NMR and Fluorescence Studies of DNA Binding Domain of INI1/hSNF5

Dongju Lee, Sunjin Moon, Jihye Yun, Eunhee Kim,[†] Chaejoon Cheong,[†] and Weontae Lee*

Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea

*E-mail: wlee@spin.yonsei.ac.kr

[†]Magnetic Resonance Team, Korea Basic Science Institute (KBSI), Ochang, Chungbuk 363-883, Korea

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Integrase Interactor 1 protein (INI1/hSNF5) or BRG1-associated factor 47 (BAF47) is a SWI/SNF-related matrix associated actin dependent regulator of chromatin subfamily B member. DNA binding domain of INI1/hSNF5 is cloned into *E.coli* expression vectors, pET32a and purified as a monomer using size exclusion chromatography. NMR data show that INI1^{DBD} has folded state with high population of α -helices. By fluorescence-quenching experiments, binding affinities between INI1^{DBD} and two double stranded DNA fragments were determined as 29.9 ± 2.6 μ M (GAL4_1) and 258.7 ± 5.8 (GAL4_2) μ M, respectively. Our data revealed that DNA binding domain of INI1/hSNF5 binds to transcriptional DNA sequences, and it could play an important role as a transcriptional regulator.

Key Words : INI1/hSNF5, DNA binding domain, NMR, Purification, Fluorescence

Introduction

Integrase interactor 1 protein (INI1)/BRG-1 associated factor 47 (BAF47) is known as a hSNF5 or SMARCB1 and it is a core subunit of ATP-dependent SWI/SNF chromatin remodeling complexes.¹⁻³ Chromatin remodeling complexes promote gene expression, transcriptional repression.⁴⁻⁶ INI1/hSNF5 regulates expression of numerous eukaryotic genes by altering DNA-histone interactions and it is the first host protein identified as an IN-interacting factor by two-hybrid screenings.² In previous studies, INI1/hSNF5 interacts with virus proteins, cellular factors such as human immunodeficiency virus type 1 integrase,⁷ human papillomavirus E1,⁸ Kaposi's sarcoma-associated herpesvirus K8,⁹ Epstein-Barr virus nuclear antigen,¹⁰⁻¹² c-MYC(cheng), ALL1, GADD34 and p53.¹³⁻¹⁵ INI1/hSNF5 is involved in regulation of cell proliferation and cell cycle by prohibiting activation of E2F-dependent genes through p16ink41-CDK4/cyclin D-Rb pathway.¹⁶ In addition, INI1/hSNF5 plays a role as a tumor suppressor because mutations of INI1 gene lead to aggressive paediatric atypical teratoid and malignant rhabdoid tumors.¹⁷

INI1/hSNF5 has functional and conserved regions which are two direct imperfect repeats (repeat 1 and repeat 2) and C-terminal coiled-coiled domain, and a homology region 3 (HR3).¹⁸ The functional domains of INI1/hSNF5 are not well characterized except for Rpt1 and Rpt2 motifs, which interact with proteins such as viral or cellular proteins.¹⁸ Previous reports show that INI1/hSNF5 interacts with the minor groove of double-stranded DNA and then, conforms positive supercoils in relaxed plasmid DNA. It implies that SWI/SNF complex plays an important role in chromatin remodeling through protein-protein interaction. It has been proposed that INI1/hSNF5 could bind DNA at the minor groove while hBRG1 has been reported to possess an AT-

hook motif which appears to play a role in DNA binding, although its biological functions of DNA binding domain are still unknown.^{18,19} Here, we performed high-yield protein purification and isotope-labeling of DNA binding domain of INI1/hSNF5 (INI1^{DBD}) to initiate structural study by NMR spectroscopy. Data from fluorescence spectrophotometry supports that INI1^{DBD} binds double stranded GAL4 DNA sequences.

Materials and Methods

Cloning and Over-expression of INI1^{DBD}. Human INI1/hSNF5 was amplified to INI1^{DBD} fragment by polymerase chain reaction (PCR). XhoI/BamHI (NEB) cut each side of amplified fragment. Mixture was incubated 8 h in 37 °C heat block. Sub-cloning into modified expression vector pET32a (Novagen Inc.) contains an N-terminal hexa-histidine [(His)₆] affinity tag, Thioredoxin (TRX), tobacco etch virus (TEV) protease recognition sites, which were transformed into *Escherichia coli* (*E. coli*) (Strain BL21 DE3) (Novagen Inc). In order to obtain INI1^{DBD}, cells were grown on LB broth media at 37 °C until optical density reached around 0.6 at 600 nm and then, added 1 mM isopropyl β -D-thiogalactoside (IPTG) from 1 M solution stock. Cells were incubated for 20 hours at 25 °C, 160 rpm shaker incubator and harvested by centrifugation at 6000 $\times$ g for 30 minutes. Cell pellets were stored at -80 °C freezer.

