Journal of the Korean Magnetic Resonance Society **2019**, *23*, 20-25
DOI 10.6564/JKMRS.2019.23.1.020

Structural characterization of the putative DNA-binding domain of CP2c and its relevance to zinc binding

Ki-Sung Ryu[1†], Ku-Sung Jo[1†], Na-Young Kim[1], Eun-Jae Jeon[1], Sung Jean Park[2], Hyun-Hwi Kim[2], Eun-Hee Kim[3], Chan-Gil Kim[1], Chul Geun Kim[4], and Hyung-Sik Won[1,*]

[1]Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju, Chungbuk 27478, Republic of Korea
[2]College of Pharmacy and Gachon Institute of Pharmaceutical Sciences, Gachon University, Incheon 21936, Republic of Korea
[3]Protein Structure Group, Korea Basic Science Institute, Ochang, Chungbuk 28119, Republic of Korea
[4]Department of Life Science and Research Institute for Natural Sciences, College of Natural Sciences, Hanyang University, Seoul 04763, Republic of Korea

Received Mar 12, 2019; Revised Mar 17, 2019; Accepted Mar 19, 2019

Abstract The transcription factor CP2c has been recently validated as an oncogenic protein that can serve as a promising target for anticancer therapy. We have recently documented that a recombinant protein corresponding to the putative DNA-binding region (residues 63-244) of CP2c adopted two different conformers, one of which is dominated by zinc binding. However, in the present study, a longer construct encompassing residues 63-302 appeared to form a single structural domain. This domain could be considered to adopt a functionally relevant fold, as the known specific binding of a dodecapeptide to this protein was evident. Hence, the residues 63-302 region rather than 63-244 can be regarded as a natively folded structural domain of CP2c. In addition, it was confirmed that zinc ions can bind to this putative DNA-binding domain of CP2c, which resulted in reduced stability of the protein. In this context, it is suggested that the mode of action of CP2c would resemble that of tumor suppressor p53.

Keywords CP2c, DNA-binding domain, NMR, p53, structural domain, zinc binding

Introduction

CP2c (also known as TFCP2, LSF and LBP-1c), belonging to the Grainyhead (Grh)/CP2 transcription factor family, was originally identified as the murine α-globin gene regulator.[1] Afterwards, CP2c has been revealed to be a central transcription factor of the CP2-subfamily members, which is conserved in multicellular organisms and ubiquitously expressed to participate in diverse cellular processes by regulating the expression of various target genes.[2,3] In addition, CP2c has been recently validated as an oncogenic protein, of which regulation is effective for treating cancer.[4-6] In this context, it is strongly believed that detailed structural information of CP2c would provide a valuable strategy for development of novel anticancer agents specifically targeting the protein. Therefore, we recently initiated a structural study of CP2c.

Fig. 1A shows the putative domain organization of CP2c. In particular, the residues 63-244 region is called Elf-1 domain (Elf1D), as it shares a sequence homology with the Grh (also known as Elf-1 or

†These authors contributed equally to this work.
*Address correspondence to: **Hyung-Sik Won,** Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju-si, Chungbuk-do 27478, Republic of Korea, Tel: 82-43-840-3589, E-mail: wonhs@kku.ac.kr

