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Preferential binding of IFI16 protein to cruciform structure and superhelical DNA

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ABSTRACT

Interferon (IFN)-inducible HIN-200 proteins play an important role in transcriptional regulation linked to cell cycle control, inflammation, autoimmunity and differentiation. IFI16 has been identified as a target of IFNα and γ and is a member of the HIN-200 protein family. Expression level of IFI16 is often decreased in breast cancers, implicating its role as a tumor suppressor. As a potent transcription factor, IFI16 possesses a transcriptional regulatory region, a PYD/DAPIN/PAAD region which associates with IFN response, DNA-binding domains and binding regions for tumor suppressor proteins BRCA1 and p53. It is also reported that IFI16 protein is capable of binding p53 and cMYC gene promoters. Here, we demonstrate that IFI16 protein binds strongly to negatively superhelical plasmid DNA at a native superhelix density, as evidenced by electrophoretic retardation of supercoiled (sc) DNA in agarose gels. Binding of IFI16 to supercoiled DNA results in the appearance of one or more retarded DNA bands on the gels. After removal of IFI16, the original mobility of the scDNA is recovered. By contrast, IFI16 protein binds very weakly to the same DNA in linear state. Using short oligonucleotide targets, we also detect a strong preference for IFI16 binding to cruciform DNA structure compared to linear DNA topology. Hence, this novel DNA-binding property of IFI16 protein to scDNA and cruciform structures may play critical roles in its tumor suppressor function.

1. Introduction

Among interferon (IFN)-inducible genes, the HIN-200 gene family plays important roles in regulation of cell proliferation, tissue differentiation, apoptosis and senescence [1]. Recently, it has been suggested that HIN-200 genes also function in apoptosis and inflammatory response by acting as mediators of IFN's activity in autoimmune diseases [2]. HIN-200 genes encode a class of homologous proteins that share a 200-amino acid signature motif (HIN) [3]. Four human (IFI16, MNDA, AIM2, and IFIX) and seven mouse (p202a, p202b, p203, p204, p205, MNDA, and AIM2) members of this family have been identified [4,5]. In addition to possessing one or two copies of the HIN domain, these HIN-200 proteins (with the exception of p202) also contain a PYRIN (PYD/DAPIN/PAAD) domain in the N-terminus [6].

As a member of the IFN-inducible HIN-200 family, the IFI16 gene is localized on chromosome 1 [7] and encodes a 729-amino acid gene product organized into a PYRIN domain and two HIN domains. In accordance with its potential role as a scaffolding protein for modulating multiple protein–protein and protein–DNA interactions, the full-length structure of IFI16 adopts an extended overall shape with the three domains oriented relatively independent of one another [8]. Indeed, IFI16 has been implicated in transcriptional regulation by modulating various protein–protein interactions with the tumor suppressor protein p53 and other transcription factors. It has been shown that IFI16 is capable of enhancing p53–DNA complex formation and transcriptional activation [8,9]. The HIN-A domain of IFI16 binds to the basic C-terminus of p53 and stimulates p53's sequence-specific DNA binding properties [8]. Consistent with the presence of two consecutive oligonucleotide/oligosaccharide-binding folds in the HIN repeats, IFI16 protein can also bind to DNA [9]. IFI16 is involved, too, in transcriptional repression and is a component of the BASC DNA repair multi-protein complex which forms after UV-induced DNA damage [10]. Several recent studies have demonstrated that Aim2 and p202 proteins can recognize cytoplasmic DNA and that they form a caspase-1-activating complex upon sensing DNA [11]. IFI16, on the other hand, can detect both cytosolic and nuclear double-stranded (ds) DNA, and it also plays an important role in DNA-responsive inflammasomes [12,13]. Further evidence supporting IFI16's protein–DNA interaction is its *in vivo* binding to the promoters of the oncogene cMYC and p53. Indeed, IFI16 binds to the G-rich fragments in both promoters and functions as a transcriptional repressor [14]. The presence of NM23 and IFI16 on the same DNA fragments also suggests their common involvement in the reduced development of some tumors [15,16]. In addition to binding G-rich DNA regions, IFI16 protein has been reported also to bind single-stranded (ss) DNA, dsDNA and RNA [17]. Preference of IFI16

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protein for other DNA sequences and structures remains to be further investigated.

