New insights into the structural basis of DNA recognition by HINa and HINb domains of IFI16

Xiangmin Ni1,2,*, Heng Ru2,*, Feng Ma3,*, Lixia Zhao2,*, Neil Shaw2, Yingang Feng4, Wei Ding2, Weibin Gong9, Qiaofeng Wang1, Songying Ouyang2,*, Genhong Cheng3,*, and Zhi-Jie Liu1,2,5,*

1 Institute of Molecular and Clinical Medicine, Kumming Medical University, Kumming 650500, China
2 National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
3 Department of Microbiology, Immunology and Molecular Genetics, University of California Los Angeles, Los Angeles, CA 90095, USA
4 The Qingdao Engineering Laboratory of Single Cell Oil and Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China
5 Human Institute, ShanghaiTech University, Shanghai 201210, China
* These authors contributed equally to this work.
† These authors contributed equally to this work.
* Correspondence to: Zhi-Jie Liu, E-mail: zjliu@ibp.ac.cn; Genhong Cheng, E-mail: gcheng@mednet.ucla.edu; Songying Ouyang, E-mail: ouyangsy@moon.ibp.ac.cn

Interferon-gamma-inducible protein 16 (IFI16) senses DNA in the cytoplasm and the nucleus by using two tandem hematopoietic interferon-inducible nuclear (HIN) domains, HINa and HINb, through the cooperative assembly of IFI16 filaments on double-stranded DNA (dsDNA). The role of HINa in sensing DNA is not clearly understood. Here, we describe the crystal structure of the HINa domain in complex with DNA at 2.55 Å resolution and provide the first insight into the mode of DNA binding by the HINa domain. The structure reveals the presence of two oligosaccharide/nucleotide-binding (OB) folds with a unique DNA-binding surface. HINa uses loop Lα5 of the canonical OB2 fold to bind to the DNA backbone. The dsDNA is recognized as two single strands of DNA. Interestingly, deletion of HINb compromises the ability of IFI16 to induce IFN-β, while HINa mutants impaired in DNA binding enhance the production of IFN-β. These results shed light on the roles of IFI16 HIN domains in DNA recognition and innate immune responses.

Keywords: interferon-gamma-inducible protein 16 (IFI16), hematopoietic interferon-inducible nuclear (HIN) domain, DNA recognition, innate immune responses

Introduction
Host cells possess intricate innate immune machinery for the detection of microbial DNA and RNA. Upon sensing the conserved pathogen-associated molecular patterns (PAMPs) associated with nucleic acids, a cascade of signaling steps is activated, culminating in the production of type I interferons (IFNs) and pro-inflammatory cytokines (Ishii and Akira, 2006; Muruve et al., 2008; Hornung and Latz, 2010). A large number of DNA sensors operating in various cell types have been identified and characterized. These sensors include TLR9 functioning in the endosomes of plasmacytoid dendritic cells (Blasius and Beutler, 2010; Kawai and Akira, 2010), DAI in mouse embryonic fibroblasts (Takaoka et al., 2007), RNA polymerase III in BMDMs, HEK, and other cell types (Ablasser et al., 2009; Chiu et al., 2009), and LRRFP1 in primary peritoneal macrophages (Yang et al., 2010). In addition to these distinct DNA sensors that function in different cell types, several cytosolic DNA sensors share DNA-binding domains and belong to a family. For example, the DExD/H-box domain-containing family of helicases includes members such as DHX36 that recognizes the CpG A oligodeoxyribonucleotide (ODN) (Kim et al., 2010), DHX9 that detects the CpG B ODN (Zhang et al., 2011c), DXD1 and DXH9 that sense poly I:C (Zhang et al., 2011a), and DDX41 that senses double-stranded DNA (dsDNA) (Zhang et al., 2011b). Recently, Sun et al. (2013) identified cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) as a cytosolic DNA sensor. The recognition of cytoplasmic DNA by cGAS activates the enzyme to catalyze the noncanonical cyclic dinucleotide from GTP and ATP (Diner et al., 2013; Gao et al., 2013a; Zhang et al., 2013). Subsequently, cGAMP activates the receptor STING to recruit TBK1, which then activates the transcription factors IRF3 and NF-κB, resulting in the production of type I interferon and other cytokines (Gao et al., 2013b; Zhang et al., 2013).

