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RPA nucleic acid-binding properties of IFI16-HIN200

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ABSTRACT

*I*nter*F*eron-γ *I*nducible protein *16* (IFI16) belongs to the interferon inducible HIN200 protein family that contains transcriptional regulators linked to cell cycle regulation and differentiation. All family members contain at most two domains of 200 amino acids, called HIN200, each containing two Oligonucleotide/Oligosaccharide Binding (OB) folds. IFI16 is involved in transcriptional repression and is a component of the DNA repair multi-protein complex known as BASC, which forms after UV-induced DNA damage. In this study, we used fold recognition and biophysical approaches as a tool to infer and validate functions to the HIN200 domain. Since the best template to model IFI16-HIN200 is Replication Protein A (RPA) in complex with single-stranded nucleic acids, we tested six RPA nucleic acid-binding characteristics for IFI16-HIN200. Our results indicate that IFI16-HIN200 is an RPA-like, OB-fold, nucleic acid-binding protein that binds to ssDNA with higher affinity than to dsDNA, recognizes ssDNA in the same orientation as RPA, oligomerizes upon ssDNA binding, wraps and stretches ssDNA, but does not destabilize dsDNA. We finally propose a framework model explaining how the HIN200 domain could prevent ssDNA from re-annealing.

1. Introduction

The *I*nter*f*eron-γ *I*nducible protein *16* (IFI16) is a member of the human and murine mammalian gene family (p200) or HIN200 [2,4,5,17]. The products of these genes were initially shown to be *H*ematopoietically expressed, *I*nterferon-inducible, nuclear antigen with *200* amino acid repeats (HIN200). More recently, HIN200 proteins have been shown to be transcriptional regulators linked to cell cycle regulation and differentiation. HIN200 gene products are scaffolding proteins that interact with and modulate the activities of multiple transcriptional factors [24,42]. There are also significant genetics and functional studies that link HIN200 proteins to the incidence of cancer [14].

HIN200 gene expression is induced by type I (α and β) and type II (γ) interferon. IFI16 is induced by interferon α and γ [17] and contains the apoptotic and inflammatory PAAD/DAPIN/Pyrin domain [32] at its N-terminus followed by the repetition of two HIN200 domains. The full-length IFI16 protein can form a dimer [23], interacts with p53 and is linked to transcriptional regulation, cell cycle arrest and apoptosis [23,24]. Immunological analyses of breast cancer specimens have shown that the levels of IFI16 are decreased suggesting a role in tumor development [31]. IFI16 has been linked to DNA repair [1] since it interacts with the multimeric *B*RCA1 *A*ssociated *S*urveillance protein *C*omplex (BASC). BASC forms after UV damage by radiation and contains DNA repair proteins that sense and repair damaged DNA. In the same study, the authors demonstrated a physical interaction between IFI16, BRCA1 and p53 suggesting that IFI16 may have a DNA repair function. In addition, the DNA-binding properties of IFI16 have suggested that it binds to double-stranded DNA [29,23,13,16]. Thus, to gain further insight into IFI16-HIN200 functions, comparative modeling of this domain using a 3D jury method [22] identified the *O*ligonucleotide/*O*ligosaccharide *B*inding (OB) fold domain of human *R*eplication *P*rotein *A* (RPA; PDB: 1JMC) as the best template. A model of the HIN200 domain of IFI16 was built from this template (Pio, 2003, unpublished data), [3] showing that the HIN200 domain contains 2 OB folds. More recently, the structure of the first and second HIN200 domains of IFI16 was solved by X-ray crystallography (PDB: 2oQ0, 3B6Y) confirming that this domain contains two OB folds. The weak similarity of sequence predicted by fold recognition between RPA and IFI16-HIN200 (less than 10%) makes it difficult to infer a function with confidence to this domain since the OB-fold superfamily members have become highly diverged, share little sequence conservation and perform a wide variety of functions (see Structural Classification of Proteins, http://scop.mrc-lmb.cam.ac.uk/scop) [38]. Consequently, the RPA function of IFI16-HIN200 needs to be further verified experimentally.

RPA is a major eukaryotic ssDNA-binding protein involved in DNA replication, recombination and repair [41]. This protein protects ssDNA from nucleolytic damage, prevents hairpin formation, and blocks DNA re-annealing until the processing pathway step is completed. To complete these processes evolution has selected protein domains, such as OB folds, that can interact with each other and allow proteins to associate and dissociate [20]. RPA containing OB-fold domains can adopt different DNA-binding modes that can be switched from one to the other

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through protein interactions and phosphorylation [6]. This remodeling insures the integrity of ssDNA and its orderly processing through the pathway [20].

