Small-Molecule Switches for Zinc Finger Transcription Factors

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Small molecules that can switch on and off endogenous genes and transgenes are invaluable tools in studying a wide variety of cellular processes. Current approaches involve: (1) the use of single transgenes having promoters inducible by hormones, such as insect molting hormone ecdysone and retinoic acids; (2) fusion proteins of zinc fingers that contain steroid hormone binding domains; and (3) binary transgenic systems consisting of an "effector" transgene which is inducible by small molecules, and whose gene product regulates transcription of a "target" transgene, for example, the tetracycline-based "tet-on" and "tet-off" systems.[1−5] Here we are pursuing a novel approach in which a small-molecule effector site is directly engineered into the DNA binding domain of a transcription factor.

Eukaryotic transcription factors typically are composed of two domains: a DNA binding domain and an activation domain. The binding of transcription factors to response elements in the promoter region enables activation domains to recruit general transcriptional factors to the TATA box and initiate transcription. Our design of small-molecule-regulated transcription factors involves the introduction of cavity-generating mutations in the DNA binding domain that lead to a loss in DNA binding affinity and, as a consequence, transcriptional activity. Small molecules that are structurally complementary to and bind the cavity might be expected to functionally rescue the mutant (Figure 1). Herein we report our design of small-molecule transcriptional switches based on this notion of structural complementation.[6−8]

A structural variant of the murine zinc finger zif268, C7, was chosen as our initial target since extensive biochemical and structural studies have been reported.[9] Moreover, zinc fingers are the most ubiquitous family of transcription factors in the human genome with more than 1000 members.[10] The C7 zinc finger protein comprises three consecutive fingers, each one having a $\beta\beta\alpha$ fold stabilized by Cys_2His_2-Zn^{2+} coordination (Figure 2A, 2B). Two critical residues, His125 and Phe116, which participate in coordination of the zinc ion and hydrophobic packing in finger 1, respectively, were mutated to generate a structurally compromised C7 mutant, F116A/H125G. This mutant is expected to have a cavity around the zinc ion (Figure 2C), which should significantly perturb the structure of the associated DNA binding helix, and as a result, transcriptional activity.

To assess the functional effects of these mutations and screen for ligands that can rescue the function of the mutant zinc finger protein, a luciferase-based reporter assay was used.[11] Specifically, the reporter plasmid was constructed by inserting six copies of the C7 recognition element, GCGTGGGCG, upstream of a luciferase reporter in the vector pGL3. The expression plasmids for the zinc finger transcription factors were obtained by fusing the C7 and F116A/H125G mutant genes, respectively, with a virus-derived activation domain VP16 (see Supporting Information for details).
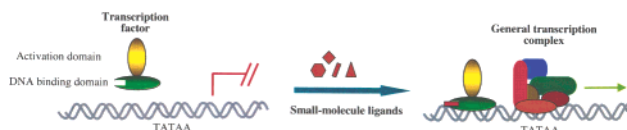
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Figure 1. Design of a small-molecule switch for gene transcription.