In contrast, members belonging to the second family of cytosolic DNA sensors, the PYHIN family, contain a pyrin (PYD) and one or more hematopoietic interferon-inducible nuclear (HIN) protein domains containing 200 amino acids (Schattgen and Fitzgerald, 2011). Thus far, four proteins—absent in melanoma 2 (AIM2), interferon-gamma-inducible protein 16 (IFI16), interferon-inducible protein X (IFIx), and myeloid cell nuclear differentiation antigen (MNDA)—in humans and 13 proteins in mice have been shown to...
be members of this family (Brunette et al., 2012). Together, these proteins are called AIM2-like receptors (ALRs) (Unterholzner et al., 2010). Except for murine p202, all these receptors contain a PYD. P202 contains two HIN domains, HINa and HINb, but lacks a PYD. The innate immune responses originating from AIM2 are exerted via the formation of a large complex called the inflammasome (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Upon the binding of DNA by the HIN domain, the PYD recruits the adaptor protein ASC via PYD–PYD interactions. ASC in turn recruits pro-caspase 1 through CARD–CARD interactions. Multiple molecules of IFI16 bound to DNA ensure filament formation as a broad host defense strategy (Morrone et al., 2014), bringing caspases into proximity for inter-molecular proteolytic activation. Activated caspase 1 processes IL-1β and IL-18 into mature pro-inflammatory forms, ultimately leading to pyroptosis. P202 has been shown to function as an antagonist of AIM2 function by sequestering DNA or heterodimerizing with AIM2 and preventing molecular crowding (Roberts et al., 2009). This activity results in the termination of AIM2-mediated responses.

Among the known DNA sensors, thus far, only IFI16 has been shown to be capable of sensing DNA in both the nucleus and the cytoplasm and of activating innate immune responses (Unterholzner et al., 2010; Kerur et al., 2011). These IFI16 functions are executed via a domain architecture consisting of an N-terminal PYD followed by two tandem HIN domains: HINa and HINb (Figure 1A). Whereas the sensing of Kaposi Sarcoma-associated herpes virus (KSHV) dsDNA in the nucleus by IFI16 results in the assembly of an AIM2-independent inflammasome and maturation of IL-1β and IL-18, the detection of ssDNA by IFI16 in the cytoplasm leads to the activation of the STING–TBK1–IRF3 pathway and the production of type I IFNs (Shaw et al., 2012). Details of the mechanism of inflammasome assembly by IFI16 are currently not fully understood. The nature of the PYD of IFI16 is different from the PYD of AIM2. Therefore, it is quite likely that IFI16 employs a different mechanism for the assembly of the inflammasome (Liu et al., 2003). Similarly, the molecular mechanism behind the activation of STING upon the binding of DNA by IFI16 is not yet known. In addition to its role as a DNA sensor in innate immune responses, IFI16 has been shown to regulate the activity of the transcription factor p53 and to participate in DNA repair (Liao et al., 2011). All these functions of IFI16 involve the binding of DNA (Yan et al., 2008).

Interestingly, both HIN domains of IFI16 are known to bind to DNA, and the crystal structure of the HINb domain bound with dsDNA has been described (Jin et al., 2012). However, the two HIN domains of IFI16 have different affinities for DNA (Unterholzner et al., 2010). In addition, the HINa domain, but not the HINb domain, is known to interact with the C-terminus of the transcription factor p53 and enhance its sequence-specific DNA binding during transcription (Liao et al., 2011). Therefore, a structural view of the HINa domain bound with DNA likely not only fills the gap in our understanding of the mechanism of DNA sensing by IFI16 but also sheds light on distinct roles of the two HIN domains in mediating the IFI16 functions.

Here, we describe the crystal structure of the HINa domain of IFI16 in complex with DNA at a resolution of 2.55 Å. The structure reveals a unique DNA-binding surface, which is different from that observed for the HINb domain of IFI16. Consequently, the differences in the mode of DNA binding confer distinct roles of the two HIN domains of IFI16 in sensing DNA and activating IFI16-mediated innate immune responses.

Results

*IF*16 HINa and HINb domains differ in their DNA-binding abilities

HINa and HINb domains of IFI16 bind to both ssDNA and dsDNA in vitro (Yan et al., 2008; Unterholzner et al., 2010). Our initial studies on the characterization of the DNA-binding abilities of HIN domains of IFI16 using GHV 50mer, HSV 60mer and VACV 70mer dsDNA suggested that the protein:DNA interactions were mostly electrostatic in nature (Supplementary Tables S1, S2 and Figure S1). Although the transfection of >70 base pair (bp)-long dsDNA has been shown to be necessary for the optimal induction of interferon by IFI16 in cells (Unterholzner et al., 2010), to determine whether the HIN domains can bind to short stretches of DNA, we examined DNA-binding affinity of the HIN domains of IFI16 using short oligonucleotides (≥20 bp) (Supplementary Table S3). The bacterially expressed HINa or HINb domain of IFI16 could bind to different types of DNA efficiently (Figure 1A and Supplementary Table S1). HINa binds to single- as well as double-stranded DNA with similar affinity, as the HINb domain and both domains together showed a lower affinity for AT-rich ssDNA (Supplementary Tables S2 and S3). Interestingly, the HINa domain showed a slightly faster rate of complex formation (Kc) than HINb for most of the DNA tested. However, the HINb domain seemed to bind to GC-rich DNA more tightly than HINa. This point is indicated by the slower rates of dissociation (Kd) of HINb from DNA (Supplementary Table S3). Thus, the HIN domains of IFI16 seem to differ in their DNA-binding abilities. To explain these differences at the molecular level, we investigated the mode of DNA binding by HINa and compared it with the DNA binding of HINb.