The three different subunits of human RPA (RPA70, RPA32 and RPA14) form a heterotrimer [41] and each contains at least one OB fold motif that recognizes nucleic acids *in vivo*. RPA70 plays a major role in recognizing ssDNA. [8]. The human RPA heterotrimer displays different binding modes triggered by the oligomerization of its OB folds. It can also recognize dsDNA and RNA but 100–1000 times more weakly, and its binding affinity to ssDNA shows a strong dependence on the length of the oligonucleotides [25,11]. Due to its ssDNA-binding ability, RPA can destabilize and unwind dsDNA during the initiation of DNA replication intrinsically or in association with helicase [36,12]. Remodeling of OB fold was recently shown by swapping the OB fold in RPA from archaea [33]. They showed that the OB folds, when interchanged, could still perform their functions. In addition to human RPA, most OB fold proteins reveal a prevalent 3′–5′ ssDNA-binding polarity: 5′ end of ssDNA directs to the C-terminus of the protein and 3′ end extends towards the N-terminus [35].

Since human RPA can physically interact with many of the individual BASC components, including p53, BLM, RFC and RAD50-MRE11-NBS1 [18–20,37] and IFI16 and RPA have identical structure and interacting partners, we hypothesize that IFI16 may be an RPA-like ssDNA-binding protein. We have therefore designed further biochemical experiments to examine oligomerization, ss-nucleic acid-binding, stretching/wrapping, binding polarity and DNA destabilization properties of IFI16-HIN200. Our results indicate that IFI16-HIN200 possesses most of the RPA-like ssDNA-binding and OB fold properties tested but does not destabilize dsDNA as does RPA.

2. Experimental procedures

2.1. Cloning and site-directed mutagenesis

The first IFI16-HIN200 domain cDNA (194–425 amino acid numbering) was PCR-amplified from the full-length cDNA template (Uniprot accession number: Q16666) and cloned into a pET100/D-TOPO vector (Invitrogen, CA). For DNA polarity assay (see below), the multiple mutants HIN$^{C351/356S}$, HIN$^{C351/356/366S}$, and HIN$^{Q238C+C351/356/366S}$ were obtained using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, USA). The plasmid sequences were verified by sequencing (Macrogen, Korea). See also Table 1 for the PCR primers.

2.2. Production of recombinant proteins

IFI16-HIN200 wild-type and mutant fusion proteins were prepared as described previously [15].

2.3. Spectroscopic analysis

Tertiary structure of IFI16-HIN200 was determined by intrinsic fluorescence using an SLM4800 spectrofluorimeter (AMICON) at a 3.7 μM protein concentration in 20 mM Na Cacodylate, pH 7.25 for the native protein. The same buffer with 8 M urea for the denatured protein was used and the protein samples were denaturated overnight at room temperature followed by heating at 80 °C for 4 h. Tyrosines of the protein were excited at λ_{ex}=275 nm. The emission spectra were recorded from 250 nm to 350 nm at room temperature. Baseline correction was performed by removing the fluorescence contribution of the buffer under the same experimental conditions as the protein samples.

Far UV circular dichroism spectra of wt IFI16-HIN200 were obtained as described previously [15] but at 22 μM protein concentration in 20 mM Na Cacodylate, pH 7.25, 20 mM Na Acetate, pH 4.0, and 20 mM HEPES, pH 7.5. Baseline correction was obtained by removing the buffer contribution to the spectra. The secondary structure content was calculated from the subtracted spectra using CONTINLL algorithm in cdPRO (http://lamar.colostate.edu/~sreeram/CDPro/main.html). The amount of the secondary structure obtained from the CD measurement was then compared to that obtained from the structural model of IFI16-HIN200. The thermo-stability of IFI16-HIN200 was measured under the same conditions but in 20 mM Na Cacodylate, pH 7.25, at 216 nm from 20 °C to 100 °C.

Table 1
Oligonucleotides used in this study

Name	Oligonucleotide sequence
IFI16-HIN200F	5′-CACCACTCCCAGAAGAAATGTT
IFI16-HIN200R	5′-TTATTAGTTTGTTTTTTTCTTTATCTGG
HIN$^{C351/356S}$-F	5′-GGACAGGACAATCTCACAATATCCCCTCTGAAGAAGGAG
HIN$^{C351/356S}$-R	5′-CTCCTTCTTCAGAGGGGATATTGTGAGATTGTCCTGTCC
HIN$^{C351/356/366S}$-F	5′-GATAGCTCCACATTTTCTCCTTTCGACTTAGAAAAAAGAACC
HIN$^{C351/356/366S}$-R	5′-GGTTCTTTTTTCTAAGTCGAAAGGAGAAAAGTTGGAGCTTATC
HIN$^{Q238C+C351/356/366S}$-F	5′-CATGCTACAGTGGCTACACAGACATCGTTCTTCCATGTGAAGG
HIN$^{Q238C+C351/356/366S}$-R	5′-CCTTCACATGGAAGAAGCATGTCTGTGTAGCCACTGTAGCATG
GC-5	5′-GGAAGAAGGAAGTGGGATCAGGATCCGCTGGCTCC
GC-3	5′-GGAGCCAGCGGATCCTGATCCCACTTCCTTCTTCC
A_{25}	Poly(dA)$_{25}$
T_{25-75}	Poly(dT)$_{25,30,35,70,75}$
G-rich 15mer	5′-GGTTGGTGTGGTTGG
FRET-18	5′-QUASAR670-GCCTCGCTGCCGTCGCCA
FRET-58	5′-TGGCGACGGCAGCGAGGC-(T)$_{40}$-QUASAR570-T

