Fluorescence Detection of Protein/Z-DNA Interactions

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A left-handed helical conformer of double-stranded DNA (dsDNA), Z-DNA, is observed in crystals of a synthetic hexamer $(dC\text{-}dG)_3$ (dC: deoxycytosine, dG: deoxyguanosine)[1] and is formed best *in vitro* when DNAs consisting of alternating dG and dC, poly(dG-dC), are placed in high salt conditions.[2] The Z-DNA conformation is stable at physiological salt concentrations when poly(dG-dC) is either methylated or brominated.[3] The *in vivo* relevance of Z-DNA, *i.e.*, the role of Z-DNA inside a cell, is still unclear, but the discovery that negative supercoiling would stabilize Z-DNA indicated biological involvement of Z-DNA *in vivo*.[4] The first two questions to be answered in order to elucidate the *in vivo* role of Z-DNA would be (1) the presence of a class of nuclear proteins that recognize the Z-DNA conformer and (2) the role(s) of the proteins in a cell. Rich and collaborators reported that an RNA-editing enzyme, human dsRNA adenosine deaminase (ADAR1), contains a domain ($Z\alpha$) that binds specifically to the Z-DNA conformation with high affinity.[5] Subsequently, Rich and Jacobs reported that the ability of a viral protein to bind to the Z-DNA conformation was essential for pathogenesis of vaccinia virus - a poxvirus that is used in smallpox vaccines - in mice: the N-terminal domain of viral E3L protein of vaccinia virus has a sequence similarity to the $Z\alpha$ family, and mutational studies clearly demonstrated that the Z-DNA-binding capability was necessary for pathogenicity in vaccinia virus.[6] These recent reports imply that blocking of the binding of viral proteins to Z-DNA would prevent the lethality associated with vaccinia infection and it would be possible to design a class of antiviral agents, including agents against variola (smallpox), which has an almost identical E3L protein.[7]

The binding of $Z\alpha$ family to Z-DNA has been assessed by electrophoretic mobility shift assay (EMSA), analytical ultracentrifuge, and surface plasmon resonance (SPR) spectroscopy.[5,8] However, to identify possible candidates for antiviral reagents (*e.g.*, against smallpox) by targeting Z-DNA-binding proteins, it is needed to find more general and robust high-throughput techniques for detecting protein/Z-DNA interactions. One of the promising approaches for the high-throughput screening is the microarray technology, where biospecific interactions (DNA/DNA, protein/protein, protein/DNA, small molecule/protein, etc) can be screened and evaluated at a time. Another approach would be the microplate-based detection, the principle of which is intrinsically the same as the microarray technology and could be incorporated into the microarray technology. Herein, we investigated a possibility of fluorescence detection of protein/Z-DNA interactions in the microplates, onto which Z-DNA was attached, with the ultimate goal of establishing a high-throughput detection of protein/Z-DNA interactions.

Our approach to detecting the protein/Z-DNA interactions is based on glutathione S-transferase (GST)-mediated dimerization of proteins. Multiple and simultaneous interactions, polyvalent interactions, have unique collective properties that are qualitatively different from properties displayed by their constituents,[9] and bidentate ligands have an affinity that can approach the product of the individual binding constants.[10] We used a dimerized $Z\alpha$ protein ($Z\alpha^{GST}$), formed by the dimerization of GSTs, as a model of Z-DNA-binding proteins and GST as a background control to access the specific interaction between Z-DNA-binding proteins and Z-DNA, and compared the results with the monomeric counterpart.

The procedure of fluorescence detection is depicted in Figure 1. Biotinylated Z-DNA, $Z\text{-}DNA^{biotin}$, was attached to the surface of microplate wells, which had been coated with streptavidin. We chose the interaction between biotin and streptavidin as a method for attaching Z-DNA, because the interaction is biospecific and strong ($K_D = 10^{-15}$ M), and the association is very rapid and unaffected by extremes of pH, organic solvents, and other denaturing agents.[11,12] The dissociation of a biotin from streptavidin requires harsh chaotropic agents and low pH (6.0 M guanidine hydrochloride, pH 1.5). The interactions between $Z\text{-}DNA^{biotin}$ and proteins were then detected by fluorescence.