Structure of the HINa domain in complex with DNA

The purified, nucleic acid-free HINa domain of IFI16 was mixed with the different long dsDNA and ssDNA for crystallization. Finally, a 12mer-long ssDNA 5’AGGCCGGCGTGA3’ was screened to diffract X-ray over 3 Å and a protein:DNA binary complex crystal was obtained. The HINa:DNA complex crystallized in the P2₁2₁2₁ space group with two molecules of protein and two strands of DNA in the asymmetric unit (Figure 1B). The initial phases were determined as described in the Methods section. The quality of the electron density was good and permitted the unambiguous fitting of residues 199–389 and the 12mer strand of DNA. The density of seven amino acids at the N-terminus and four at the C-terminus was missing. Iterative rounds of model building followed by refinement resulted in a model with good statistics and geometry (Table 1). The final model, containing 380 amino acids (two molecules of HINa) and 22mer (two strands of DNA), was refined to 2.55 Å resolution (Rwork = 23.17%, Rsigma = 25.74%).

Interestingly, although ssDNA was mixed with the HINa domain of IFI16 for crystallization, in each asymmetric unit, the DNA seems to have undergone self-annealing to form a pseudo
right-handed helical spiral dsDNA architecture during the course of crystallization (Supplementary Figure S2). Therefore, the structure of the HINa domain of IFI16 looks like two protein molecules bound to a dsDNA (Figure 1B). The overall structure of the HINa domain of IFI16 bound to DNA reveals the presence of two OB folds, referred to hereafter as OB1 (AA 199–281) and OB2 (AA 314–389) (Figure 1A), joined by a long linker and is similar to the unliganded structure (PDB_ID 2OQ0) (Figure 1C and D). The density of residues 222–223, which is not visible in the unliganded structure, is visible in the DNA bound structure. These amino acids are in close proximity to the DNA. Further, deviations are observed at the positions of the Co atoms of R370–K377. These amino acids are observed to contact DNA in the ligand-bound structure. Superimposition studies reveal that the overall topology of the HINa domain of IFI16 (this study) is similar to that of the HINb domain of IFI16 (Jin et al., 2012), the HIN domain of AIM2 (Jin et al., 2012), and the HINa domain of p202 (Ru et al., 2013) (Figure 1D). Thus, the HIN proteins use a highly conserved topology to carry out their functions. However, the distribution of the charge on the surface is remarkably different, which is consistent with previous report (Liao et al., 2011), resulting in different DNA-binding surfaces and affinities.

IFI16 HINa domain binds to DNA using a different mode from the known HIN domains

Two HINa domain molecules are observed in the asymmetric unit. There are three hydrogen bonds interactions and non-bonded contacts between these two HINa domain molecules with the DNA. Therefore, the structure of the HINa domain of IFI16 bound to DNA is shown by the blue arrow. The asymmetric unit shows two molecules of the HINa domain, each bound to one strand of DNA. The secondary structural elements of the 2 OB folds are labeled according to the canonical OB fold convention. The density of residues 222–223, which is not visible in the unliganded structure, is visible in the DNA bound structure. These amino acids are in close proximity to the DNA. Further, deviations are observed at the positions of the Co atoms of R370–K377. These amino acids are observed to contact DNA in the ligand-bound structure. Superimposition studies reveal that the overall topology of the HINa domain of IFI16 (this study) is similar to that of the HINb domain of IFI16 (Jin et al., 2012), the HIN domain of AIM2 (Jin et al., 2012), and the HINa domain of p202 (Ru et al., 2013) (Figure 1D). Thus, the HIN proteins use a highly conserved topology to carry out their functions. However, the distribution of the charge on the surface is remarkably different, which is consistent with previous report (Liao et al., 2011), resulting in different DNA-binding surfaces and affinities.

