The Interaction between Z-DNA and the Zab Domain of Double-stranded RNA Adenosine Deaminase Characterized Using Fusion Nucleases*

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Zab is a structurally defined protein domain that binds specifically to DNA in the Z conformation. It consists of amino acids 133-368 from the N terminus of human double-stranded RNA adenosine deaminase, which is implicated in RNA editing. Zab contains two motifs with related sequence, Za and Zβ. Za alone is capable of binding Z-DNA with high affinity, whereas Zβ alone has little DNA binding activity. Instead, Zβ modulates Za binding, resulting in increased sequence specificity for alternating (dCdG)n as compared with (dCdA/dTdG)n. This relative specificity has previously been demonstrated with short oligonucleotides. Here we demonstrate that Zab can also bind tightly to (dCdG)n stabilized in the Z form in supercoiled plasmids. Binding was assayed by monitoring cleavage of the plasmids using fusion nucleases, in which Z-DNA-binding peptides from the N terminus of double-stranded RNA adenosine deaminase are linked to the nuclease domain of FokI. A fusion nuclease containing Za shows less sequence specificity, as well as less conformation specificity, than one containing Zab. Further, a construct in which Zβ has been replaced in Zab with Za, cleaves Z-DNA regions in supercoiled plasmids more efficiently than the wild type but with little sequence specificity. We conclude that in the Zab domain, both Za and Zβ contact DNA. Za contributes contacts that produce conformation specificity but not sequence specificity. In contrast, Zβ contributes weakly to binding affinity but discriminates between sequences of Z-DNAs.

The biological role of Z-DNA has long awaited elucidation. New hypotheses were formed upon the discovery that the N terminus of ADAR1 binds to Z-DNA with high affinity (1). ADAR1 deaminates adenosines to inosine (functionally G) in double-stranded RNA in vitro (2), and has been suggested to edit pre-mRNA in vivo (3). The substrates for A to G RNA editing remain to be enumerated; they include modulating central nervous system function by changing glutamic acid receptors (4) and serotonin receptors (5). These editing events may serve to target ADAR1 to regions of active transcription and thereby to nascent mRNA (6).

Although editing events have been characterized primarily in mammals, double-stranded RNA deaminase activity has also been shown in many animal phyla (3). ADAR1 has been identified from a variety of vertebrates (3); in every case the Z-DNA-binding motifs at the N terminus are conserved (1). These motifs, called Za and Zβ, are separated by a linker of conserved size but divergent sequence; a single known exception is in the human protein (hADAR1), where the linker has been precisely duplicated (1, 7).

The Z-DNA-binding region of hADAR1 has been extensively characterized (1, 8–10). Za binds to Z-DNA stabilized by bromination (1) and in supercoiled plasmids with (dCdG)n inserts (9, 11). When Za is titrated into a solution containing short oligonucleotides with alternating purine-pyrimidine sequences, the CD spectrum converts from a B-DNA to a Z-DNA form (8–10, 12). It has recently been shown by limited proteolysis that the Z-DNA-binding region of hADAR1 is structurally organized into a bipartite domain (12). This domain, named Zab, contains the originally identified Z-DNA-binding region, Za; a second motif, Zβ, identified by sequence similarity (1); and the intervening linker. Although this entire region fulfills the definition of a protein domain, it is also possible to identify a proteolytically stable subdomain containing only Za. The DNA binding of this subdomain, named Za, has been compared with that of Za. Za and Zab were overexpressed in Escherichia coli and tested for DNA binding by gel mobility shift assays and by the ability to stabilize short oligonucleotides in the Z conformation as shown by CD (12). Both bind to Z-DNA in the presence of large amounts of competing B-DNA. Za binds to short stretches of both (dCdG)n and (dCdA/dTdG)n, and oligonucleotides of either sequence adopt the Z form in the presence of a 2:1 base pair:peptide ratio of protein. In contrast, Zab does not bind to (dCdA/dTdG)n in these assays.

Fusion nucleases have a particular utility for the study of Z-DNA-binding proteins, because they have separate domains for DNA binding and cleavage. Z-DNA in cells must exist embedded in long stretches of B-DNA. It is likely to be stabilized by supercoiling or by protein binding rather than base modification and high salt. A fusion nuclease with a specificity for binding Z-DNA will cut Z-DNA in a supercoiled plasmid at physiological salt concentrations and at modest protein:DNA ratios. The cleavage can easily be detected (11). This assay has

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§ The abbreviations used are: ADAR1, double-stranded RNA adenosine deaminase; hADAR1, human ADAR1; Fκ, the nuclease domain of FokI endonuclease.

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allowed us to characterize the binding of several different Z-DNA-binding peptides.