Figure 2 shows a graph of relative fluorescence intensities (after divided by the background fluorescence) *vs.* the concentrations of the proteins. The initial concentration of the proteins was 25 μM and varied with a three-fold serial dilution. While the reaction wells containing GST gave very low levels of fluorescence signals, the significant fluorescence intensity from $Z\alpha^{GST}$ was observed above micromolar concentrations. These results indicate that $Z\alpha^{GST}$ proteins remained in the reaction wells by binding to Z-DNA and the

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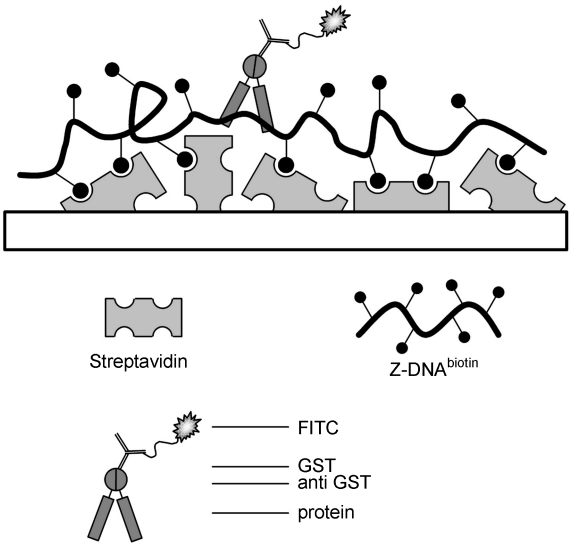

Figure 1. Schematic diagram of the detection of Z-DNA binding by dimeric Zα^{GST}. Z-DNA^{biotin}, which is Z-conformation of biotinylated poly(dG-dC) stabilized by bromination, was immobilized onto the streptavidin-coated surface. Dimerized Zα through the fused GST domain, Zα^{GST}, was complexed with fluorescein-conjugated anti-GST antibody, and was applied to the Z-DNA-presenting surface. The Zα bound to Z-DNA was detected by fluorescence from the fluorescein moiety.

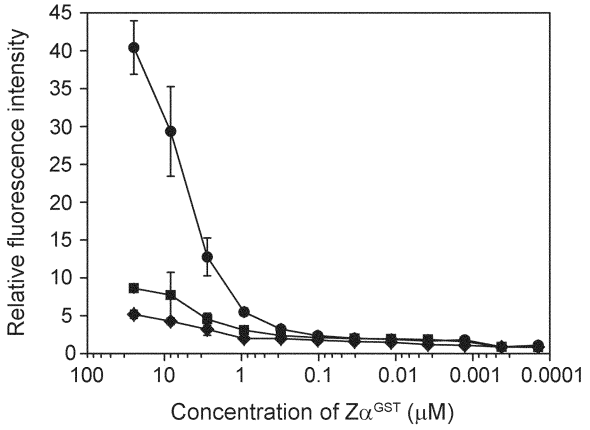

Figure 2. Fluorescence detection of Z-DNA binding by Zα. Each well of the 96-well microtiter plate, presenting Z-DNA^{biotin}, was incubated with varied concentrations of dimerized Zα (Zα^{GST}) (circles). As control experiments, the binding of GST to Z-DNA (squares) and the binding of monomeric Zα (Zα^{FITC}) to Z-DNA (diamonds) were also evaluated by fluorescence detection. The fluorescence intensity was recorded as a function of the concentration of Zα. The relative fluorescence intensity represents the observed fluorescence intensity divided by the background fluorescence.

binding of Zα proteins is specific. As a control experiment, we used monomeric Zα labeled with FITC through a cysteine residue (Zα^{FITC}), instead of the dimerized counterparts. Any noticeable fluorescence intensity was not observed even at a high concentration of Zα (40 μM), which clearly shows that the bivalent binding is crucial in the enhancement of the detection limit.