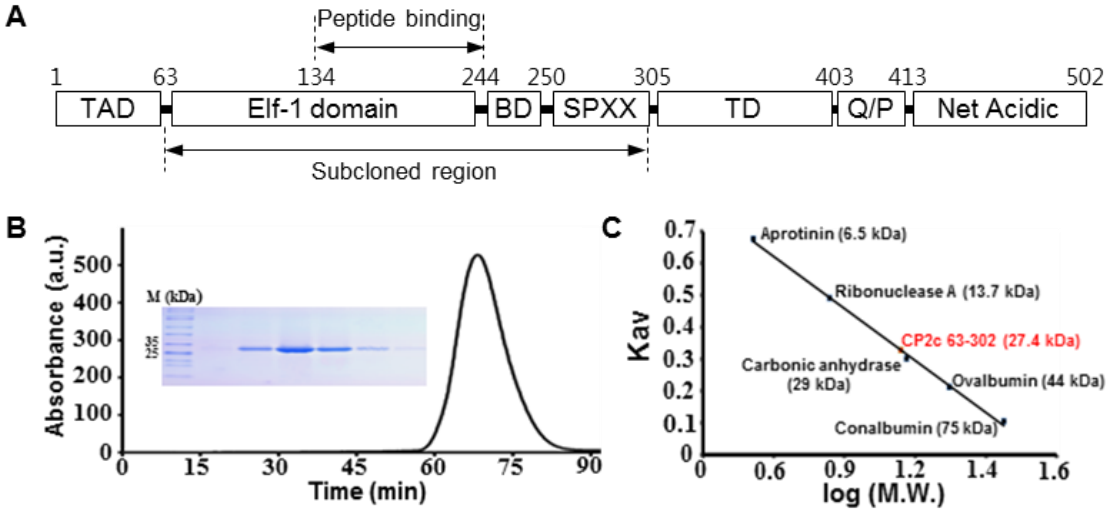

Figure 1. Preparation and biochemical characterization of CP2c(63-302). (A) Schematic diagram showing the putative domain organization of CP2c.[2] Constructed region in this study and the known binding site of the dodecapeptide are illustrated by arrows. TAD, transactivation domain; BD, basic domain; TD, tetramerization domain. (B) Gel-filtration elution profile of the purified CP2c(63-302). Inset shows SDS-PAGE image for the elution fractions containing the protein. (C) Hydrodynamic size of CP2c(63-302) as estimated by analytical gel-filtration. Black dots indicate the protein standards used. Red dot designates the K_{av} value of CP2c(63-302), of which apparent molecular weight was calculated using the standard curve.[9,10]

NTF-1) protein.[2] Furthermore, as this region has been annotated as a putative DNA-binding region for both the two homologous proteins, we have previously attempted structural characterization of the CP2c Elf1D.[7] As results, the recombinant protein corresponding to this region adopted two different conformers, one relatively well-ordered and the other less-ordered. However, it could not be ensured whether this conformational equilibrium would be also relevant in intact protein, or it is an artifact appearing only in the isolated form. Likewise, the novel observation of zinc binding to the Elf1D construct[6] needed to be validated and further characterized. Meanwhile, crystal structures have been recently solved for the DNA-binding domain (DBD) of a Grh-like (Grhl) protein and its complex with DNA.[8] This DBD construct for crystal structures was comprised of the residues 248-485 of Grhl1. Although its N-terminal part corresponds to the homologous Elf1D of CP2c, the full size of the construct is matched with approximately 63-302 region of CP2c, of which C-terminal part beyond Elf1D contains the so-called BD and SPXX region (Fig. 1A) and shares no significant sequence homology to Grhl1. In the present study, alternatively to the Elf1D (residues 63-244), we constructed the 63-302 region of CP2c as a putative DBD of the protein. Subsequently, the preliminary results of structural characterization suggest that this region can fold into a single species of conformation, of which relevance to zinc binding is also validated.

Experimental Methods

DNA construct and protein preparations – DNA recombination, protein expression and purification for the CP2c(63-302) followed the protocols previously established for the Elf1D. Briefly, the subcloned DNA fragments were inserted into pCold1-1 vector, followed by transformation into the *E. coli* BL21(DE3)pLysS cells. The proteins expressed with an N-terminally tagged hexahistidine were purified by application of Ni^{2+}-affinity chromatography, followed by proteolytic removal of the hexahistidine tag. After final purification by

gel-filtration, the protein concentrations were spectrophotometrically deduced using the molar absorptivity at 280 nm, which was predicted from the amino acid sequence.