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Interface area of 511 Å² (Supplementary Table S4). Interestingly, many hydrogen bond and hydrophobic interactions are observed between the two DNA chains (Supplementary Figure S3). However, although the ssDNA backbone forms a pseudo dsDNA helical spiral in the asymmetric unit, the interactions between bases of the two DNA chains, including hydrogen bond and hydrophobic interactions, do not obey the standard DNA double helix that is held by complementary base pairs together (Supplementary Figure S3). To determine whether these two monomers of HINa in the asymmetric unit constitute a homodimer, we subjected the structure to PISA analysis (Krissinel and Henrick, 2007). The analysis returned a CSS score of zero and ∆G P-value of >0.5 for the specificity of the interfaces between the two monomers generated by all possible symmetry operations, suggesting that the monomers of HINa of IFI16 do not form an oligomer in the crystals. This result is in agreement with the size exclusion chromatography (SEC) elution profile of the unliganded HINa protein during purification, where the protein eluted as a monomer, and previous description (Liao et al., 2011).

The HINa domain of IFI16 uses a large, positively charged surface, primarily made up of amino acids from the strand β4 and the L45 loop of the OB2 fold connecting strand β4 with β5, to bind to DNA (Figure 2A and B). Although the ssDNA seems to have annealed into dsDNA during the course of crystallization, one molecule of HINa contacts only one strand of DNA (Figure 2C and D). A cluster of positively charged residues from the OB2 fold tether the backbone of one strand of DNA, forming a pseudo helical spiral after base pairing with another strand (Figure 2D and E). An examination of the HINa-DNA contacts of this DNA-binding surface reveals that the protein makes contact with the DNA backbone (Supplementary Figure S3). A stretch of positively charged potential contributed primarily by the side chains of positively charged amino acids (R368–K377) binds to the negatively charged DNA backbone (Figure 2C). The DNA spiral is clamped into position using two pairs of side chains. The side chains of K371 and K372 form one clamp, and the side chains of N373 and Q374 form another clamp. The DNA spiral is held between the side chains of these two pairs of positively charged residues. The N-terminal loop comprising V199–R203 is in the vicinity of the DNA backbone. The amino acids immediately preceding V199 but not observed in the structure—R196, R197, and N198—are also likely to be part of this DNA-binding surface.

A detailed analysis of the structure revealed the presence of a second DNA-binding surface, belongs to another asymmetric unit, located on the opposite face of the protein (Supplementary Figure S4). This DNA-binding surface consists primarily of amino acids from the canonical loop L34 of the OB1 fold and the C-terminus of the HINa domain. Residues K252, N254, K256, and K257 from L34 are close to the DNA backbone, with K256 donating a hydrogen bond to the oxygen of the phosphate group from the DNA backbone (Supplementary Figure S4). In addition, K214 from the loop connecting strand β1 with β2 interacts with the DNA backbone. The last amino acid observed in the structure, K391, is close to the DNA. Therefore, the C-terminal 392–KXTN–393 amino acids preceding K391, which are not visible in the structure, are likely to bind to DNA.

Thus, the HINa domain of IFI16 tethers DNA using a distinct DNA-binding surface that is different from the DNA-binding surfaces observed for the HINb domain of IFI16, HIN domain of AIM2, and HINa domain of p202 (Figure 1D and Supplementary Figure S5). Comparing the amino acids of the HINb domain of IFI16, HIN domain of AIM2, HINa domain of p202, and replication protein A (RPA) involved in DNA binding with the amino acids of the HINa domain of IFI16 reveals that none of the residues of the HINa domain of IFI16 involved in DNA binding are conserved (Supplementary Figure S6). The HINa domain of IFI16 can specifically bind to single strands of DNA. The contacts with the DNA are electrostatic in nature and primarily involve the side chains of positively charged residues and the negatively charged atoms of the DNA backbone.

**Different DNA-binding mode of IFI16 HIN domains examined by NMR**

To validate the DNA-binding interface observed in the crystal structure, we performed nuclear magnetic resonance (NMR) titrations by the addition of DNA to the isotope-labeled protein solution. Both single- and double-stranded AT-rich and GC-rich DNA sequences (Supplementary Table S1) were used for the NMR titration experiments (Supplementary Table S1). HINa and HINb domains of IFI16 were titrated under identical conditions. The chemical shifts for >90% of the backbone resonances could
Figure 2 DNA-binding surfaces of the HINa domain of IFI16. (A) Surface electrostatic potential representation of the HINa domain of IFI16 with DNA bound to the positive potential. Blue indicates positive potential; red indicates negative potential. (B) Cartoon representation depicting the location of the DNA-binding site. Electron density for the DNA contoured at 1.0σ is shown in A and B. (C) Amino acids involved in the binding of DNA are depicted as sticks. Oxygen atoms of amino acids are colored in cyan; nitrogen atoms are colored in blue; carbon atoms are colored in magenta. (D) One molecule of the HINa domain of IFI16 binds to one strand of DNA. The cartoon shows HINa molecules using identical sets of residues to bind to DNA. (E) The HINa domain of IFI16 has a putative second DNA-binding surface. The cartoon (left panel) and surface electrostatic potential representation (right panel) depict the location of the DNA-binding surfaces. The putative second DNA-binding surface is spatially separated and located on the opposite face of the protein. The DNA is shown as ribbons.
assigned for both HINa and HINb suspended in 20 mM HEPES, 150 mM NaCl (pH 7.5) (Supplementary Figure S7).