Purification of INI1^{DBD}. Harvested cells were suspended in lysis buffer [25 mM Sodium Phosphate, 300 mM NaCl, 5 mM β -mercaptoethanol, protease inhibitor cocktail-(Roche) pH 7.5] and lysed by sonicator [90% amplify, 1s pulse, 10 cycle]. The cell lysates were centrifuged for 30 minutes, 14000 $\times$ g at 4 °C. After centrifugation, Ni²⁺ affinity chromatography was used for protein binding (Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were further wash-

ed with 60 mM imidazole washing buffer and eluted with 500 mM imidazole elution buffer. TRX-tag was cleaved with Tobacco etch virus (TEV) protease at 25 °C.²⁰ Final purification was performed by size exclusion column chromatography using a HiLoad Superdex 75 column in 10 mM HEPES, 100 mM NaCl, 10 mM Dithiothreitol (DTT), 0.01% NaN₃ solution at pH 7.0.

Size exclusion chromatography with four reference proteins [albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and aprotinin (6.5 kDa)] was used to confirm the molecular weight of INI1^{DBD} by the following equation: $\log Y = -1.2177 X + 6.263$ (X = elution fraction/45.85 mL, $R^2 = 0.9942$). Purified protein was concentrated to 1.0 mM using an Amicon Ultra centrifugal filter device (Millipore).

NMR Spectroscopy. For hetero-nuclear NMR experiments, we prepared isotope labeled proteins. Cells were cultured in

M9 minimal medium containing ¹⁵N labeled NH₄Cl (¹⁵N, 99%, Cambridge Isotope Laboratories, Inc.). Protein sample was prepared in NMR buffer solution (10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% NaN₃, at pH 7.0 containing 90% H₂O/10% D₂O). All NMR experiments were performed at 25 °C on a Bruker DRX900 spectrometer equipped with a z-shielded gradient triple-resonance cryo-probe. The ¹H chemical shifts were referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The ¹H-¹⁵N HSQC experiment was acquired using a uniformly ¹⁵N-labeled sample. Pulsed-field gradient (PFG) techniques with a WATERGATE pulse sequence were used for all H₂O experiments, resulting in a good suppression of the solvent signal.²¹ All NMR data were processed using NMRpipe and NMRDraw software.²²

Fluorescence Experiments. Two double-stranded oligonucleotides, (GAL4_1 (5'-CGATGATGAAGATACC-3'), GAL4_2 (5'-GTGCACGATGCACAG-3')) were designed

Figure 1. Schematic representation of the vector map and structural domains of INI1/hSNF5. (a) The vector map of INI1^{DBD} expressed in *E. coli* with TEV cleavage site is shown. (b) Structural domains of INI1/hSNF5 are drawn by data from previous studies.¹⁸ The vector consists of a Thioredoxin, (His)₆ tag and TEV enzyme cleavage site (ENLYFQG). (c) Secondary structures and primary sequence of INI1^{DBD} were shown. Secondary structures were predicted by psi-pred server (<http://bioinf.cs.ucl.ac.uk/psipred/>).

based on data from Morozov *et al.*¹⁸ and purified using polyacrylamide gel electrophoresis (PAGE) (Operon). Binding affinities between GAL4 DNA and INI1^{DBD} were measured on a model RF-5301PC spectro-fluorophotometer (Shimadzu, Kyoto, Japan). INI1^{DBD} was titrated up to a molar ratio of 1:2.5 (INI1^{DBD}: GAL4 DNA) using a 2 mL thermostat cuvette. Samples were excited at 280 nm for side-chain of the aromatic residues of INI1^{DBD}, and emission spectra were recorded for light scattering effects from 270 to 500 nm.²³ The K_d values were calculated by the equation $\log(F_0 - F/F) = \log(1/K_d) + n \log[\text{ligand}]$, where F_0 and F represent fluorescence intensity of the protein at 355 nm in the absence and presence of DNA,²⁴ respectively. The number n represents the ligand binding site of the protein.

Results and Discussion

Cloning and Expression of INI1^{DBD}. DNA binding domain of INI1/hSNF5 corresponding residues from 106 to 183 was successfully cloned by PCR amplification and sub-cloned into pET32a (Novagen Inc.) at the BamHI and XhoI site (Fig. 1(a)). INI1/hSNF5 consists of three structural domains which are DNA binding, Repeats1 and Repeats2 domain (Fig. 1(b)). The construct contains TRX, hexahistidine [(His)₆] affinity tag with TEV protease recognition sequence (ENLYFQG) in the N-terminus (Fig. 1(b)). INI1^{DBD}

protein was successfully expressed in both 250 mL LB and M9 media using 1 mM IPTG at 25 °C.