Analytical gel-filtration – Gel-filtration experiments were performed on a HiLoad 16/600 Superdex™ 75 (GE Healthcare) column at a flow-rate of 1 ml/min in a standard buffer: 20 mM HEPES-NaOH (pH 6.7), 125 mM NaCl, 2 mM DTT. Hydrodynamic size (apparent molecular weights) of the protein was derived from its elution volume in comparison with molecular weight standards of a gel-filtration calibration kit, LMW (GE Healthcare).[9,10]

Circular dichroism (CD) spectroscopy – A 0.1-cm path-length cell was used for the CD experiments of CP2c(63-302) in 10 mM sodium phosphate buffer (pH 6.7) containing 25 mM NaCl. Standard far-UV CD spectra of the protein (20 µM) were recorded at room temperature, on a Jasco J-710 spectropolarimeter, with a 1 nm bandwidth and a 4 sec response time. Three individual scans were summed and averaged, followed by the subtraction of blank buffer CD signals. Thermal stability was assessed using an Applied Photophysics Chirascan CD spectrometer equipped with a temperature controller. Temperatures were controlled to gradually increase at a rate of 1 °C/min from 20 to 80 °C, while the CD signals at 222 nm were recorded at every 0.2 °C, with a 1 nm bandwidth.

Nuclear magnetic resonance (NMR) spectroscopy – Conventional 2D-[^{1}H/^{15}N]-TROSY spectra were obtained with the [^{15}N]-enriched CP2(63-302) (250 µM), which was produced by cultivating the transformed *E. coli* cells in M9 minimal media supplemented with [^{15}N]NH_4Cl as a sole nitrogen source. The NMR measurements were performed on a Bruker Biospin Avance II 800 spectrometer equipped with a cryoprobe, at 298 K. The NMR buffer contained 20 mM HEPES-NaOH (pH 6.7), 125 mM NaCl, 2mM DTT, and 7% D_2O. Stock solutions of zinc chloride and a dodecapeptide (sequence: N-acetyl-HKFHQHRLPHLA-amide)[2,7] dissolved in the NMR buffer were used for titration experiments. The NMR spectra processed using NMRPipe[11] software were visualized and analyzed using NMRViewJ[12] program.

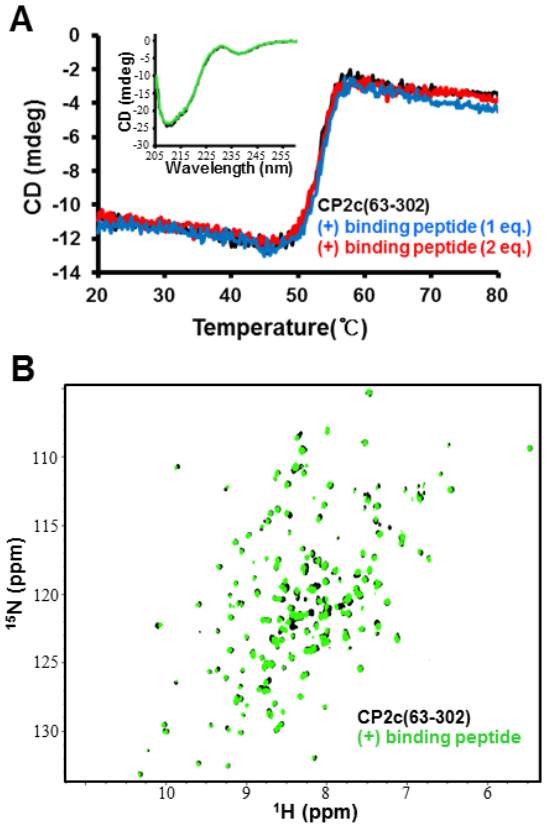

Figure 2. Examination of a dodecapeptide binding to CP2c(63-302). (A) Thermal denaturation of CP2c(63-302) (20 µM), monitored by CD at 222 nm, in the absence (black) and presence of the binding peptide (blue and red for 1 and 2 equivalent titration, respectively). Inset shows standard far-UV CD spectra in the absence (black) and presence (green) of 2 equivalents of the peptide. (B) 2D-[^{1}H/^{15}N]-TROSY NMR spectra of [^{15}N]CP2c(63-302) (250 µM), in the absence (black) and presence of 2 equivalents of the binding peptide (green).