The titration of HINa with the GC-rich ssDNA used in the crystallization experiments caused both significant chemical shift perturbations (CSP) and intensity decreases in the $^1$H-$^1$N HSQC spectra of HINa (Figure 3). The addition of B-type dsDNA or Z-type dsDNA (Supplementary Table S1) caused similar CSPs and intensity decreases. Upon the addition of ssDNA at molar ratios as low as 1:0.3, many NH signals disappeared quickly or attenuated, with peak intensities decreasing to <20% of the original (Figure 3A and B). Residues showing decreased intensities are not clustered in the sequence (Figure 3B), suggesting that the addition of DNA leads to the formation of large, stable protein–DNA complexes that decrease the NH signals in the $^1$H-$^1$N HSQC spectra. This interpretation is further supported by the fact that signals from many residues forming the $\beta$-barrels of the two OB folds, which are located far from the interaction interface, disappeared in the $^1$H-$^1$N spectra after the addition of DNA (Supplementary Figure S8), as these signal intensities are more sensitively dependent on molecular weight. The residues with the largest CSP values are obviously clustered in the sequence, and mapping of the CSP results onto the structure of the binary complex reveals that these amino acids interact with DNA in the crystal structure (Figure 3C and D). Almost all the residues with CSP shifts are located around the DNA-binding site (Figure 3D, Supplementary Table S5). Thus, a combination of NMR titration studies and the crystal structure of the HINa domain in complex with DNA reveal that the HINa domain of IFI16 binds ssDNA to form a stable protein–DNA complex in solution. The DNA-binding interface of the HINa domain of IFI16 observed in the crystal structure is similar to the DNA-binding interface formed in solution upon the addition of DNA to a protein solution containing the HINa domain (Supplementary Table S5).

NMR titration results of the HINb domain of IFI16 with DNA were different from that of HINa (Supplementary Figure S9). Although the AT-rich or GC-rich ssDNA or dsDNA caused significant chemical shift perturbations in the HINb $^1$H-$^1$N HSQC spectra, the signal intensities were less weakened than in the case of HINa (Supplementary Figure S9A and B). Most of the signals retained >50% of their intensities when compared with the intensities from DNA-free signals. In contrast to HINa, the HINb domain could not form a large stable complex with DNA in solution. The intensities of the signal for N598, N602, T616, S688, R708, and K709 were enhanced. Amongst these residues, the signal intensities for T616, R708, and K709 were enhanced by 3–5 times, suggesting that DNA binding stabilizes the conformation of these residues. Mapping of these residues on the structure of HINb bound with DNA (PDB ID 3RNU) reveals that T616, R708, and K709 interact with the DNA, whereas N598, N602, and S688 do not contact the DNA. Further, the NMR titration results show that residues with large CSP values are mainly located at the DNA-binding interface observed in the crystal structure of the HINb:DNA complex described by Jin et al. (2012). In addition, a few residues with large CSP values are observed to interact with neighboring HINb molecules (Supplementary Figure S9C and D). Although HINb and dsDNA form large complexes in the crystal structure (Jin et al., 2012), the addition of DNA to the HINb solution does not cause a sharp decrease in signal intensity. This result may be attributed to the shorter length of DNA (12 bp) used in our NMR titration experiments as opposed to the longer length of DNA (16 bp) used to crystallize the HINb:DNA complex. Thus, the NMR titration experiments reveal differences in the behavior of the two HIN domains in the presence of DNA, which may be a result of the differences in the modes of DNA binding of these domains. The HIN domains bind to DNA in solution using the same DNA-binding interface observed in their respective crystal structures.

In addition, AIM2 is known to use DNA as an oligomerization platform to initiate signaling (Robert et al., 2009; Unterholzner et al., 2010; Jin et al., 2012). To determine whether IFI16 uses a similar mechanism to activate signaling, we performed a small-angle X-ray scattering (SAXS) analysis of the HINab domain (AA 192–685) construct alone and in the presence of 42mer dsDNA (Supplementary Figure S10). The molecular weight estimated from the $p(R)$ value (79.6 kDa) suggested that the unliganded HINab construct existed as a monomer in solution (Supplementary Figure S10A and B). This result is in agreement with the previously published SAXS analysis of full-length IFI16 (Liao et al., 2011). The low-resolution envelope generated for the shape of the protein indicated that the HINab domain assumes an elongated shape in solution with a linker separating the two domains spatially (Supplementary Figure S11). In the presence of the 42mer dsDNA, HINab forms a large oligomer with an estimated molecular weight of 349.6 kDa (Supplementary Figure S10C and D), which is consistent with the result of NMR. Thus, the structural view of HINa in complex with DNA and the results of NMR and SAXS analysis of the HIN domains alone and in the presence of DNA suggest that similar to AIM2 (Jin et al., 2012), IFI16 might also use DNA as a platform for oligomerization to activate signaling.