It has been demonstrated that cruciform structures are targets for many architectural and regulatory proteins, including histones H1 and H5, HMG proteins, HU, p53 [18,19], 14-3-3 proteins [20,21], the proto-oncogene protein DEK and others (reviewed in [22]). A number of DNA-binding proteins, such as the HMGB-box family members, Rad54, BRCA1 protein, as well as PARP-1 polymerase, possess weak sequence-specific DNA binding yet bind preferentially to cruciform structures. In addition, DNA negative supercoiling often stabilizes local non-B DNA structures including cruciforms, Z-DNA or intramolecular triplexes which can occur in regulating regions of numerous genes. Importantly, a number of DNA–protein complexes involve DNA's wrapping around the protein, and such binding is often promoted by negative supercoiling of DNA [23]. In contrast, other proteins have been reported to interact with the supercoiled (sc) DNA at crossing points or longer segments of the interwound supercoil [24,25]. Moreover, the eukaryotic genome has been shown to contain unconstrained supercoiling, part of which can be attributed to the process of transcription [26]. The spontaneous presence of DNA supercoiling is a requirement for genome organization [27], and formation of superhelical DNA segments is an integral part of transcription processes. Intermediate supercoils are formed during DNA replication, and superhelical stress is distributed throughout the entire replicating DNA molecule [28]. Recent data have provided evidence that DNA binding of p53 is strongly dependent on the structural features of the target DNA [18,29–31]. In fact, full-length p53 is able to bind sequence-specifically to target oligonucleotides adopting stem-loop structure [32,33]. Moreover, p53 protein has been reported to exhibit a strong preference for p53 target sequence in cruciform structure [19]. Here, we examine the binding properties of IFI16 protein to different DNA targets. Using various structures of short DNA oligonucleotides, we demonstrate a strong preference of IFI16 protein binding to cruciform structure. Furthermore, we also evaluate IFI16 binding to long DNA targets with superhelical and linear topology.

2. Materials and methods

2.1. Preparation of DNA substrates

Synthetic oligonucleotides with and without FAM-labeling were purchased from VBC-Biotech. We used identical oligonucleotides as did Naseem et al. to prepare linear double-stranded and junction double-stranded DNA [34]. Complementary oligonucleotides were annealed by incubation at 94 °C for 10 min, with subsequent cooling to 4 °C at a rate of 1 °C/min in annealing buffer (10 mM Tris–HCl, 50 mM NaCl). Supercoiled plasmid DNAs of pBluescript II SK (-), pE9 [35] and pCFNOCON [18] were isolated from bacterial strain DH5α as described in the QIAGEN protocol (QIAGEN GmbH, Germany). *HindIII* restriction enzyme (New England BioLabs) was used for linearization of plasmids.

2.2. Preparation of IFI16 protein

IFI16 gene was amplified from human IFI16 cDNA (Mammalian Gene Collection, Structural Genomic Consortium) by PCR and subcloned into pET15b (Novagen) expression vector. Protein was expressed in *Escherichia coli* BL21-CondonPlus cells (Stratagene) with N-terminal His6-tag. Protein purification was performed by Co^{2+} affinity column (TALON).

2.3. Monoclonal antibodies

Monoclonal antibody against IFI16 protein (IFI-230, ab50004, synthetic peptide KDILNPDSSMETSPDF corresponding to C terminal amino acids 768–783 of human IFI16 as immunogen) was purchased from Abcam (UK).

2.4. Competition gel retardation assays on polyacrylamide gel

Binding reactions typically contained 100 ng labeled DNA, 75 ng IFI16 protein, 50 mM KCl, 5 mM Tris–HCl (pH 7.6), 0.5 mM EDTA, 0.01% Triton X-100 and increasing amount of competitor DNA in a final reaction volume of 20 μl. Competitor DNAs (50, 100, 150, 200 ng and 1 μg double-strand DNA or junction DNA were added at the start of the incubation reaction. Samples were incubated on ice for 10 min. The reactions were analyzed in 8% non-denaturing polyacrylamide gels in 0.33x Tris–borate-EDTA buffer, and electrophoresed at 90 V for 120 min at 4 °C. Protein–DNA complexes were visualized on an LAS-3000 image analyzer (Fujifilm) by Blue LED (460 nm) incident light source and processed digitally.

