP, the triple mutant (V2A/L50A/L106A) is not significantly more destabilizing than the V2A or L50A mutants, although stabilization of the triple mutant by Shield-1 is attenuated. Transduced cells were treated with either vehicle or Shield-1, and lysates were immunoblotted with antibodies against either FKBP12 or YFP (supplemental Fig. 4A). As expected, protein levels by Western blot correlate well with YFP levels measured by analytical flow cytometry, and no evidence of partial degradation was observed. Inhibition of the proteasome with lactacystin prevented degradation of the V2A-YFP fusion protein (supplemental Fig. 4B), which is consistent with our previous findings (7).

To determine if additional destabilizing mutations would result in an additive effect on FKBP stability, we returned to the biophysical analyses and made further mutations at the N ter-
minus of FKBP. The V4A and I7V mutations are destabilizing by 2.78 and 0.92 kcal/mol (8), respectively, so we incorporated these into the FKBP F36V background in all possible combinations with the V2A mutation. These single, double, and triple mutants were fused to the N terminus of YFP and individually transduced into NIH3T3 cells, and YFP expression levels were measured in the absence and presence of 1 μM Shield-1 (Fig. 4A). Treatment with Shield-1 stabilized the expression levels of all seven mutants, and the most efficiently rescued mutants were the single point mutants, V2A and V4A, and the double mutants, V2A/I7V and V4A/I7V. These four mutants were further characterized to determine the rates of protein degradation upon removal of Shield-1 (Fig. 4B). The mutants that displayed the lowest expression levels in the absence of Shield-1 showed the fastest rate of protein degradation, with both double mutants returning to basal levels after 4 h.

As a simple test of the potential generality of this destabilizing activity, we fused the V2A mutant to the N terminus of firefly luciferase. Firefly luciferase is stabilized by Shield-1 in a dose-responsive manner (supplemental Fig. 5). As observed

FIGURE 3. Shield-1 stabilizes the FKBP-YFP fusion proteins. NIH3T3 cells stably expressing the indicated fusion proteins were split into two populations that were either mock-treated (−) or treated with 1 μM Shield-1 (+) for 24 h. FKBP mutants were fused to the N terminus (A) or the C terminus (B) of YFP, and expression levels were measured by flow cytometry and normalized to cells expressing the parent F36V-YFP protein treated with 1 μM Shield-1. Data represent the average of three experiments ± S.E.

FIGURE 4. Characterization of additional FKBP mutants. A, FKBP-YFP fusions were either mock-treated (−) or treated with 1 μM Shield-1 (+), and YFP expression levels were monitored by flow cytometry. Data are presented as the average MFI ± S.E. relative to that of the parent F36V FKBP. B, NIH3T3 cells stably expressing FKBP-YFP fusion proteins were treated with 1 μM Shield-1 for 24 h. The cells were then washed with medium to remove Shield-1, and decreases in fluorescence intensity were monitored by flow cytometry: V2A-YFP (squares), V4A-YFP (triangles), V2A/I7V-YFP (diamonds), and V4A/I7V-YFP (circles).
with the YFP reporter, Shield-1 treatment provides modest stabilization to the fusion of F36V to luciferase. In contrast, fusion of V2A to luciferase results in destabilization of the fusion protein, with expression levels and, in turn, luminescence rescued upon the addition of Shield-1 (supplemental Fig. 5B). A kinetic analysis showed that Shield-1 treatment causes maximum stabilization in 2–3 h and, further, that removal of Shield-1 from stabilized cells results in loss of luciferase expression in 1–2 h (supplemental Fig. 5, C and D).

The ideal system to conditionally regulate protein expression levels using small molecules would utilize a stabilizing ligand that does not exhibit any off-target effects in a biological environment. In order to assess the ability of Shield-1 to perturb gene expression patterns, we treated NIH3T3 cells with vehicle or one of three concentrations of Shield-1 (10, 100, and 1000 nM). Each set of conditions was performed in triplicate, and following 24 h of Shield-1 treatment, RNA was isolated, processed, and used to probe mouse exonic evidence-based oligonucleotide arrays. We used significance analysis of microarray (SAM) to identify genes that were differentially expressed following 24 h of Shield-1 treatment, RNA was isolated, probed mouse exonic evidence-based oligonucleotide arrays. The false discovery rate was obtained using unpaired two-class SAM, and genes whose mRNA levels changed significantly upon Shield-1 treatment were identified.