Purification of INI1^{DBD}. Data from Ni-NTA affinity chromatography shows that INI1^{DBD} is soluble in elution buffer. Fusion protein was eluted at imidazole concentration of 500 mM and purified as a purity of > 90% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2(a)). INI1^{DBD} was obtained after cleavage of Trx tag followed by Ni-NTA column work. Molecular weight of the purified fusion protein was determined as a 27 kDa and INI1^{DBD} is ~9 kDa after cleavage of Trx tag. To increase protein purity and determine oligomeric state of INI1^{DBD}, size-exclusion chromatography was performed using HiLoad 16/60 superdexTM75. Data from size-exclusion chromatography shows that INI1^{DBD} forms a monomer (~9 kDa) in solution (Fig. 2(b)) by comparing reference proteins as a standard molecular marker. The final yields of the INI1^{DBD} protein are 10-15 mg per 250 mL of cultured cell.

NMR Spectroscopy. One-dimensional NMR (1D-NMR) was used to confirm folded state of the purified INI1^{DBD}. A number of high-field methyl proton resonances ranged from 0.5 to 1.0 ppm as well as dispersion of amide proton resonances (7.5-9.5 ppm) strongly suggested that INI1^{DBD} has a stable folded structure under our experimental condition (Fig. 3(a)). 2D ¹H-¹⁵N HSQC spectrum shows that most of

Figure 2. SDS page and elution profile of the size exclusion chromatography. (a) The SDS pages identify INI1^{DBD} during each purification steps. Lane 1 and 2 indicate supernatant and pellet of the cell lysate. Lane 3, 4 and 5 show the elution profiles, flow-through, washing and elution, respectively. Lane 7, 8-11 and 12 indicate profile after tag cleavage, flow-through, washing and elution, respectively. (b) Elution profile of the size exclusion chromatography with SDS page. INI1^{DBD} was eluted from 80 to 90 mL. Molecular weight of INI1^{DBD} was calculated by the equation, $\log Y = -1.2177 X + 6.263$ ($R^2 = 0.9942$, X = elution fraction/45.85 mL).

Figure 3. One-dimensional NMR and ¹H-¹⁵N 2D HSQC spectra of INI1^{DBD}. (A) One-dimensional ¹H NMR spectrum of INI1^{DBD} is shown. NMR sample was prepared in 10 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Na₃N solution, at pH 7.0. NMR experiment was performed on a Bruker DRX900 spectrometer. (b) ¹H-¹⁵N 2D HSQC spectrum was recorded using uniformly ¹⁵N labeled INI1^{DBD}.

Figure 4. Fluorescence spectra of INI1^{DBD} in the presence of double-stranded DNA. (a) Fitting plot of INI1^{DBD} is shown in the presence of difference mixing ratio of double-stranded DNA (GAL4-1) (1:0 (red), 1:0.2 (green), 1:0.4 (purple), 1:0.6 (black), 1:0.8 (orange), 1:1 (blue), 1:2 (magenta)). (b) Fitting plot of INI1^{DBD} with mixing ratio of double-stranded GAL4-2 DNA (1:0 (red), 1:0.2 (green), 1:1 (purple), 1:1.5 (black), 1:2 (orange), 1:2.5 (blue)).

backbone NH resonances were spread over the spectrum in NMR solution (10 mM HEPES, 100 mM NaCl, 2 mM DTT and 0.01% NaN₃ at pH 7.0) (Fig. 3(b)). Based on chemical shift information of the backbone amide proton and nitrogen resonances, we expect that INI1^{DBD} has high population of α -helices.

DNA Binding of INI1^{DBD}. Previous report suggested that DNA binding domain of INI1/hSNF5 could interact with double strand DNA.¹⁸ Data from fluorescence-quenching experiments provide that the binding affinities between INI1^{DBD} and two double stranded DNA fragments were determined as 29.9 ± 2.6 μ M (GAL4_1) and 258.7 ± 5.8 (GAL4_2) μ M, respectively (Fig. 4(a)). The binding stoichiometry between the protein and DNA is calculated as 1:1 based on data from fluorescence spectroscopy. It is of interest to know that GAL4_1 binds 10 times stronger than GAL4_2. Taken together, these results revealed that DNA binding domain of INI1/hSNF5 binds to transcriptional DNA sequences, and INI1^{DBD} could play an important role as a transcriptional regulator. Since it has also been suggested that INI1^{DBD} would mediate protein-protein interaction

with HIV-1 integrase,⁷ INI1/hSNF5 could modulate viral replication of HIV-1 by binding to minor groove of DNA. This hypothesis will be revealed by structural information of INI1/HIV-1 integrase complex which is currently in progress.