A short peptide containing Za (residues 121–197), which has been described previously (1). Two molecules of Za appear to bind together on Z-DNA and display conformational but not sequence specificity (9). In addition, it has been shown previously that a construct with two Za segments connected by a short flexible linker bind Z-DNA more tightly than the Za construct alone and that two molecules of Za bind to a 6-base pair Z-DNA substrate (9). When Za is linked to ZpI by the same flexible linker, the binding differed from that of the linked Za-Za construct in character, with less sensitivity to competing Z-DNA (9). Under low salt conditions, which emphasize non-specific binding, Za (residues 133–209) binds slightly more specifically than the Za construct. In contrast, Zab binds DNA with a much higher affinity than Za, we conclude sequence specificity. Zaa nuclease may thus be used as a rea-

The construction of pET15b:Za was cleaved with

The DNA fragments for primer extension were

Expression and Purification of Fusion Nucleases—The expression of fusion nuclease has been described previously (11). Briefly, expressed proteins were expressed in E. coli strain BL21 (DE3) and were found in the supernatant after lysis. His-tagged proteins were enriched using His-Bind resin (Novagen). Fusion nuclease were further purified on MonoS cation exchange columns (Amersham Pharmacia Biotech) and, if necessary, by Superose 12 (Amersham Pharmacia Biotech) gel filtration chromatography. Nucleases were concentrated and stored at ~80 °C in 50% glycerol. Protein concentrations were determined spectrophotometrically by measurements of the absorbance maximum near 280 nm. Extinction coefficients were calculated according to Gill and Von Hippel (14).

Cleavage of Plasmid Substrates—Plasmids were isolated from XL1 Blue using the alkaline lysis method followed by Wizard Plus Miniprep (Promega). DNA was further extracted with phenol/CHCl3; to remove residual RNAse. DNAs were then precipitated and dissolved at ~80 °C in 50% glycerol. Protein concentrations were determined spectrophotometrically by measurements of the absorbance maximum near 280 nm. Extinction coefficients were calculated according to Gill and Von Hippel (14).

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plasmids were cut with \( Pst \)I. B-Z transition for \((dCdA/dTdG)_{30}\) than for \((dCdG)_{13}\) (13). In some of the plasmids contain Z-DNA at bacterial superhelical tension equally well (9, 12). In contrast, Zab binds poorly, if at all, concentrations. ZabFN cuts much less efficiently near the homologous plasmid with a \((dCdA/dTdG)_{30}\) insert. Although as shown in Fig. 2. Fig. 2 shows a comparison of the cleavage by ZaFN, ZabFN, and ZaaFN, as well as the previously characterized ZaFN. Plasmids containing a \((dCdG)_{13}\) insert were incubated with enzymes and then subsequently cut with \(Pst\)I. Two diagnostic bands (open arrows) are produced when the plasmid is also cleaved next to the Z-DNA insert by the fusion nuclease. ZaFN cleaves at the insert but also produces a number of other bands as the result of cleavage at non-Z-DNA sites. ZaFN cleaves considerably less than that seen for ZabFN; however, the nonspecific product bands are absent, and the overall level of nonspecific cleavage is dramatically reduced. ZaaFN shows increased activity; even at the lowest enzyme concentration, more than 50% of the substrate is specifically cleaved. Like ZabFN, ZaaFN shows decreased nonspecific cleavage.

Unlike ZabFN, ZaaFN cleaves at Z-DNA formed by different sequences (Fig. 4). Like ZaFN, ZaaFN cleaves a plasmid with a \((dCdA/dTdG)_{30}\) insert, albeit only to a limited extent. Both ZaFN and ZaaFN produce noticeable degradation of this plasmid at nonspecific sites, as visualized by the other bands near the full-length band. This nonspecific cutting is more pronounced on the \((dCdA/dTdG)_{30}\) containing plasmid than on the same plasmid without the insert (Fig. 4, pDHg16). The explanation for this is unknown; however, it may be the result of an interaction between Zaa and Za and the insert sequence, which is of insufficient duration or affinity to allow specific cleavage but still enough to raise the level of background cleavage.

Two Copies of a Z-DNA-binding Motif Result in Increased Conformational Specificity; \(Zb\) Is More Effective than \(Za\)—As demonstrated above, both ZaFN and ZaaFN cut plasmids with less specificity than either ZabFN or ZaaFN in high salt cond-
Results in the loss of all activity. In contrast, ZaaFN has less single-stranded breaks. Whereas primer extension experiments show both double- and single-stranded breaks, sequencing lanes using the same primers. The experiments results in preferential stop sites, which are mapped relative to the midpoint with the (dCdG)13 insert. The primer is positioned at the mids were used as templates for primer extension. Two primers might be expected if the binding domain were situated at random within the insert; however, the simplest interpretation is that the FokI nuclease, like many others, is unlikely to cut within Z-DNA. The differences in the extent of cleavage with the different nucleases are not unexpected in light of the results shown in Fig. 3. Sites on both sides of the insert can be seen using the SalI primer (Fig. 6B); using the EcoRI primer, only upstream sites are evident (Fig. 6A). This may be the result of extensive upstream cutting, as will be discussed for ZaaFN.

ZafN and ZabFN show nearly identical patterns, with preferred sites visible on both sides of the insert in both directions. The total extent of cleavage is moderate. There are some differences in intensity between different sites when these two nucleases are compared. ZabFN cleaves distant sites more strongly than the other nucleases (Fig. 6, A and B, designated by asterisks). These differences are reproducible. They may be the result of differences in the size of the binding domain construct and the position on Z-DNA. It is also possible that the difference in preference reflects a difference in ability to bind to junction sequences that may adopt the Z-conformation.