Results

Structural characterization – Like the previous construct, Elf1D (CP2c residues 63-244),[7] the present CP2c(63-302) protein was well expressed as a

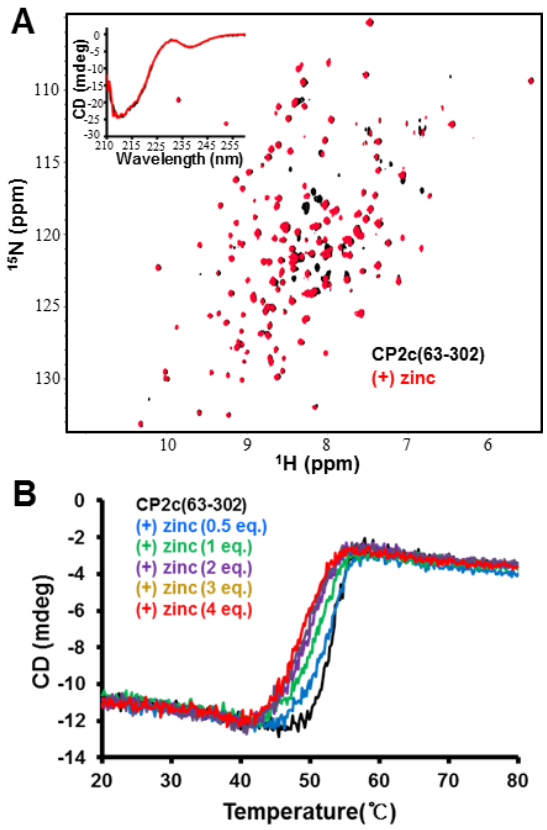

Figure 3. Examination of zinc binding to CP2c(63-302). (A) 2D-[^{1}H/^{15}N]-TROSY NMR spectra of [^{15}N]CP2c(63-302) (250 μM), in the absence (black) and presence of 2 equivalents of zinc (red). Inset shows standard far-UV CD spectra in the absence (black) and presence (red) of 2 equivalents of zinc. (B) Thermal denaturation of CP2c(63-302) (20 μM), monitored by CD at 222 nm, in the absence (black) and presence of zinc (molar ratios are indicated for individual colors).

soluble form in our expression system established. However, unlike Elf1D that showed a poor stability readily precipitating after purification, the purified CP2c(63-302) was stable without precipitation during storage. Furthermore, gel-filtration profile of the purified CP2c(63-302) showed a single species of eluate (Fig. 1B), which was distinctive from the two elution peaks previously observed for Elf1D. The hydrodynamic size of CP2c(63-302) deduced from its elution volume was approximately 27.4 kDa, which was broadly consistent with the theoretical molecular weight (27.8 kDa) of the protein (Fig. 1B). Therefore, the recombinant CP2c(63-302) protein could be considered to behave in solution as a monomer with a homogeneous conformation, whereas conformational equilibrium previously observed for the isolated Elf1D might not be practical in intact CP2c.

Far-UV CD spectrum of CP2c(63-302) showed a somewhat unusual trace characterized by a negative minimum at 210 nm and a distinctive negative band centered at approximately 240 nm (inset in Fig. 2A), of which reliable curve fitting could not be obtained using conventional software analyzing secondary structure contents (data not shown). Therefore, significant portion of irregular folds such as unstructured and/or diverse turn regions were presumable in the overall conformation of CP2c(63-302), although the CD signals in lower-wavelength region could not be ensured due to poor reproducibility; *i.e.*, CD signals at below 205 nm entailed severe noises, probably due to the UV light scattering. However, at the level of tertiary structure, a globular conformation could be presumable for the isolated CP2c(63-302), given that the hydrodynamic size estimated by gel-filtration was consistent with the theoretical value for monomeric state. The 2D [^{1}H/^{15}N]-TROSY NMR spectrum of CP2c(63-302) also supported that the protein structure contained an appreciable portion of well-ordered region, by showing an apparently well-dispersed resonances (Fig. 2B).