HINa mutants impaired in DNA binding show an enhanced induction of IFN-β

The binding of DNA by IFI16 has been shown to either evoke the production of type I IFNs via the STING–TBK1–IRF3 pathway or result in the assembly and activation of the inflammasomes. Mutation of the residues of the HINb domain of IFI16 involved in DNA binding resulted in a significant decrease in the ability of the protein to induce the IFN-β promoter upon the transfection of HEK293T cells with DNA in a luciferase reporter gene-based assay (Jin et al., 2012). Therefore, the binding of DNA to the HINb domain has been proposed to activate innate immune signaling. However, the effect of DNA binding by the HINb domain on the function of IFI16 is not clear. To address this issue, we first identified amino acids involved in DNA binding using our structure of the HINa:DNA complex and mutated them in clusters to abolish DNA binding. A cluster of mutations designated M3 (K252A/N254A/K256A/K257A) probed the interface of one of the DNA-binding surfaces; a cluster of mutations designated M5 (R368A, K370A, K372A, N373A, Q374A) probed the interface of the other DNA-binding surface located on the opposite side (Figure 4A, Supplementary Table S6). Both M3 and M5 decreased the affinity of the protein
Figure 3 Validation of the DNA-binding interface by NMR titration experiments. (A) $^1$H-$^15$N HSQC spectra for the HINa domain of IFI16 titrated with GC-rich ssDNA. (B and C) Bar diagram depicting intensity decreases (B) and chemical shift perturbations (C) versus the residue number of HINa when the protein was titrated at a 1:1 molar ratio with DNA. (D) Mapping of chemical shift perturbations (CSPs) on the crystal structure of the HINa:DNA complex. Residues with CSPs (shown as red sticks) are in close proximity to the DNA in the crystal structure.
for GHV 50mer dsDNA, HSV 60mer dsDNA and VACV 70mer dsDNA by >10-fold compared to the wild-type HINa domain (Supplementary Table S6). Thus, as observed in the crystal structure and validated by NMR titration experiments, mutagenesis studies also suggest the presence of two spatially separated positively charged surfaces with the ability to bind to DNA.

Next, to investigate the in vivo effect of impairing the DNA-binding ability of the HINa domain on the function of IFI16, we introduced mutations, including M3 and M5, in full-length IFI16 and evaluated the ability of these mutants to induce the IFN-β promoter activity in a HEK293T cell-based luciferase reporter assay. STING was essential for the IFI16-based activation of IFN-β promoter; therefore, IFI16 or its mutants were co-transfected with STING (Figure 4B). Using this assay, we also studied the effect of the deletion of the HINa or HINb domain on the function of the protein (Figure 4C). Quite unexpectedly, in response to transfection by plasmid DNA, the HINa domain deletion mutant of IFI16 had almost no effect on the activation of the IFN-β promoter. In contrast, under identical conditions, deletion of the HINb domain impaired the activation of the IFN-β promoter, which is comparable with the GFP control (Figure 4C). The HINa domain region of IFI16 (AA 192–685) could fully activate the IFN-β promoter as the wild-type IFI16. STING, wild-type IFI16 (IFI16-WT), and HIN domain-deficient IFI16 (IFI16-ΔHINA, IFI16-ΔHINB) were co-transfected with IFN-β promoter firefly luciferase reporter and pTK-Renilla luciferase reporter genes. Data are shown as relative luciferase activity and are representative of three independent experiments (mean ± SD, n = 6). (D) Only the HINa domain (AA 192–685) of IFI16 could fully activate the IFN-β promoter as the wild-type IFI16. STING, wild-type IFI16 (IFI16-WT), and IFI16 domains (IFI16-PYRIN, IFI16-HINA, IFI16-HINB, IFI16-HINA-B) were co-transfected with IFN-β promoter firefly luciferase reporter and pTK-Renilla luciferase reporter. Data are shown as relative luciferase activity and represent three independent experiments (mean ± SD, n = 6). (E) HINa mutants increased IFI16-activated IFN-β promoter activity. STING, wild-type IFI16 (IFI16-WT), and HINa domain mutants (M3, M5) were co-transfected with IFN-β promoter firefly luciferase reporter and pTK-Renilla luciferase reporter. Data are shown as relative luciferase activity and represent three independent experiments (mean ± SD, n = 6).