In summary, we applied a concept of "multi- or poly-valency" to the detection of the binding between Z-DNA and proteins in the microplate format. The result clearly showed an advantage of using dimerized Zα over monomeric Zα for a reliable detection of Z-DNA/protein interactions. The work demonstrated here would be a basis of general and robust techniques to detect protein/Z-DNA interactions and lead to the identification or examination of possible candidates for antiviral reagents that block the binding to Z-DNA.

Experimental Section

Purification of proteins. GSTs are dimeric with subunits of 26 kDa. Using the recombinant DNA technology, the Zα domain gene from human ADAR1[8] was fused to the GST gene from *Schistosoma japonicum* encoded in pET41b expression vector (from Novagen) at *Nco*I/*Hind*III restriction sites (pET41b-ZaGST). Zα protein fused with GST at its N terminus (Zα^{GST}) was expressed from *E. coli* harboring pET41b-ZαGST and purified to homogeniety by affinity purification using GST·Bind™ Resin (from Novagen) and subsequent cation-exchange chromatography on a Mono-S column (from Amersham Biosciences). Recombinant GST protein purified from *E. coli* was purchased from United States Biological and was used as a control. Fluorescence labeling of proteins was achieved by complexing Zα^{GST} or GST with equal moles of fluorescein isothiocyanate (FITC)-conjugated goat anti-GST antibody (from United States Biological). Monomeric Zα was labeled at a cysteine residue by using fluorescein-5-maleimide (Molecular Probes).

Preparation of Z-DNA^{biotin}. Z-DNA^{biotin} was prepared from poly(dG-dC) (from Amersham Biosciences) by a two-step process: bromination and biotinylation. Briefly, poly(dG-dC) was stabilized in the Z-DNA conformation by bromination,[3b] and the brominated poly(dG-dC) was subsequently biotinylated with EZ-Link™ photoactivatable biotin (from Pierce).[12] An aqueous solution (100 μL) of the brominated poly(dG-dC) (50 μg in 50 μL of the water) and EZ-Link™ photoactivatable biotin (50 μg in 50 μL of the water) was placed in an ice bath and irradiated with 366-nm light for 20 min. After the irradiation, 300 μL of 0.1 M Tris-HCl (pH 9.0) was added to the resulting solution and excess biotin was extracted with *n*-butanol (400 μL × 2).

Fluorescence detection. The purified Z-DNA^{biotin} was incubated in the streptavidin-coated 96-well plate (from Pierce). Bovine serum albumin (BSA) solution was prepared by dissolving BSA (50 mg/L) in an aqueous buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 1 mM EDTA. Before the attachment of Z-DNA^{biotin} to the microplate, the microplate was passivated with the BSA solution (200 μL) for 30 min at room temperature. Z-DNA^{biotin} (100 μL) was then added and incubated for 1 h at room temperature. Unbound Z-DNA^{biotin} was removed and the wells were washed with the BSA solution (150 μL × 2). The target proteins complexed with FITC-conjugated anti-GST were separately prepared by three-fold serial dilution, and 100 μL of each protein aliquot was added to the wells and

incubated for 12 h at 4 °C. After incubation, the unbound proteins were removed, and the wells were washed with the BSA solution (200 μL × 2). Finally, 100 μL of the BSA solution was added and fluorescence was detected with a microplate fluorescence reader (Spectramax plus 384, Molecular Device; excitation: 485 nm, emission: 535 nm). The light intensity was 10,000 and the exposure time was 0.1 sec.