A
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               →           →          ━━━
Finger 1 105 YACPVESCDRRFSKSADLKRHIRIH 129
Finger 2 135 FQCRI--CMRNFSRSDHLTTHIRTH 157
Finger 3 163 FACDI--CGRKFARSDERKRHTKIH 185
```
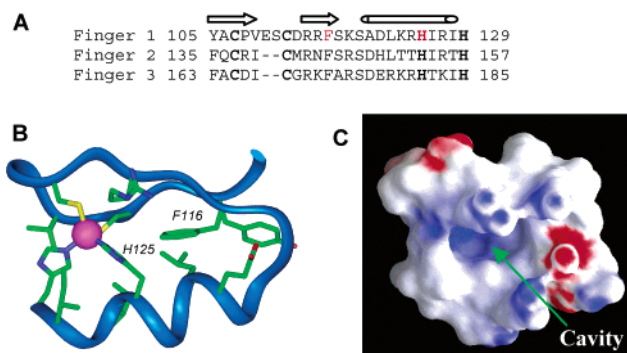
Figure 2. Sequence and structure of the zinc finger C7 protein: (A) Sequence of the 3-finger C7. The β-strands are marked with the arrow while the α-helices are marked with the tube. The residues that coordinate the zinc ion are in bold. The intended mutation sites are in red. (B) Structure of finger 1 from zif268 (PDB code: 1AAY) showing the residues involved in Zn^{2+} coordination and packing of the hydrophobic core. (C) Surface rendering of a model of the F116A/H125G mutant finger 1 in the same orientation as in B.

Transfection of HeLa cells with the wild-type C7 gene resulted in a 100-fold increase in luciferase activity. However, the luciferase activity remained at basal level when HeLa cells were transfected with the mutant F116A/H125G-C7 zinc finger, presumably because the mutations disrupt the binding of the mutant zinc finger to the response element (data not shown).

To identify a small molecule that complements the F116A/H125G mutation, a 250-membered heterocycle library containing such diverse scaffolds as imidazoles (**1**), indoles (benzoxazole, benzothiazole, or benzimidazole) (**2**), quinolines (**3**), isoquinolines (**4**), quinazolines (**5**), and quinoxalines (**6**) was screened for activation of the reporter gene after transfection with the mutant zinc finger protein (Figure 3). Among the 16 compounds that showed at least 2-fold activation at 100 μM, eight of them share the common scaffold, **2**. A small library of 2-aryl benzimidazole analogues was then synthesized by reacting 1,2-phenylenediamine with a variety of aryl aldehydes in acetonitrile with an excess of DDQ.[12] Screening of this new set led to a more potent compound, 2-(4′-quinoline)benzimidazole (**7**), which induces luciferase activity by about 18-fold at 100 μM concentration. This same compound has no effect on the luciferase gene activation of either WT C7 or control vector-transfected HeLa cells (Figure 4A). In addition, concentration-dependent reporter gene activation by
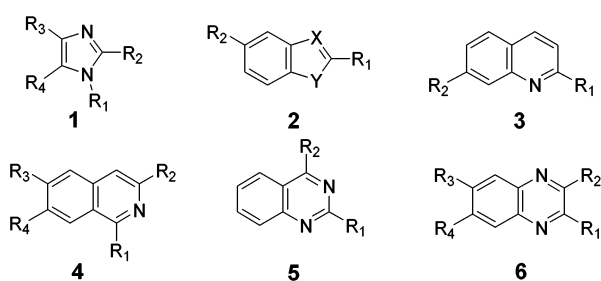
Figure 3. Representative scaffolds in the heterocycle library: R_1, R_2, R_3, and R_4 are either alkyl or aryl substituents; X = C, N; Y = N, O, S.
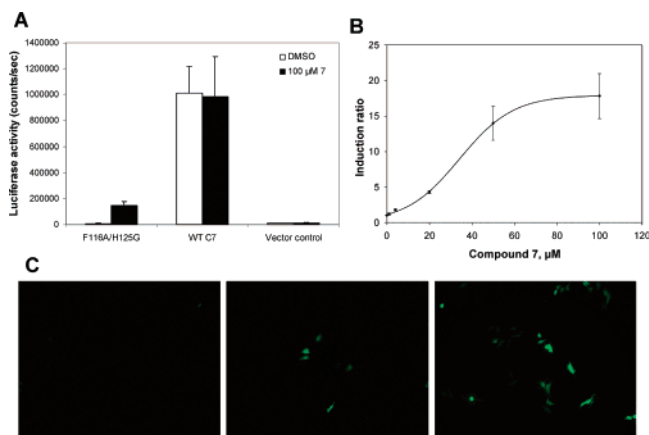
Figure 4. Activation of F116A/H125G-C7-mediated reporter gene expression by compound **7**. (A) Compound **7** selectively activated the mutant over the wild-type zinc finger in a luciferase-based reporter assay. (B) Concentration-dependent behavior of compound **7**; the data were fitted to a standard sigmoidal equation. (C) Rescue of the mutant zinc finger by **7** in a GFP-based reporter assay imaged with fluorescence microscope: F116A/H125G-C7 transfected HeLa cells (left); F116A/H125G-C7 transfected HeLa cells treated with 50 μM **7** (middle); and WT C7 transfected HeLa cells (right).

compound **7** was also observed with an EC_{50} value of 35 μM (Figure 4B). At concentrations higher than 100 μM significant cytotoxicity was observed. Furthermore, in a similar GFP-based transactivation assay using HeLa cells transfected with the mutant zinc finger, treatment with 50 μM compound **7** led to an increase in the number of fluorescent cells relative to untreated cells indicating that **7** partially restores the activity of the structurally compromised zinc finger transcription factor (left and middle fluorescence images in Figure 4C). A larger portion of HeLa cells are fluorescent when transfected with wild-type zinc finger.
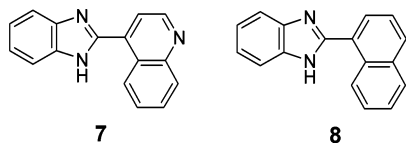
The effect of compound **7** on the DNA binding affinities of the zinc finger proteins was measured by surface plasmon resonance.[13] The dissociation constant (K_D) between the mutant zinc finger and a biotinylated hairpin containing the C7 recognition element was measured to be 5.7 μM; for comparison the wild-type protein binds the DNA with a K_D of 1.0 nM. In the presence of 100 μM compound **7**, the binding affinity (K_D) of the mutant C7 protein is

350 nM, a 16-fold increase, which is consistent with the results of the functional assay. In a control experiment with an inactive but structurally similar compound, 2-(2′-naphthyl)benzimidazole (**8**), the K_D was 4.0 μM, similar to that of the mutant zinc finger. Taken together, these results indicate that there is a specific interaction between compound **7** and the mutant zinc finger, which in turn increases the binding of the mutant C7 to its cognate DNA sequence. Presumably the partial affinity recovery of the mutant C7 by compound **7** leads to the functional rescue of the zinc finger transcription factor.

In summary, we have developed what should be a general approach to the generation of selective small-molecule switches for zinc finger transcription factors. Initial biochemical studies suggest that the switch functions by enhancing the affinity of the transcription factor toward its cognate DNA sequence. Further improvement of this system may be possible by screening a larger collection of ligands as well as optimizing the shape of the cavity using directed protein evolution.

Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) The MBP-C7 and MBP-C7-F116A/H125G proteins were expressed in XL-1 Blue cells following the manufacturer's instructions (New England Biolabs, Beverly, MA). The crude proteins were passed through an amylose column followed by a heparin affinity column to give homogeneous proteins, as judged by SDS-PAGE. A biotinylated hairpin DNA containing C7 recognition elements, biotin-ATAGGCCGCCCACGC-GGGTTTTCCCGCGTGGGCGGCCTAT, was immobilized onto the Sensor Chip SA (Biacore, Uppsala, Sweden) as the ligand while a random DNA hairpin ligand, biotin-GGCCGAGACTATATGCTATATAGCATAT-AGTCTCGGCC, was immobilized as a control. The zinc finger proteins were allowed to flow through the chip surface at varying concentrations, and the sensorgrams were recorded. The sensorgrams were fitted with the steady-state binding equation: $R_{eq} = K_A C R_{max}/(1 + K_A C n)$ within the BIAevaluation (version 3.0) program. The dissociation constants (K_D) were derived as $K_D = 1/K_A$.

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