2.5. Gel electrophoretic mobility shift assay on agarose gel

DNA (usually 300 ng) and IFI16 proteins were mixed at different ratios in 10 μl of DNA binding buffer (5 mM Tris–HCl, pH 7.0, 1 mM EDTA, 50 mM KCl and 0.01% Triton X-100). Samples were incubated for 10 min at 4 °C and loaded onto a 1% agarose gel containing 0.33× Tris–borate-EDTA buffer. Agarose electrophoresis was performed for 4 h at 100 V (usually 4 V/cm) at 4 °C. The gels were stained with ethidium bromide and snapped on UV transilluminator by digital imaging system (Herolab) and processed digitally.

2.6. Proteinase K treatment

To deproteinize the IFI16–DNA complex, 2 μl of buffer containing 5% SDS, 50 mM EDTA and 1 μg of proteinase K was added to each sample. Samples were incubated for 20 min at 52 °C, cooled and applied to the agarose gel.

2.7. Supershifting of the IFI16–DNA complexes by monoclonal antibodies

DNA and IFI16 protein were mixed in 10 μl of the DNA binding buffer. Samples were incubated for 10 min at 4 °C. IFI16–DNA complexes were incubated with MAb IFI-230 (Abcam, molar ratio MAb:IFI16 was 2:1) at 4 °C for another 10 min and loaded onto a 1% agarose gel.

3. Results and discussion

3.1. Structure-specific DNA binding of IFI16 protein

To investigate DNA binding properties of IFI16, we employed gel shift assay with short oligonucleotides on polyacrylamide gel. We analyzed linear ds oligonucleotide targets and substrates that mimic those formed during the repair of ds breaks by homologous recombination (e.g., four-way junction DNA, which has a similar topology to cruciform structures *in vivo*). Although we observed no binding of IFI16 to ssDNA or dsDNA, we did observe strong affinity for four-way junction DNA (data not shown). To study the relative binding affinities of IFI16 in more detail, competition gel retardation assays were performed in which IFI16 was bound to FAM-labeled four-way junction DNA and competed with unlabeled linear and four-way junction-containing DNA (Fig. 1). It is clear from Fig. 1 that linear dsDNA of the competitors (Fig. 1A, lanes 10–14) were unable to disrupt the IFI16 four-way junction complex even when present at 10-fold molar excess (Fig. 1A, lane 14). The most effective competitor was four-way junction DNA (Fig. 1A, lanes 3–7), which reduced the binding of IFI16 at 10-fold

