

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-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 α) 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 α 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 α 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 α protein (Z α ^{3ST}), 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-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-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 μ M 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 α ^{3ST} was observed above micromolar concentrations. These results indicate that Z α ^{3ST} proteins remained in the reaction wells by binding to Z-DNA and the

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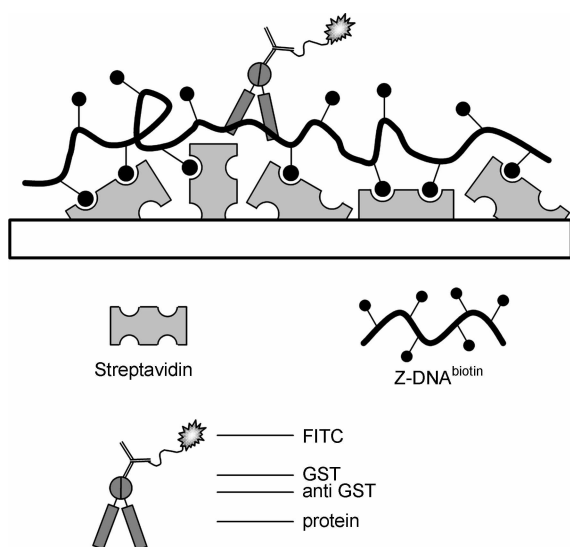


Figure 1. Schematic diagram of the detection of Z-DNA binding by dimeric $Z\alpha^{GST}$. Z-DNA^{biotin}, which is Z-conformation of biotinylated poly(dG-dC) stabilized by bromination, was immobilized onto the streptavidin-coated surface. Dimerized $Z\alpha$ through the fused GST domain, $Z\alpha^{GST}$,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

binding of $Z\alpha$ proteins is specific. As a control experiment, we used monomeric $Z\alpha$ labeled with FITC through a cysteine residue ($Z\alpha^{FITC}$), instead of the dimerized counterparts. Any noticeable fluorescence intensity was not observed even at a high concentration of $Z\alpha$ (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 polyvalency" to the detection of the binding between Z-DNA and proteins in the microplate format. The result clearly showed an advantage of using dimerized $Z\alpha$ over monomeric $Z\alpha$ 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 $Z\alpha$ domain gene from human ADARI⁸ was fused to the GST gene from *Schistosoma japonicum* encoded in pET41b expression vector (from Novagen) at *NcoI/HindIII* restriction sites (pET41b-Z α GST). $Z\alpha$ protein fused with GST at its N terminus ($Z\alpha^{GST}$) was expressed from *E. coli* harboring pET41b-Z α GST and purified to homogeneity by affinity purification using GST-BindTM 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

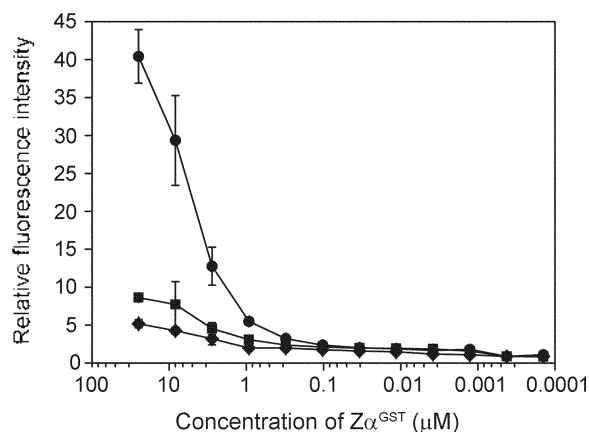


Figure 2. Fluorescence detection of Z-DNA binding by $Z\alpha$. Each well of the 96-well microtiter plate, presenting Z-DNA^{biotin}, was incubated with varied concentrations of dimerized $Z\alpha$ ($Z\alpha^{GST}$) (circles). As control experiments, the binding of GST to Z-DNA (squares) and the binding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

States Biological and was used as a control. Fluorescence labeling of proteins was achieved by complexing $Z\alpha^{GST}$ or GST with equal moles of fluorescein isothiocyanate (FITC)-conjugated goat anti-GST antibody (from United States Biological). Monomeric $Z\alpha$ 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-LinkTM 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-LinkTM 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 $\mu\text{L} \times 2$).

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 $\mu\text{L} \times 2$). 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 μ L \times 2). Finally, 100 μ L 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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