TABLE 1

| Clone ID   | Gene symbol | Gene description | Fold change (false discovery rate) |
|------------|-------------|------------------|-----------------------------------|
| mSQ003922  | Ebp4.1I2    | Erythrocyte protein band 4.1-like 2 | 2.2 (<0.1%)                        |
| mSQ001430  | Cankt1c     | Casein kinase 1, ε | 2.7 (<0.1%)                        |
| mSQ000375  | Tcfe3       | Transcription factor E3   | 1.9 (27.5%)                         |

- Up-regulated genes
- Down-regulated genes

The data shown in Fig. 3 suggest that there is a strong context dependence with respect to both the sites of mutation and the orientation of the fusion protein. FKB mutants fused at the N terminus of YFP are more strongly destabilizing than C-terminal fusions. The higher efficiency with which the N-terminal fusions are degraded may reflect mechanistic differences in the quality control machinery monitoring protein stability. The nature of the amino substitution is also important. When fused at the N terminus of YFP, the L106P mutant is strongly destabilizing (1–2% expression in the absence of Shield-1) (7). However, the L106A mutation is only moderately destabilizing (25–30% expression in the absence of Shield-1).

Within a given fusion protein orientation, (i.e. FKB fused to the N terminus of YFP), we observed a positive, but weak, correlation between in vitro thermodynamic stability and intracellular stability in the context of a fusion protein. All of the mutants are destabilizing, but the extent of destabilization differs. The V2A, L50A, and L106A mutations are similarly destabilizing in terms of Δ∆G_{U-V}; however, the expression levels of their YFP fusion proteins are 5, 11, and 25%, respectively, in the absence of Shield-1.

Mammalian cells must be able to unfold proteins for degradation by ATP-dependent proteases as well as for translocation across some membranes (20). When analyzing the susceptibility of proteins to protease-mediated degradation, Matouschek and co-workers (21) have shown that the local structure of the
substrate is more important than overall protein stability. They have also demonstrated that efficient proteasome-mediated degradation requires an unstructured region within the target protein to serve as an initiation site (22). Similar context-dependent effects have been observed by Kenninston et al. (23) as well as Canet et al. (24). In light of these studies, it is possible that the mutations of the single domain FKBP protein regulate the different intracellular rates of degradation through specific local effects rather than global effects on protein stability.

The effects of our destabilizing mutations appear to be additive. When we made additional mutations to the FKBP domain of the V2A-YFP fusion protein, we observed that a greater degree of instability was conferred relative to V2A alone (Fig. 4A). As we have observed previously, mutants that are strongly destabilizing exhibit faster degradation rates when Shield-1 is withdrawn (7). Upon the addition of Shield-1, the rates of synthesis for the mutants are indistinguishable. In this respect, these FKBP-derived DDs function as ligand-dependent degrons that modulate the rate of degradation. It is the ratio of the rates of synthesis and degradation that govern the expression level of a given protein.

Several new FKBP ligands were synthesized and tested as stabilizing ligands, and we observed that their potencies differed widely (Fig. 2B). The affinity of each ligand for the F36V protein was measured, and the dissociation constants are very similar (supplemental Fig. 1). This observation suggests that the range of stabilizing potencies results from different cellular permeabilities rather than underlying biophysical differences. Shield-1 binds equally well to the V2A, L50A, and L106A mutants, as expected.

It is difficult to prove that Shield-1 is not binding to other proteins and causing cellular responses that are unrelated to FKBP fusion proteins. To probe the specificity of the interaction between Shield-1 and other cellular constituents, we used an unbiased microarray analysis to look for perturbations in gene expression patterns in NIH3T3 cells treated with different concentrations of Shield-1. We were particularly interested in genes whose expression exhibited dose-dependent changes in response to different concentrations of Shield-1. Using fairly liberal thresholds for significance, we found only a very small number of genes whose RNA levels changed appreciably relative to mock-treated control cells. Importantly, none of the gene expression changes were observed in all three concentrations of Shield-1 that were tested. These data support the notion that Shield-1 is relatively free of off-target perturbations to the cellular environment. Our findings are consistent with those of the Ariad group (25) that tested a structurally similar dimeric FKBP ligand in a Phase I human clinical trial.

The ability to conditionally regulate a single protein of interest will probably provide many new insights into complex biological systems. However, many investigations would benefit from the ability to independently regulate the levels of two or more different proteins. For example, a method to separately control the intracellular expression levels of GSK-3α and GSK-3β would be useful for probing the potentially overlapping functions of these two kinases.

The existence of two or more DD systems that are regulated by orthogonal small molecules would be useful, so what is the best discovery method to identify new DDs?

Ligand-dependent stability can be engineered into FKBP using either biophysical data as detailed in this work or through screening an unbiased library of FKBP mutants (7). The biophysics-based design strategy delivers mutants that display the desired ligand-dependent stability, although the extent of destabilization conferred by the various mutants is not uniform or predictable. Additionally, the biophysical data required for this design strategy are not as widely available for other proteins as might be needed. Conversely, one can manipulate various parameters in a screening process (e.g. concentration of stabilizing ligand, incubation periods), to deliver mutants with specific characteristics. From this perspective, the unbiased screening strategy appears to be a more reliable strategy for engineering new destabilizing domains.

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