Discussion

A comparison of the structure of our HINa:DNA binary complex with the structure of the HINb domain bound to DNA (PDB_ID 3RNU) reveals that although the overall topologies of the
structures of these domains are similar, they bind to DNA at different surfaces. HINa uses residues from the other sides of both OB folds to tether DNA whereas HINb uses residues from the linker region and the OB2 fold to bind to the DNA backbone (Figure 1D, Supplementary Figure S5). These observations are supported by the crystal structures and NMR titration experiments presented here. The most remarkable difference yet between the two HIN domains of IFI16 is that whereas one domain of HINb contacts both strands of DNA (PDB_ID 3RNU), one domain of HINa specifically contacts only one strand of DNA. This observation confers a distinct role for HINa in DNA binding. The structure of the HINa domain in complex with DNA provides the first glimpse into the nature of the unique DNA-binding ability of HINa domains.

HINa has been shown to bind to ssDNA as well as dsDNA (Yan et al., 2008; Brazda et al., 2012). Our crystal structure of the HINa domain bound to DNA provides an explanation for this ability of HINa. We propose dsDNA are recognized as two single strands of DNA by HINa domains, which may explain why HINa binds to single- as well as double-stranded DNA with similar affinity. Interestingly, in addition to the ssDNA-binding surface observed in the crystal structure and validated by NMR titration experiments, a second positively charged surface located on the opposite face of the protein seems to recognize the ends of the pseudo dsDNA. Both NMR titration and mutagenesis studies suggest that this surface is capable of binding DNA. It is possible that in the full-length protein, this positively charged patch of HINa instead interacts with the N-terminal PYD.

Our studies on the HIN domains reveal that they exhibit two distinct modes of DNA binding (Supplementary Figure S5). In the AIM2-like mode of DNA binding, the HIN domain primarily uses the linker connecting the two OB folds to tether DNA (Jin et al., 2012). In the p202 HINa-like mode of DNA binding, the HIN domain primarily uses loops from the OB1 and OB2 folds to bind to DNA (Ru et al., 2013). These two distinct modes of DNA binding exhibited by HIN domains seem to result in different responses. Furthermore, the differences in the modes of DNA binding seem to have resulted in different affinities for DNA. P202 can sequester DNA from AIM2 because the HINa domain of p202 has a higher affinity for DNA than the HIN domain of AIM2. Thus, the HIN domains may have evolved with two distinct modes of DNA binding, each mode capable of eliciting a different response.

Recently, IFI16 was shown to sense KSHV and herpes simplex virus 1 (HSV-1) DNA in the nucleus (Kerur et al., 2011; Orzalli et al., 2013). Both viruses are known to activate DNA damage response pathways. IFI16 is also known to participate in the cellular response to DNA damage (Agilipay et al., 2003). In this context, IFI16 might be able to bind to ends, nicks, or gaps in foreign DNA or damaged self DNA and initiate an immune response. Our structure of the HINa domain of IFI16 bound to DNA reflects this versatile ability of IFI16 in DNA binding. The HINa domain of IFI16 can recognize different topologies of DNA (Supplementary Figures S12 and S13). For example, the crystal structure and NMR titration experiments reveal that the M3 site can potentially bind to and detect the ends of dsDNA and that the M5 site might bind to the backbone spiral of single- or double-stranded DNA. Thus, although IFI16 might not be able to differentiate between a viral infection and DNA damage, both scenarios are likely to present DNA that can be accessed by IFI16 to activate downstream signaling.

ALRs have been proposed to use DNA as an oligomerization platform to activate signaling (Hornung et al., 2009; Roberts et al., 2009; Jin et al., 2012). Our crystal structure of HINa bound to DNA together with the results of our NMR titration experiments and SAXS analysis on HIN domains of IFI16 support the DNA-mediated oligomerization model for the activation of IFI16-mediated signaling. Unliganded IFI16 exists as a monomer in solution. In the presence of DNA, IFI16 forms oligomers.

In summary, our studies on the HIN domains of IFI16 reveal differences between the abilities of HINa and HINb domains of IFI16 to bind to DNA. The crystal structure of the HINa domain bound to DNA provides the first glimpse into the nature of ssDNA binding by HINa domains. The structure further reveals that the mode of DNA binding by HINa is distinctly different from DNA binding by the HINb domain. Whereas HINb mutants exhibiting diminished DNA binding were impaired in their ability to induce IFN-β production, HINa mutants with impaired DNA-binding ability enhanced the induction of IFN-β production. Thus, our results suggest that the HIN domains of IFI16 might play distinct roles during the sensing of DNA. The structure presented here, together with the structure of the HINb domain bound to DNA, completes the molecular description of the region of IFI16 involved in sensing DNA. These results markedly advance our understanding of the role of HIN domains of IFI16 in sensing DNA.