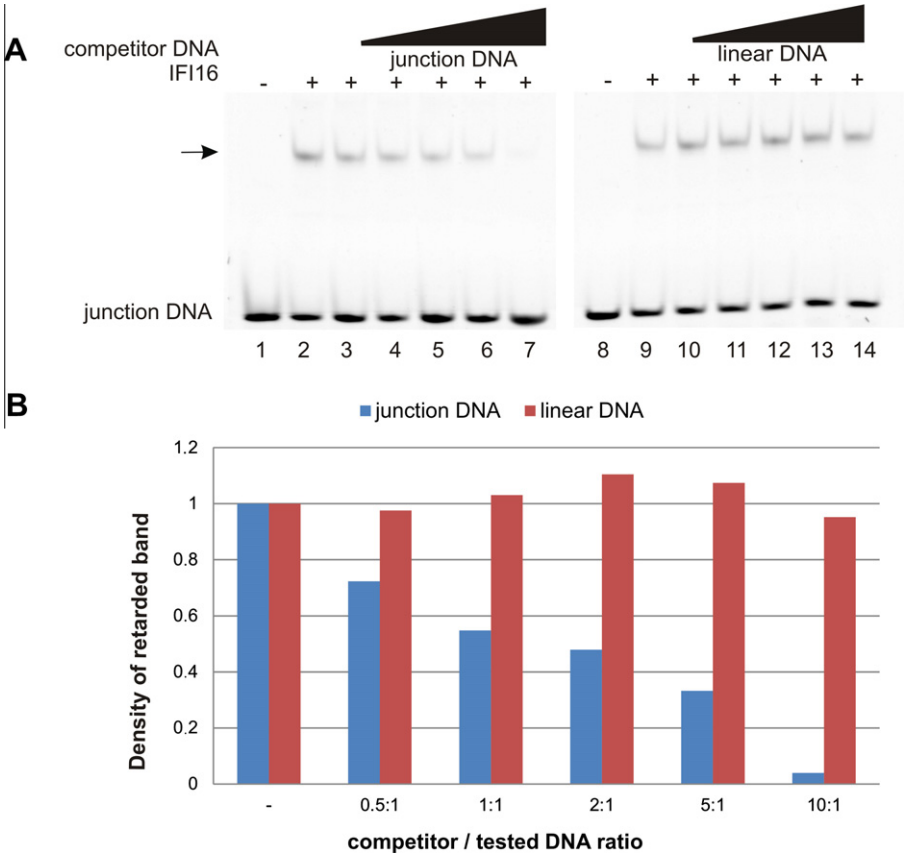

Fig. 1. IFI16 competition assay (A) 100 ng labeled four-way junction DNA (lanes 1–14) and increasing amounts of competitor four-way junction (lanes 3–7) or double-stranded DNA (lanes 10–14) were incubated with identical amount of IFI16. Competitor DNA / tested DNA ratios were 0.5:1 (lanes 3, 10), 1:1 (lanes 4, 11), 1.5:1 (lanes 5, 12), 2:1 (lanes 6, 13), and 10:1 (lanes 7, 14). Samples were incubated for 10 min on ice in binding buffer. We then loaded samples onto 8% polyacrylamide gel and separated complexes (90 V 120 min). Arrow show localization of protein/DNA complex. (B) The bar graph of relative intensity of IFI16/DNA complex is expressed as the percentage of band without competitor DNAs (lane 2, arrow).

molar excess by 93%. Densitometric comparison of retarded band also demonstrated a clear preference of IFI16 for four-way junction DNA (Fig. 1B). These data together suggest a binding preference of IFI16 for cruciform structures.

3.2. IFI16 protein binds preferentially to scDNA

Although IFI16 protein has been implicated in transcriptional regulation, little is known about the mechanism of its action. It is likely, however, that the regulation is via protein–protein interaction and/or by direct binding to DNA. To date, there has been no evidence about IFI16 binding to longer DNA substrates such as scDNA. To study the effects of IFI16 binding to long DNA, we used linear fragments and superhelical DNA of the plasmid pE9. Complexes of IFI16 with scDNAs were formed in DNA binding buffer and the binding was subsequently analyzed in agarose gels. As a result of IFI16 protein binding to scDNA, changes in the DNA electrophoretic mobility were observed (Fig. 2, lanes 2–5). At an IFI16:DNA ratio of 2:1 (Fig. 2, lane 2), a weak retarded band was noticed. As IFI16:DNA ratio increased from 4:1 to 28:1 (Fig. 2, lanes 3–5), the intensity of the free scDNA band was decreased and a strong retarded band was also observed. In contrast to scDNA (Fig. 2, lanes 1–5), the mobility of the linear DNA upon IFI16 protein binding (Fig. 2, lanes 6–10) was unaffected under the same conditions. When a higher concentration of IFI16 protein was used, part of the linear pBluescript was also slightly affected (Fig. 2, lane 10). At higher IFI16/DNA ratios, formation of smeared bands and corresponding decrease of the free scDNA band were evident. Importantly, deproteinization of the IFI16/DNA complex with proteinase-K led to restoration of the

