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Destabilizing Domains Derived from the Human Estrogen Receptor

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Supporting Information

ABSTRACT: Methods to rapidly and reversibly perturb the functions of specific proteins are desirable tools for studies of complex biological processes. We have demonstrated an experimental strategy to regulate the intracellular concentration of any protein of interest by using an engineered destabilizing protein domain and a cell-permeable small molecule. Destabilizing domains have general utility to confer instability to a wide range of proteins including integral transmembrane proteins. This study reports a destabilizing domain system based on the ligand binding domain of the estrogen receptor that can be regulated by one of two synthetic ligands, CMP8 or 4-hydroxytamoxifen.

There are a number of perturbation techniques to study gene and protein function in living biological systems. RNA interference has been used to achieve posttranscriptional gene silencing.[1] This approach is widely used in cultured cells for elucidating the function of the target protein or gene. In animal studies the Cre recombinase is commonly used as a gene perturbation method.[2] By using tissue- or cell-specific promoters, Cre expression can be controlled temporally and spatially in order to disrupt targeted genes. However, these approaches are neither reversible nor tunable. Tunable regulation of protein function is desired to mimic pharmaceutical inhibition for the purpose of target validation. All of the techniques that target the DNA or mRNA precursor molecules that encode a protein under study, since existing functional protein molecules must be degraded, suffer from inevitable delays following the perturbation.

Small molecule inhibitors are the most effective reagents to target proteins directly and rapidly. Cell permeable small molecules remain the most widely used inhibitors or activators of specific proteins, and most drugs fall into this category of molecules. For studies of biological processes, small molecules are valued for their speed, dose-dependent manner, and reversibility of their activities that provide a useful complement to genetic techniques. However, the specificity of these agents for their target proteins is always a concern. Small molecules may bind not only to the target protein but also to one or more off-target proteins.

To overcome the trade-off between genetic and chemical perturbation, Shokat and co-workers developed a method by which a specific kinase can be inhibited using a small molecule.[3] They made mutations in the protein of the interest to modify the binding pocket for the ligand. Furthermore, a known chemical inhibitor was also redesigned to fit the modified binding pocket in target kinase. The method has been successful both in cells and in mice. However, this approach is presently limited to ATPases and GTPases, and thus a more widely applicable method is desired. A temperature-sensitive mutant of mammalian dihydrofolate reductase (DHFRts), a destabilizing residue by the N-end rule, was found to be stable at a permissive temperature but unstable at 37 °C.[4,5] The addition of methotrexate, a high-affinity ligand for mammalian DHFR, to cells expressing DHFRts inhibited degradation of the protein partially. This was an important demonstration that a small molecule ligand can stabilize a protein otherwise targeted for degradation in cells. Years later, a rapamycin derivative was used to stabilize an unstable mutant of the FRB domain of mTOR (FRB*) and restore the function of the fused kinase, GSK-3β.[6,7] This system demonstrated that ligand-dependent stability represented an attractive strategy to regulate the function of a specific protein in a complex biological environment. The Muir group also developed a system to control protein activity. In this system, the target protein becomes functional when the ubiquitin complementation occurs by rapamycin-induced dimerization of FK506-binding protein and FKBP12.[8]

We previously developed a strategy in which a cell-permeable ligand is used in conjunction with a genetically encoded protein domain to regulate any protein of interest (Figure 1a).[9,10] Mutants of the human FKBP12 or ecDHFR protein were engineered to be metabolically unstable in the absence of their high-affinity ligands, Shield-1 or trimethoprim (TMP), respectively. We call these mutants destabilizing domains (DDs) and observed that the instability of a DD conferred to any fused partner protein results in degradation of the entire fusion protein by the proteasome. Shield-1 and TMP bind to and stabilize the DD in a dose-dependent manner. The genetic fusion of the DD to the gene of interest ensures specificity, and small-molecule control confers reversibility and dose dependence to protein stability and function.