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References

1. Wang, A. H.-J.; Quigley, G. J.; Kolpak, F. J.; Crawford, J. I.; van Boom, J. H.; van der Marel, G.; Rich, A. *Nature* **1979**, *282*, 680.
2. Pohl, F. M.; Jovin, T. M. *J. Mol. Biol.* **1972**, *67*, 375.
3. (a) Behe, M.; Felsenfeld, G. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 1619. (b) Möller, A.; Nordheim, A.; Kozlowski, S. A.; Patel, D. J.; Rich, A. *Biochemistry* **1984**, *23*, 54.
4. Peck, L. J.; Nordheim, A.; Rich, A.; Wang, J. C. *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 4560.
5. Herbert, A.; Alfken, J.; Kim, Y.-G.; Mian, I. S.; Nishikura, K.; Rich, A. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 8421.
6. Kim, Y.-G.; Muralinath, M.; Brandt, T.; Pearcy, M.; Hauns, K.; Lowenhaupt, K.; Jacobs, B. L.; Rich, A. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6974.
7. (a) Rich, A.; Zhang, S. *Nature Rev.* **2003**, *4*, 566. (b) Xu, Y.; Zhang, Y. X.; Sugiyama, H.; Umano, T.; Osuga, H.; Tanaka, K. *J. Am. Chem. Soc.* **2004**, *126*, 6566.
8. (a) Schwartz, T.; Rould, M.; Lowenhaupt, K.; Herbert, A.; Rich, A. *Science* **1999**, *284*, 1841. (b) Scwartz, T.; Loewenhaupt, K.; Kim, Y.-G.; Li, L.; Brown, B. A., II; Herbert, A.; Rich, A. *J. Biol. Chem.* **1999**, *274*, 2899.
9. Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem. Int. Ed.* **1998**, *37*, 2757.
10. (a) Cowburn, D.; Zheng, J.; Xu, Q.; Barany, G. *J. Biol. Chem.* **1995**, *270*, 26738. (b) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531. (c) Maly, D. J.; Choong, I. C.; Ellman, J. A. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2419. (d) Profit, A. A.; Lee, T. R.; Niu, J.; Lawrence, D. S. *J. Biol. Chem.* **2001**, *276*, 9446. (e) Jahnke, W.; Florsheimer, A.; Blommers, M. J.; Paris, C. G.; Heim, J.; Nalin, C. M.; Perez, L. B. *Curr. Top. Med. Chem.* **2003**, *3*, 69. (f) Erlanson, D. A.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Simmons, R. L.; DeLano, W. L.; Choong, I. C.; Burdett, M. T.; Flanagan, W. M.; Lee, D.; Gordon, E. M.; O'Brien, T. *Nat. Biotechnol.* **2003**, *3*, 308. (g) Braisted, A. C.; Oslob, J. D.; Delano, W. L.; Hyde, J.; McDowell, R. S.; Waal, N.; Yu, C.; Arkin, M. R.; Raimundo, B. C. *J. Am. Chem. Soc.* **2003**, *125*, 3714. (h) Bachhawat-Sikder, K.; Kodadek, T. *J. Am. Chem. Soc.* **2003**, *125*, 9550.
11. (a) Park, T. J.; Lee, K.-B.; Lee, S. J.; Park, J. P.; Lee, Z.-W.; Lee, S. Y.; Choi, I. S. *J. Am. Chem. Soc.* **2004**, *126*, 10512. (b) Lee, K.-B.; Kim, D. J.; Lee, Z.-W.; Woo, S. I.; Choi, I. S. *Langmuir* **2004**, *20*, 2531. (c) Park, J. P.; Lee, S. J.; Park, T. J.; Lee, K.-B.; Choi, I. S.; Lee, S. Y.; Kim, M.-G.; Chung, B. H. *Biotechnol. Bioprocess Eng.* **2004**, *9*, 137. (d) Lee, K.-B.; Yoon, K. R.; Woo, S. I.; Choi, I. S. *J. Pharm. Sci.* **2003**, *92*, 933.
12. Lee, K.-B.; Lee, Y.-W.; Kim, D. J.; Lee, S.; Woo, S. I.; Kim, Y.; Kim, Y.-G.; Choi, I. S. *Bull. Korean Chem. Soc.* **2003**, *24*, 1702.