Finally, binding of a dodecapeptide that is known to bind at the middle region of CP2c(63-302) (Fig. 1A) was examined. The CD spectrum of CP2c(63-302) was not perturbed in the presence of the peptide (inset in Fig. 2A), indicating no significant alteration of the protein at least at the level of secondary structure. In addition, the stability of CP2c(63-302) was unlikely affected by the addition of the peptide, as indicated by the thermal denaturation profiles of the protein (Fig. 2A). However, the NMR spectrum of CP2c(63-302) showed clear perturbation (*i.e.* chemical shift change and/or line broadening) of some specific resonances upon the addition of the peptide (Fig. 2B). Therefore, it could be reasonably concluded that the peptide specifically bound at

certain site of the protein. In turn, the observed specific binding of the peptide supported that the isolated CP2c(63-302) likely adopted a native fold as in intact CP2c.

Characterization of zinc binding – As our previous investigation has unexpectedly found that zinc ion can bind to the isolated Elf1D,[7] we also examined the zinc binding of CP2c(63-302). Although no significant alteration was observed in the far-UV CD spectrum (inset in Fig. 3A), limited number of resonances in the NMR spectrum of CP2c(63-302) was significantly perturbed upon the addition of zinc (Fig. 3B), thereby confirming that zinc ions specifically bound at certain sites of the protein. Interestingly, however, addition of zinc more than two equivalents of zinc to the NMR sample (250 μM) induced severe precipitation of the protein (data not shown). Accordingly, we examined the effect of zinc binding on the protein stability by CD spectroscopy. Under the CD experimental condition employing low concentration (20 μM) of the protein, no detectable precipitation occurred in the presence of zinc up to four equivalents. However, denaturing temperature of CP2c(63-302) was gradually decreased with increasing concentrations of zinc. This observation not only confirmed that zin ion can bind to CP2c(63-302) but also indicated that the zinc binding decreases the protein stability.

Discussion

Our initial approach to the CP2c structure previously paid attention to the Elf1D region (residues 63-244),[7] as it has been appreciated to be its putative DBD.[2] However, recent crystal structure of the DBD of a CP2c homologue, Grhl1, indicated that a longer region might be required for independent folding into a stabilized domain structure.[8] Finally, the present results suggests that the Elf1D for itself does not fold into a structurally stabilized domain, but alternatively the 63-302 region can serve as a structural domain that is stabilized as a single homogeneous conformation. In addition, based on the present results, the overall conformation of CP2c(63-302) is expected to resemble that of the Grhl1 DBD, of which globular conformation contains a significant portion of unstructured or irregular folds,[8] although their C-terminal parts (residues 245-301 for CP2c) share no significant sequence homology. Unfortunately, as a proper oligomerization is also essential for DNA binding of intact CP2c,[13-15] it is quite tricky at present to certify that the isolated CP2c(63-302) adopted a functional structure compatible with DNA binding. However, as the specific binding of a known dodecapeptide was corroborated by NMR (Fig. 2B), we postulate that the CP2c(63-302) protein could adopt a native fold as in its intact form.

Although it has not been reported that metal ions are involved in the function of CP2c, the present results verified that the putative DBD of CP2c can bind zinc ions. Meanwhile, recent crystal structure revealed that the Grhl1 DBD shares a structural homology with the DBD of the tumor suppressor p53.[8] Therefore, given the sequence homology in DBD between Grhl1 and CP2c, it is reasonably postulated that the CP2c DBD also resembled the p53 DBD. Although zinc ion was not involved in the DNA binding of Grhl1,[8] the functional p53 tetramer coordinates a single Zn^{2+} in each of its four identical subunits at a loop region of DBD.[16] Therefore, the observed zinc binding of CP2c(63-302) suggests that the zinc binding might be also involved in regulation of CP2c. The bound zinc in p53 DBD plays an important role in its DNA-binding activity and conformational stability. In contrast, it is unlikely that CP2c requires zinc for its DNA binding, since the zinc binding to CP2c(63-302) resulted in reduced stability of the protein. However, it is also known that improper binding of excess zinc promotes misfolding of the p53 DBD, which can be associated with development of cancer.[16] Therefore, the present results that showed zinc-induced destabilization of CP2c(63-302) suggest that the zinc-induced misfolding of CP2c might also be physiologically relevant. Collectively, our results strongly suggest that the functional and/or conformational regulation of CP2c by zinc ion would be worthy of in-depth

investigation.