Based on our experiences with previous DDs, we chose the estrogen receptor ligand binding domain (ERLBD, residues 305−549 of ERS1) as a candidate protein to engineer a novel destabilizing domain. Since the estrogen receptor signaling pathway is involved in a variety of diseases, such as breast cancer, the pathway has been widely studied and numerous agonist and antagonists of estrogen receptor have been developed. Thus, compatible pairs of ERLBD and drugs are known, and ERLBDs are often used as the base for making new biological tools (Figure 1b).[11] Furthermore, previous studies disclosed ligands that bind to mutant but not wild-type forms of the ERLBD.[12] By using one of these mutant domains encoding

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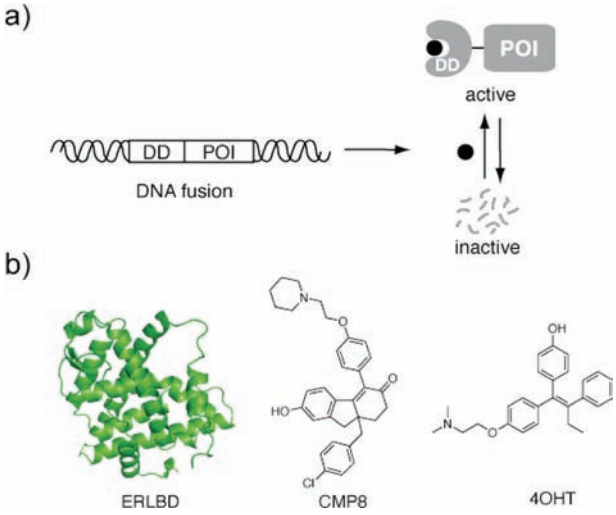

Figure 1. (a) Strategy for conferring ligand-dependent stability to a protein of interest (POI) by fusing the DNA sequence encoding DD to the gene encoding the POI. (b) Structure of wild-type ERLBD and the ligands, CMP8 and 4OHT.

three mutations (L384M, M421G, G521R),[12] we anticipated that it would be possible to regulate the stability of an ERLBD-derived DD using a ligand that does not perturb endogenous estrogen-sensitive networks. An additional mutation (Y537S) was introduced to further destabilize the ERLBD and to configure it as a potential DD candidate.[13] This tetra mutant represented a promising starting point for DD development, as our experiments showed a seven-fold dynamic range regulated by ligand [Figure S1, Supporting Information (SI)].

To engineer mutants that display drug-dependent stability, we designed a cell-based screen using yellow fluorescent protein (YFP) as a reporter for ERLBD stability. The strategy was designed to identify mutants possessing the desired characteristics of a destabilizing domain: low fluorescence signal levels in the absence of ligand, large dynamic range, robust and predictable dose–response behavior, and rapid kinetics of degradation. We used error-prone PCR to generate libraries of ERLBD mutants based on the four parental mutations (L384M M421G G521R Y537S) sequence.[14,15] For the ligand, we synthesized a known compound, CMP8, that has been reported to bind only to the mutant ERLBD.[16] The library was prepared with the ERLBD mutants cloned in-frame at the 3′-end of the YFP gene, and a retroviral expression system was used to stably transduce the library into NIH 3T3 fibroblasts. Fluorescence activated cell sorting (FACS) was used to screen libraries of candidate DDs. First, we sorted the cells treated with 5 μM CMP8 for 43 h, at which point YFP-expressing cells were isolated by FACS. Next, this population was sorted in the absence of CMP8, where we selected cells that exhibited low levels of YFP expression. Cells were dosed with 5 μM CMP8 again, after which point a third sort was performed to select bright cells (Figure S2, SI). This sorted population of cells showed a significant increase in YFP signal compared to parental cells (Figure S2, SI). After three rounds of sorting, the cells were allowed to recover, their genomic DNA was extracted, and candidate DDs were amplified by PCR and isolated.