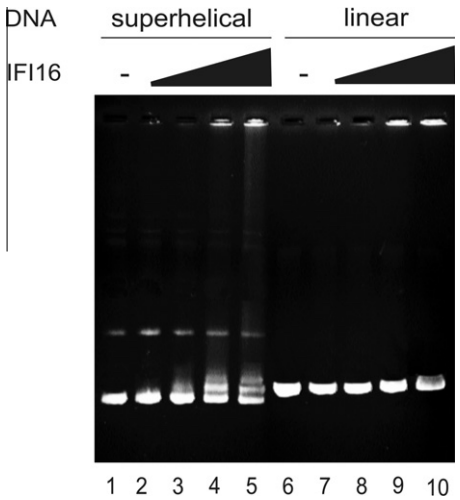

Fig. 2. Comparison of IFI16 protein binding to supercoiled and linear DNAs 300 ng supercoiled pE9 (lanes 1–5) and linear pE9 (lanes 6–10) were incubated with increasing concentration of IFI16 (25 ng [2:1], lanes 2 and 7; 50 ng [4:1], lanes 3 and 8; 100 ng [8:1], lanes 4 and 9; and 350 ng [28:1], lanes 5 and 10) in binding buffer (5 mM Tris–HCl [pH 7.6], 1 mM EDTA, 50 mM KCl and 0.01% Triton X-100) on ice for 10 min. Lanes 1 and 6 contain DNAs of supercoiled pE9 and linear pE9, respectively, without IFI16 protein. Samples were electrophoresed on 1% agarose gel at 100 V and 4 °C for 3–4 h.

original electrophoretic mobility of the DNA (data not shown). These results indicate that the observed shifts in scDNA mobility were caused by binding of IFI16 protein and not by partial

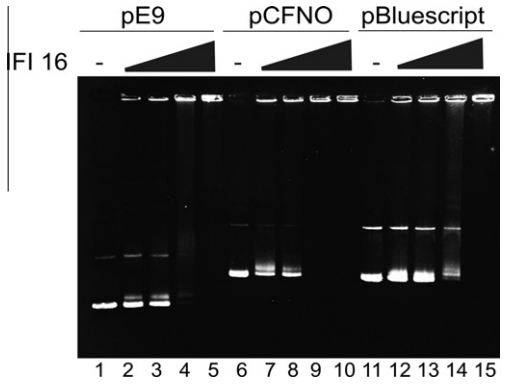

Fig. 3. Binding of IFI16 protein to supercoiled DNAs. 300 ng supercoiled pE9 (lanes 1–5), pCFNO (lanes 6–10) and supercoiled pBluescript (lanes 11–15) were incubated with increasing concentration of IFI16 (50 ng [4:1], lanes 2, 7 and 12; 125 ng [10:1], lanes 3, 8 and 13; 350 ng [28:1], lanes 4, 9 and 14; and 900 ng [70:1], lanes 5, 10 and 15) in binding buffer (5 mM Tris–HCl [pH 7.0], 1 mM EDTA, 50 mM KCl and 0.01% Triton X-100) on ice for 10 min. Lanes 1, 6 and 11 contain DNAs of supercoiled pE9, pCFNO and pBluescript, respectively, without IFI16 protein. For details, see Fig. 2.

relaxation of the superhelices by topoisomerase activity possibly present in the protein preparation.

To test the binding of IFI16 protein to different scDNA targets, we performed further gel shift analyses (Fig. 3). We used the superhelical plasmid pE9 as well as plasmids pBluescript and pCFNO. pCFNO DNA constructs are derived from pBluescript and contained a perfect inverted-repeat at the HindIII site [18]. Plasmids were isolated in native superhelical state (as described in the QIAGEN protocol) and tested for binding with IFI16 protein. At higher IFI16:DNA ratios, the intensity of free scDNA band decreased notably. In addition, higher IFI16:DNA ratios ultimately led to the formation of discrete bands with corresponding disappearance of the free scDNA band. Incubation of IFI16 with all tested superhelical DNAs led to a similar pattern of retarded bands. All tested DNAs in superhelical conformation showed reasonable binding to IFI16 protein. The majority of pCFNO DNA was already in complex with IFI16 protein at lower IFI16 concentrations, consistent with its being the best DNA target of IFI16 (Fig. 3, lane 7). In addition, lower IFI16 protein concentration also led to complete disappearance of scDNA.

3.3. Monoclonal antibodies (MAb) supershift complexes of IFI16 with superhelical DNA

Thus far, we have clearly demonstrated that IFI16 can form protein–DNA complexes with scDNA. In order to show that IFI16 protein is indeed present in the IFI16–scDNA complexes, monoclonal antibody (MAb) against IFI16 (Abcam, ab50004) was used. After addition of MAb, the retarded bands corresponding to IFI16–scDNA complex were supershifted (Fig. 4, lanes 3, 5 and 7), confirming that these bands were indeed IFI16–scDNA complexes. Importantly, supershifted bands could be observed even at lower IFI16 protein concentration. The intensity of these supershifted bands was slightly weaker compared to the original IFI16–scDNA band, suggesting that the IFI16 epitope for MAb may be partially masked by IFI16 binding to scDNA and/or the MAb may slightly destabilize the IFI16–scDNA complex.