2.4. Electrophoretic Mobility Shift Assay (EMSA)

GC-5, GC-3, A_{25}, T_{25}, G-rich 15mer and dsDNA GC 5–3 formed by annealing GC-5 and GC-3 (Table 1) were end-labelled by T4 polynucleotide kinase (Invitrogen, CA) with γ-^{32}P ATP (Amersham Bioscience, USA) and gel purified. A 2-fold serial dilution of wt IFI16-HIN200, starting from 106.3 μM to 0.0012 μM, was incubated with 360 nM ^{32}P-labelled nucleic acids at room temperature in 20 mM Na Cacodylate, pH 7.25. After electrophoresis the wet gels were exposed to a phosphor screen (Amersham Bioscience, CA) overnight and visualized on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Amersham Phamacia Biotech, CA).

2.5. Protein–DNA UV cross-linking

Wild-type IFI16-HIN200 (3.7 μM) was incubated with single-stranded oligo A_{25}, T_{25}, T_{30}, T_{35}, T_{70}, T_{75}, GC-5, GC-3, dsDNA GC 5–3 and $(A–T)_{25}$ annealed by A_{25} and T_{25} (Table 1) at DNA:protein molar ratios of 0, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 in 20 mM Na Cacodylate, pH 7.25. Each reaction was exposed to 254 nm UV light at a distance of 20 cm on a UV Stratalinker 2400 (Stratagene, CA) at 1000 J/min for 15 min. Cross-linked products were analyzed on 15% SDS-PAGE and visualized by silver staining. Each oligonucleotide was cross-linked to IFI16-HIN200 at 1:1 DNA: protein molar ratio in parallel and subjected to Western blot with mouse monoclonal Penta-His Antibody (Qiagen, CA) to detect the His-tagged IFI16-HIN200.

2.6. Experimental molecular weight determination

For each SDS-PAGE gel, a calibration curve was plotted from the migration distance (R_f) of each low range molecular weight standard (BioRad) to estimate the molecular weights of the protein monomer and protein–DNA complexes from their R_f.

2.7. Tyrosine fluorescence quenching assay

Wild-type IFI16-HIN200 has 6 tyrosines that may be quenched by oligonucleotides and a fluorescence quenching assay can be performed to obtain the binding constants for different oligonucleotides. IFI16-HIN200 (3.7 μM) was titrated by A_{25}, T_{25}, GC-5, GC-3, dsDNA $(A–T)_{25}$ and GC 5–3 (Table 1) in 20 mM Na Cacodylate, pH 7.25. The DNA: protein molar ratio was increased from 0.01 to 0.1 at an increment of 0.01 and from 0.1 to 3.0 at an increment of 0.1. At each titration point, the sample was excited at 275 nm (λ_{ex} of tyrosine) and the emission intensity at 304 nm (λ_{em} of tyrosine) was recorded and plotted against the corresponding oligonucleotide concentration. Spectra of oligonucleotides alone were recorded under the same conditions as the negative control.

The data were analyzed by curve fitting to a one-site ($Y=B_{max}X/(K_d+X)$) or two-site ($Y=B_{max1}X/(K_{d1}+X)+B_{max2}X/(K_{d2}+X)$) binding model using GraphPad Prism 4.03 (GraphPad Software Inc.). The parameters B_{max} (maximal binding) and K_d (dissociation constant) were calculated from a non-linear regression fit. The statistical *F* test in GraphPad was used to determine the preferential binding models. Scatchard plots were analyzed in the GraphPad software.

2.8. Glutaraldehyde chemical cross-linking

Wild-type IFI16-HIN200 (1.8 μM) was cross-linked by glutaraldehyde alone or with T_{25} at a DNA:protein molar ratio of 0.1:1 for 10 min at 20 °C in 400 μl 20 mM HEPES, pH 7.5, as described [39]. The cross-linked species were analyzed on a 15% SDS-PAGE, together with IFI16-HIN200 without poly $(dT)_{25}$ and glutaraldehyde as the negative control. The gel was visualized by silver staining. The molecular weights of different cross-linked species were estimated by the calibration curve as described above.

2.9. Melting depression

Double stranded DNA $(A–T)_{25}$ was diluted to a final concentration of 10 μM in 100 μl 20 mM Na Cacodylate, pH 7.25, containing 100 mM NaCl. Absorbance at 260 nm (A_{260}) was measured on a Cary 300 Bio UV–Visible spectrophotometer equipped with a temperature regulator. The measurement was performed from 20 °C to 90 °C in the