Genes encoding individual YFP-ERLBD fusions were stably transduced back into NIH 3T3 cells, and YFP fluorescence levels were measured in both the absence and presence of 5 μM CMP8. Mutants displaying low basal fluorescence levels and also high dynamic range were then sequenced (Figure 2, Table

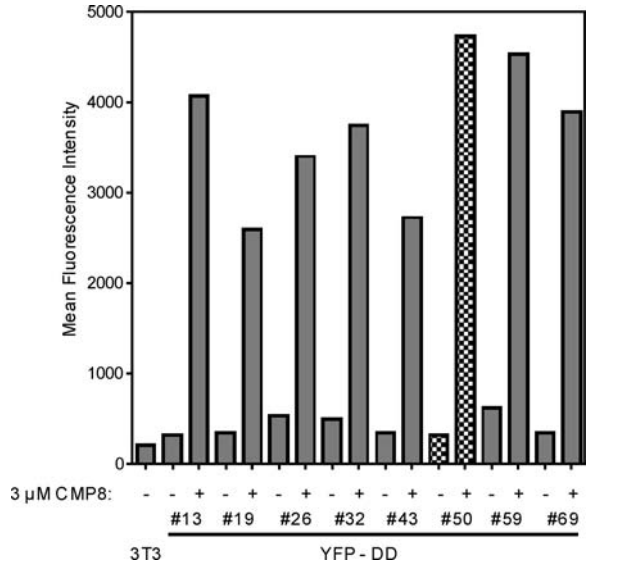

Figure 2. NIH 3T3 cells stably transduced with the indicated YFP-ERLBD fusions derived from error-prone PCR were either mock-treated or treated with 3 μM CMP8 for 24 h and YFP expression was monitored by analytical flow cytometry.

S1, SI). From the screen using ERLBD fused to the C-terminus of YFP, we chose clone no. 50 with the largest dynamic range (~80 fold) for further analysis (ER50 with 6 mutations: T371A L384M M421G N519S G521R Y437S). Treating cells with various concentrations of CMP8 caused YFP levels to increase in a dose-dependent manner as measured by analytical flow cytometry (Figure S3b, SI). DDs fused to the C-terminus of YFP were fully stabilized by 3 μM CMP8 and display wider dynamic range relative to the parental sequence (Figure 2). The enhanced dynamic range is the result of more effective destabilization in the absence of ligand as well as enhanced stabilization after binding to the ligand. Fusing these mutants to the N-terminus of YFP also conferred ligand-dependent stability (Figure S3a, SI). YFP fluorescence, measured by analytical flow cytometry, was nearly as low as the autofluorescence of untransduced NIH 3T3 cells. Ligand for the wild-type estrogen receptor, estradiol and tamoxifen, were also tested, but these did not have any affect on DD stability as predicted (Figure S3c,d, SI).

Next, we investigated the kinetics of DD rescue and degradation. Upon treatment with CMP8 or 4-hydroxytamoxifen (4OHT), cells stably expressing DD-YFP became fluorescent at a faster rate than former DDs, with YFP levels reaching nearly 100% in 12 h, whereas previous DDs took twice as long (Figure 3a). The rate of protein degradation was measured after removing the stabilizing ligand, and YFP fluorescence reached basal levels in few hours as anticipated from studies involving previous DDs (Figure 3b).

To gain insight into the cellular mechanism by which these new DDs were degraded, cells stably transduced with the ER50 fusion proteins were cultured in the presence and absence of either CMP8 or 4OHT to stabilize the YFP-DD fusion proteins. Cells were then treated with a proteasome inhibitor (MG132 or bortezomib) or a lysosome inhibitor (chloroquine). The stabilizing ligand was then removed, and YFP levels were

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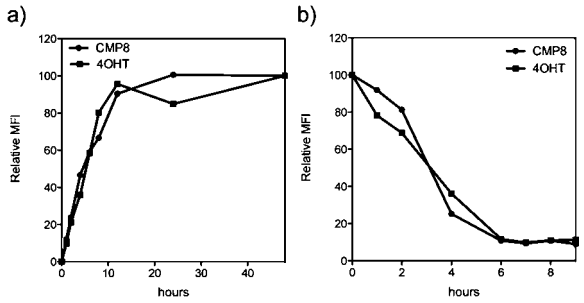

Figure 3. Flow cytometry data of the kinetics of ERLBD-DD. (a) NIH 3T3 cells stably expressing YFP-ER50 fusions were either treated with 3 μM CMP8 or 10 μM 4OHT, and increases in fluorescence were monitored. (b) Cells were treated with ligand for 24 h, at which point the cells were washed with media to remove ligand, and decreases in fluorescence were monitored.