Conclusion

DNA binding domain of INI1/hSNF5 is cloned into *E. coli* expression vectors, pET32a and purified using size exclusion chromatography. INI1^{DBD} was determined as a monomeric conformation in our experimental condition. Data from one-dimensional NMR and 2D ¹H-¹⁵N HSQC show that INI1^{DBD} has folded state with high population of α -helices. By fluorescence-quenching experiments, binding affinities between INI1^{DBD} and two double stranded DNA fragments were determined as 29.9 ± 2.6 μ M (GAL4_1) and 258.7 ± 5.8 (GAL4_2) μ M, respectively, indicating INI1^{DBD} could play an important role as a transcriptional regulator.

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References

- Kingston, R. E.; Bunker, C. A.; Imbalzano, A. N. *Genes Dev.* **1996**, *10*, 905-920.
- Kingston, R. E.; Narlikar, G. J. *Genes Dev.* **1999**, *13*, 2339-2352.
- Wang, W.; Cote, J.; Xue, Y.; Zhou, M.; Khavari, P. A.; Biggar, S. R.; Muchardt, C.; Kalpana, G. V.; Goff, S. P. & other authors. *EMBO J.* **1996**, *15*, 5370-5382.
- Wang, W.; Xue, Y.; Zhou, S.; Kuo, A.; Cairns, B. R.; Crabtree, G. R. *Genes Dev.* **1996**, *10*, 2117-2130.
- Sudarsanam, P.; Lyser, V. R.; Brown, P. O.; Winston, F. *PNAS.* **2000**, *97*, 3364-3369.
- Underhill, C.; Qutob, M. S.; Yee, S. P.; Torchia, J. J. *Biol. Chem.* **2000**, *275*, 40463-40470.
- Kalpana, G. V.; Marmon, S.; Crabtree, G. R.; Goff, S. P. *Science* **1994**, *266*, 2002-2006.
- Lee, D.; Kim, J. W.; See, T.; Hwang, S. G.; Choi, E. J.; Choe, J. J. *Biol. Chem.* **2002**, *277*, 22330-22337.
- Hwang, S.; Lee, D.; Gwack, Y.; Min, H.; Choe, J. J. *Gen. Virol.* **2003**, *84*, 665-676.
- Kwiatkowski, B.; Chen, S. Y.; Schubach, W. H. J. *J. Virol.* **2004**, *78*, 6067-6072.
- Wu, D. Y.; Kalpana, G. V.; Goff, S. P.; Schubach, W. H. J. *J. Virol.* **1996**, *70*, 6020-6028.
- Wu, D. Y.; Krumm, A.; Schubach, W. H. J. *J. Virol.* **2000**, *74*, 8893-8903.
- Rozenblatt-Rogen, O.; Rozovskaia, T.; Burakov, D.; Sedkov, Y.; Tillib, S.; Blechman, J.; Nakamura, T.; Croce, C. M.; Mazo, A.; Cannani, E. *PNAS.* **1998**, *95*, 4152-4157.
- Alder, H. T.; Chinery, R.; Wu, D. Y.; Kussick, S. J.; Payne, J. M.; Fornace, A. J., Jr.; Tkachuk, D. C. *Mol. Cell Biol.* **1999**, *19*, 7050-7060.
- Lee, D.; Sohn, H.; Kalpana, G. V.; Choe, J. *Nature* **1999**, *399*, 487-491.
- Imbalzano, A. N.; Jones, S. N. *Cancer Cell* **2005**, *7*, 294-295.
- Versteeg, I.; Sevenet, N.; Lange, J.; Rousseau-Merk, M. F.;

- Ambros, P.; Handgretinger, R.; Aurias, A.; Delattre, O. *Nature* **1998**, *394*, 203-206.
18. Morozov, A.; Yung, E.; Kalpana, G. V. *PNAS*. **1998**, *95*, 1120-1125.
19. Singh, M.; D'Silva, L.; Holak, T. A. *J. Biol. Chem.* **2006**, *387*, 1469-1478.
20. Yun, J.-H.; Kim, H.; Park, H. E.; Lee, J. S.; Lee, W. *Bull. Korean Chem. Soc.* **2012**, *33*, 3248.
21. Kay, L. E.; Keifer, P.; Saainen, T. *J. Am. Chem. Soc.* **2003**, *114*, 10663-10665.
22. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277-293.
23. Barik, A.; Mishra, B.; Shen, L.; Mohan, H.; Kadam, R. M.; Dutta, S.; Zhang, H. Y.; Priyadarsini, K. I. *Free Radic. Biol. Med.* **2005**, *39*, 811-822.
24. Jiang, M.; Xie, M.; Zheng, D.; Liu, Y.; Li, X.; Chen, X. *J. Mol. Struct.* **2004**, *692*, 71-80.
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