Material and methods

Full methods including protein production, small-angle X-ray scattering (SAXS), surface plasmon resonance (SPR), etc. can be found in Supplementary Materials and methods.

Constructs

ORFs encoding the HINa (AA 192 to 393), HINb (AA 515 to 710), and HINab (AA 192 to 710) domains of human IFI16 isoform 2 were sub-cloned in frame into the pET28a(+) vector (Novagen) using the Ndel and XhoI restriction sites with an N-terminal 6×His tag followed by a thrombin cleavage site. Mutations were introduced either with the QuikChange™ site-directed mutagenesis kit (Stratagene) or by the two-step overlapping PCR method using the wild-type construct as a template. The DNA sequences of all the constructs used in the study were verified by sequencing (GENEWIZ).

Protein production

The recombinant proteins were expressed using the Escherichia coli BL21(DE3) R1PL (Stratagene) strain. The production of recombinant proteins was initiated by adding 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubating the cultures for another 16 h at 20°C. The proteins were purified and the 6×His tag was removed by thrombin cleavage. Tagless protein was further applied onto the HiTrap heparin column (GE Healthcare) and size exclusion chromatography (SEC). To purify the protein-DNA complex,
the DNA (synthesized by GENEWIZ) was added to a dilute protein solution in a 2:1 ratio of DNA to protein. The mixture was concentrated and further purified by SEC to remove excess DNA. All the mutants were expressed and purified using the same procedure used for the wild-type protein.

**Crystallization**

Crystallization screening for HINα–DNA complex was conducted by the sitting drop vapor diffusion method as described by Zhao et al. (2014). Initial hits were further optimized by the hanging drop vapor diffusion method by manually mixing 1.0 µL of protein with 1.0 µL of reservoir solution at 16°C. Diffraction-quality crystals for the HINα:ssDNA complex were obtained in the conditions containing 20% (w/v) PEG 3350 and 0.2 M potassium fluoride; or 20% (w/v) PEG 3350 and 0.2 M sodium formate; or 20% (w/v) PEG 3350 and 0.2 M lithium acetate dehydrate. The crystals were cryo-cooled in liquid nitrogen prior to diffraction testing and data collection.

**X-ray data collection, structure determination and refinement**

The diffraction data for DNA-bound IFI16 HINα complexes were collected at 100 K using an ADSC Q315 CCD detector at Beamline BL5.0.1 (ALS, Advanced Light Source, Lawrence Berkeley National Lab, USA). All datasets were indexed, integrated, and scaled using the HKL2000 software package (Otwinowski and Minor, 1997). The structures were solved by molecular replacement (Lebedev et al., 2008) using the structure of IFI16 HINb domain (PDB_ID 3RNU) as a search model. The initial model was built using PHENIX Autobuild (Adams et al., 2010) and later manually improved in Coot (Emsley and Cowtan, 2004). Refinement was performed using Refmac (Murshudov et al., 1997) and PHENIX Refine (Adams et al., 2010; Afonine et al., 2012) alternately. Statistics for the final model are listed in Table 1. The quality of the final model was validated by MolProbity (Chen et al., 2010). Atomic coordinates and structure factor have been deposited in the Protein Data Bank under the accession number 4QGU.

**NMR spectroscopy**

All NMR experiments were performed at 303 K on a Bruker AVANCE 600 MHz spectrometer equipped with a z-gradient triple-resonance cryoprobe according to the previous description (Jiao et al., 2014).

**Small-angle X-ray scattering**

SAXS data for the HINab domain alone and in the presence of 42mer DNA were collected at beamline 12-ID-B, APS, Argonne National Lab, and the data were treated as described previously (Jiao et al., 2013).

**Surface plasmon resonance**

SPR experiments were performed in duplicate with a Biacore T100 instrument (Biacore) at 25°C in 20 mM HEPES, 100 or 150 mM NaCl, 0.5 mM EDTA and 0.05% (v/v) Tween 20, pH 7.5 as described by Ouyang et al. (2012).

**Luciferase reporter assays in HEK293T cells**

The HEK293T cells were plated in a 96-well plate overnight followed by co-transfection with a mixture of pTK-β-galactosidase reporter plasmid, IFN-β promoter firefly luciferase reporter plasmid and other indicated plasmids using lipofectamine transfection reagents (Polyplus-transfection). Post-transfection (24 h later), luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The data were normalized for transfection efficiency by dividing the firefly luciferase activity by the activity of Renilla luciferase.

**Supplementary material**

Supplementary material is available at Journal of Molecular Cell Biology online.

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**References**