Acknowledgements

This study was supported by the National Research Foundation (NRF) (grant no. 2016R1A2B4009700). The use of NMR and CD machines was supported by the Korea Basic Science Institute (Ochang, Korea) under the R&D program (project No. D38700) supervised by the Ministry of Science and ICT.

References

1. K. M. Barnhart, C. G. Kim, S. S. Banerji, and M. Sheffery, *Mol. Cell Biol.* **8**, 3215 (1988)
2. H. C. Kang, B. M. Chung, J. H. Chae, S.-I. Yang, C. G. Kim, and C.-G. Kim, *FEBS J*. **271**, 1265 (2005)
3. K.-S. Jo, H.-R. Jo, C. G. Kim, C.-G. Kim, and H.-S. Won, *J. Kor. Magn. Reson. Soc*. **18**, 30 (2014)
4. B. K. Yoo, L. Emdad, R. Gredler, C. Fuller, C. I. Dumur, K. H. Jones, C. Jackson-Cook, Z. Su, D. Chen, U. H. Saxena, U. Hansen, P. B. Fisher, and D. Sarkar, *Proc. Natl. Acad. Sci. USA* **107**, 8357 (2010)
5. T. J. Grant, J. A. Bishop, L. M. Christadore, G. Barot, H. G. Chin, S. Woodson, J. Kavouris, A. Siddiq, R. Gredler, X.-N. Shen, J. Sherman, T. Meehan, K. Fitzgerald, S. Pradhan, L. A. Briggs, W. H. Andrews, D. Sarkar, S. E. Schaus, and U. Hansen, *Proc. Natl. Acad. Sci. USA* **109**, 4503 (2012)
6. P. K. Santhekadur, D. Rajasekaran, A. Siddiq, R. Gredler, D. Chen, S. E. Schaus, U. Hansen, P. B. Fisher, and D. Sarkar, *Am. J. Cancer Res.* **2**, 269 (2012)
7. K.-S. Jo, K.-S. Ryu, H.-W. Yu, S.-N. Lee, J.-H. Kim, E.-H. Kim, C.-Y. Wang, C.-G. Kim, C. G. Kim, and H.-S. Won, *J. Kor. Magn. Reson. Soc*. **22**, 119 (2018)
8. Q. Ming, Y. Roske, A. Schuetz, K. Walentin, I. Ibraimi, K. M. Schmidt-Ott, and U. Heinemann, *Nucleic Acids Res.* **46**, 2082 (2018)
9. K.-S. Jo, D.-W. Sim, E.-H. Kim, D.-H. Kang, Y.-B. Ma, J.-H. Kim, and H.-S. Won, *J. Kor. Magn. Reson. Soc.* **22**, 64 (2018)
10. Y.-S. Lee, J. Lee, K.-S. Ryu, Y. Lee, T.-G. Jung, J.-H. Jang, D.-W. Sim, E.-H. Kim, M.-D. Seo, K.W. Lee, and H.-S. Won, *J. Mol. Biol.* **427**, 3850 (2015)
11. F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, and A. Bax, *J. Biomol. NMR* **6**, 277 (1995)
12. B. A. Johnson, *Methods Mol. Biol.* **278**, 313 (2004)
13. H. C. Kang, J. H. Chae, Y. H. Lee, M.-A. Park, J. H. Shin, S.-H. Kim, S.-K. Ye, Y. S. Cho, S. Fiering, and C. G. Kim, *Mol. Cell. Biol.* **25**, 600. (2005)
14. H. C. Kang, J. H. Chae, K. S. Choi, J. H. Shin, C. G. Kim, and C.-G. Kim, *Nucleic Acids Res.* **38**, 5456 (2010)
15. U. Hansen, L. Owens, and U. H. Saxena, *Cell Cycle* **8**, 2146 (2009)
16. S. N. Loh, *Metallomics* **2**, 442 (2010)