Several findings have demonstrated the involvement of IFI16 protein in regulating gene expression [1,14,15]. Two mechanisms have been considered for such regulation: (a) protein–protein interaction with transcription factors, and (b) direct interaction with DNA. Both hypotheses have experimental support. It has been revealed that several proteins, such as USP7 and IFI16, are capable of interacting with p53 and influence p53–DNA binding [8,36]. On the other hand, it also has been shown that IFI16 protein is able to

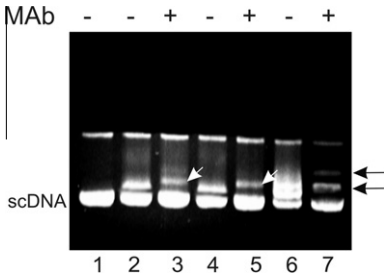

Fig. 4. Supershifting of IFI16–DNA complexes with monoclonal antibody 300 ng supercoiled pE9 (lanes 1–7) were incubated with three concentrations of IFI16 (0 ng, lane 1; 68 ng [5:1], lanes 2 and 3; 125 ng [10:1], lanes 4 and 5; and 350 ng (28:1), lanes 6 and 7) in binding buffer. Monoclonal antibody IFI-230 (lanes 3, 5 and 7) was added at molar ratio MAb:IFI16 of 2:1. We incubated the samples on ice for 10 min. Arrows show localization of the protein–DNA complex (lanes 2, 4 and 6) and/or localization of the MAb–protein–DNA complex (lanes 3, 5 and 7). For details see Fig. 2.

bind directly to ssDNA as well as to the promoter regions of cMYC and p53 [14]. We have shown that full-length IFI16 protein has a strong preference for four-way junction DNA. Crystal structure of IFI16 protein illustrates a positively charged electrostatic potential in the second subdomain of HIN-A and the first subdomain of HIN-B [8]. On the other hand, contemporary crystal structures of their HIN domains in complex with dsDNA show the HIN domains form clamshell-like configurations, with the inner surface lined by basic residues enclosing the dsDNA backbone phosphates. The structures suggest non-sequence-specific DNA recognition by IFI16, which may facilitate DNA-mediated assembly of large signaling complexes such as the inflammasomes.

To date, there has been no evidence of IFI16 binding to long DNA targets and topologically constrained DNA molecules. In this paper, we show for the first time the direct binding of IFI16 to long DNA in native superhelical DNA state and its strong preference for superhelical DNA. We also observed a higher affinity for four-way junction DNA compared to linear ds- or ss-DNA. Taken together, these results suggest that, in vitro, alternative DNA structure is the preferential target for IFI16. The concept that DNA binding properties of proteins are strongly dependent on DNA structure has been evidenced in a large number of DNA-binding proteins, including RNA polymerase [37], 14-3-3 [21], BRCA1 [35,38], HMGB1 [39], p53 [18,19,40], and several other transcription factors [41]. It also has been shown that the conformational state of the p53 target sequence is an important determinant for p53 sequence-specific binding [42,43]. Structural transitions in chromatin occur concomitantly with DNA replication or transcription, processes that involve a local separation of DNA strands and may thereby facilitate formation of alternative structures in DNA. Inverted repeats, which are capable of forming cruciform structures *in vivo*, are often localized in promoter regions and can therefore be important targets for proteins such as IFI16 involved in transcription. In this study, we present data showing IFI16 can bind strongly to scDNA and cruciform structures such as the four-way DNA junction *in vitro*. The plasmid pCFNO used in our study contains perfect palindromic sequence 16 bp long, which enable it to form cruciform structure at native superhelical conditions [18]. The ability of IFI16 to recognize local DNA structures suggests that it may play an important role in DNA damage recognition during the repair response. We believe that such a strong preferential binding of IFI16 to superhelical DNA and local DNA structures could play important roles in its regulation of transcription.

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