measured after four hours. Cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with an anti-YFP antibody (Figure S4, SI). CMP8- or 4OHT-treated cells showed strong expression of the expected fusion proteins. To further elucidate the pathway of degradation, we collected cells and quantified the fluorescence of the YFP by analytical flow cytometry (Figure 4). The data showed that the proteasome inhibitors MG132 and bortezomib slowed degradation of YFP-DD fusion protein switching to nonpermissive conditions, indicating that proteasome is involved in the degradation of the DD. However, findings also suggest that other pathways may be involved in the degradation of the DD, since YFP levels dropped 2-fold even with proteasome inhibitors present. These results are consistent with those of other DDs.

In addition, to investigate the generality of the new DD system, the ER50 DD was fused to H-ras as well as the cell cycle regulatory protein, p21. Cells that stably express these fusion proteins were cultured in the presence and absence of CMP8. Cell extracts were resolved by SDS-PAGE and immunoblotted with either an anti-Hras or anti-p21 antibody (Figure S5, SI). As anticipated, these fusion proteins exhibit ligand-dependent stability, indicating that, like the other DD systems, this DD can be used to regulate a variety of proteins other than YFP.

In conclusion, we have developed an additional general method to regulate the stability of specific proteins in a rapid, reversible, and tunable manner using a small molecule, CMP8 or 4OHT. Mutants of human ERLBD protein were engineered to be strongly unstable when expressed in mammalian cells in the absence of a stabilizing ligand. When this new DD is fused to a protein of interest, its instability is conferred to the protein of interest, resulting in rapid degradation of the entire fusion protein. CMP8 or 4OHT are high-affinity ligands for mutant ERLBD that stabilizes fusion proteins in a dose-dependent manner, and protein levels in the absence of ligands are negligible. This ERLBD-derived DD system is orthogonal to the existing FKBP- and DHFR-based DD systems, in the sense that the stabilizing ligands do not affect the stability of their noncognate DDs. This novel DD system will enable researchers to simultaneously and independently regulate three proteins in biological studies. For example, the factors for induced pluripotent stem cells could be interesting targets.[17] Another important advantage of using the ERLBD-derived DD system is that one of the ligand demonstrated here, 4OHT, is