ZaaFN cuts at the same sites as the other nucleases. Only sites upstream of the insert are visualized with the EcoRI primer (Fig. 6A), and downstream sites are underrepresented with the SalI primer as well. This can be explained if the higher level of cleavage with this nuclease results in plasmid molecules that are multiply nicked or cut. Such plasmids will only template for primer extension to the major upstream sites. In addition, cleavage with ZaaFN produces several additional bands, mapping very close to the B-Z junction (Fig. 6B, open box). Perhaps the binding of Zaa to Z-DNA is stable enough to allow less favorable, slower cleavage by FokI nuclease very close to the Z-DNA or the B-Z junction.

Fig. 6 (C and D) shows the map of cleavage sites around a (dCdA/dTdG)30 insert. Only ZaaFN cuts well enough for data to be collected. Cutting is shown flanking the insert and even encroaching into it. The superhelical density of the plasmid is probably not sufficient to maintain the entire insert in the Z form (17). In addition, there is a notable asymmetry of cleavage for the (dCdA/dTdG)30 insert (Fig. 6E). Cleavage is much stronger 5' to (dCdA) and 3' to (dTdG). This cleavage is likely to reflect an asymmetric binding between Zaa and this DNA sequence, with both copies in Zaa aligned to reinforce this asymmetry.

**DISCUSSION**

Fusion nucleases are potent tools to characterize the DNA binding of protein domains. Cleavage by the nuclease moiety is a measure of the specificity and affinity of the binding domain (11, 15, 18, 19). Here we characterize Z-DNA binding, using nucleases that combine the endonuclease domain of FokI restriction enzyme with various constructs from the N-terminal...
end of hADAR1. This region has been shown to bind Z-DNA with high affinity and specificity (1), and the domain structure has been mapped (12). Here we examine the role of subdomains in the binding to Z-DNA.

Any of several constructs containing the Z-DNA-binding motif Zα can bind to Z-DNA. High affinity binding does not require the entire subdomain, but Zα binding is more Z-DNA-specific than that of truncated constructs. The complete domain, Zab, contains both Zα and Zβ motifs with the intervening linker and binds with greater specificity as shown in experiments with B-DNA competitor. It also appears to be more selective for sequence. Our experiments make it unlikely that the specificity for sequence is the result of a requirement for different binding energies needed to stabilize different sequences in the Z form. These experiments were conducted using supercoiling to stabilize the Z conformation, a situation thought to reflect what occurs in vivo.

It has been shown previously that Zα provides most of the
binding affinity in Zab (11). When Zab is bisected with chymotrypsin to separate Za and Zβ containing subdomains, the resulting DNA binding is identical to that of the Za-containing piece alone. This result is supported by our demonstration that when Zβ is replaced in Zab with Za, the resulting construct Zaa binds much more tightly than either Za or Zab. The binding is appropriate for the additive effects of two copies of Za. Further, it had been suggested that Zβ might modify the binding of Za to produce the sequence specificity of Zab. Again, the results from Zaa support this theory; Zaa binds to Z-DNA of different sequences, as does Za. On the other hand, there is evidence that Zaa has sequence specificity; when the cleavage sites are mapped on the nonpalindromic substrate, (dCdA/dTdG)₃₀, there is an asymmetry of cleavage. It is possible that Zaa makes some base-specific contacts that result in this bias.

The specific cleavage sites in a plasmid containing a Z-DNA insert are generally the same using ZaFN, ZabFN or ZaaFN. However, some differences in the frequency with which each site is used are observed. ZaFN prefers sites that are further from the edge of the insert, whereas ZabFN and ZaaFN prefer the nearer sites. This preference may be explained by differences in the C-terminal residues between Za and Zaa, which are the same, and Zab. These residues may orient the nuclease domain differently, such that it more easily makes contact with substrate DNA at different sites. Such a result of altered C-terminal residues has been shown previously for two zinc finger DNA-binding domains in fusion nuclease constructs (16, 20). In addition, ZaaFN has unique cleavage sites, very close to the edge of the insert. These sites may result from the tighter proteolysis (data not shown). The second copy of Zab, when Zβ is replaced in Zab with Za, the resulting construct remains stably folded without major changes to the structure, as shown by limited proteolysis (data not shown). The second copy of Za probably binds Z-DNA at a position similar to that of Zβ.

One goal of research into fusion nucleases containing a Z-DNA-binding domain is the need for useful reagents to identify Z-DNA in vivo. The original construct, ZaFN, showed some promise, but the specificity and affinity were not high enough to be useful. ZabFN, which demonstrates the highest specificity for the Z conformation, may prove useful; however, the sequence specificity is likely to limit its usefulness. ZaaFN is a more likely candidate for an all-purpose reagent to detect Z-DNA. It is capable of cleaving supercoiled (dCdG)₉ nearly to completion. It can recognize (dCdA/dTdG)₉, and mixed sequences (data not shown) in the Z form. Future experiments expressing ZaaFN in eukaryotic cells and examining both reporter genes and endogenous genes may prove of interest.

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