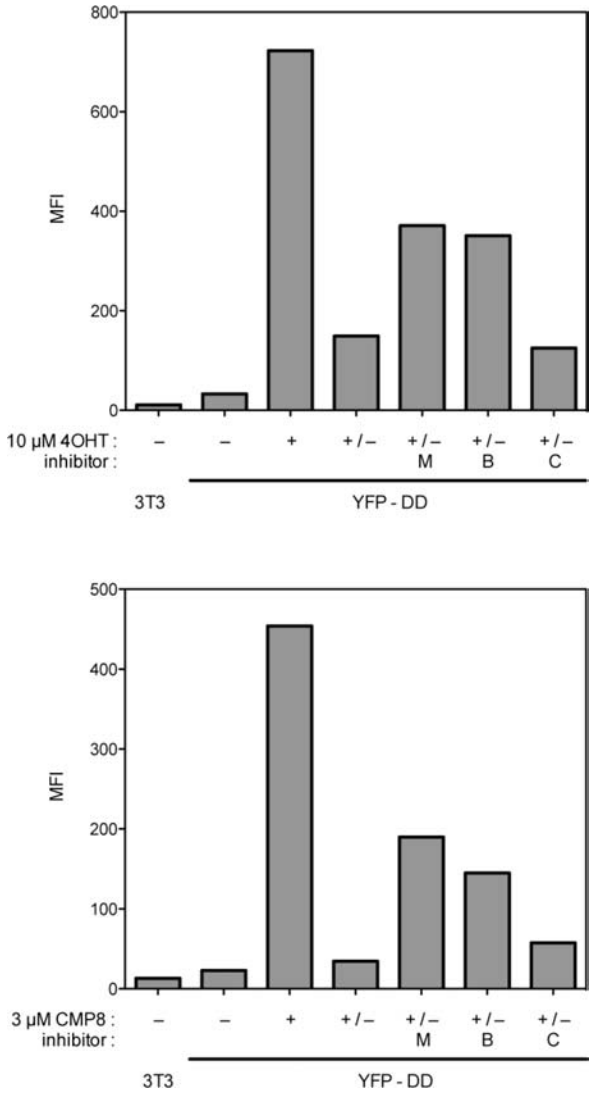

Figure 4. NIH 3T3 cells stably expressing YFP-ER50 fusions were treated with 10 μM 4OHT or 3 μM CMP8 for 24 h. Cells were then washed with media and treated with 10 μM MG132 (M), 2 μM bortezomib (B), or 100 μM chloroquine (C) for 4 h. Fluorescence was monitored using flow cytometry.

commercially available, which is significant when using the DD system in animals. Additionally, this novel DD system strongly implies that one will be able to develop any new DD systems by following our method with a high-affinity ligand and its ligand binding domain.

■ ASSOCIATED CONTENT

Supporting Information

A detailed description of experimental procedures and controls and complete synthesis of CMP8. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806−811.
(2) Gavériaux-Ruff, C.; Kieffer, B. L. *Pharmacol. Ther.* **2007**, *113*, 619−634.
(3) Shah, K.; Liu, Y.; Deirmengian, C.; Shokat, K. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3565−3570.
(4) Lévy, F.; Johnston, J. A.; Varshavsky, A. *Eur. J. Biochem.* **1999**, *259*, 244−252.
(5) Dohmen, R. J.; Wu, P.; Varshavsky, A. *Science* **1994**, *263*, 1273−1276.
(6) Stankunas, K.; Bayle, J. H.; Gestwicki, J. E.; Lin, Y.-M.; Wandless, T. J.; Crabtree, G. R. *Mol. Cell* **2003**, *12*, 1615−1624.
(7) Liu, K. J.; Arron, J. R.; Stankunas, K.; Crabtree, G. R.; Longaker, M. T. *Nature* **2007**, *446*, 79−82.
(8) Pratt, M. R.; Schwartz, E. C.; Muir, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 11209−11214.
(9) Banaszynski, L. A.; Chen, L.-C.; Maynard-Smith, L. A.; Ooi, A. G. L.; Wandless, T. J. *Cell* **2006**, *126*, 995−1004.
(10) Iwamoto, M.; Björklund, T.; Lundberg, C.; Kirik, D.; Wandless, T. J. *Chem. Biol.* **2010**, *17*, 981−988.
(11) Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A.; Greene, G. L. *Cell* **1998**, *95*, 927−937.
(12) Gallinari, P.; Lahm, A.; Koch, U.; Paolini, C.; Nardi, M. C.; Roscilli, G.; Kinzel, O.; Fattori, D.; Muraglia, E.; Toniatti, C.; Cortese, R.; De Francesco, R.; Ciliberto, G. *Chem. Biol.* **2005**, *12*, 883−893.
(13) Nettles, K. W.; Bruning, J. B.; Gil, G.; Nowak, J.; Sharma, S. K.; Hahm, J. B.; Kulp, K.; Hochberg, R. B.; Zhou, H.; Katzenellenbogen, J. A.; Katzenellenbogen, B. S.; Kim, Y.; Joachmiak, A.; Greene, G. L. *Nat. Chem. Biol.* **2008**, *4*, 241−247.
(14) Atwood-Moore, A.; Ejebe, K.; Levin, H. L. *J. Virol.* **2005**, *79*, 14863−14875.
(15) Zaccolo, M.; Williams, D. M.; Brown, D. M.; Gherardi, E. *J. Mol. Biol.* **1996**, *255*, 589−603.
(16) Kinzel, O.; Fattori, D.; Muraglia, E.; Gallinari, P.; Nardi, M. C.; Paolini, C.; Roscilli, G.; Toniatti, C.; Gonzalez Paz, O.; Laufer, R.; Lahm, A.; Tramontano, A.; Cortese, R.; De Francesco, R.; Ciliberto, G.; Koch, U. *J. Med. Chem.* **2006**, *49*, 5404−5407.
(17) Takahashi, K.; Yamanaka, S. *Cell* **2006**, *126*, 663−676.