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Intrinsically disordered fold of a PIAS1–binding domain of CP2b

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Abstract The transcription factor CP2 regulates various biological systems at diverse tissues and cells. However, none of the four CP2 isoforms has been solved in structure yet. In particular, two different regions of the CP2b isoform have been characterized to interact with the PIAS1 in nucleus to regulate the α-globin gene expression. Among them, in this study, the region encompassing residues 251-309 of CP2b was prepared as a recombinant protein and its solution structure was characterized by NMR spectroscopy. The results indicated that the CP2b(251-309) fold belongs to typical IDRs (intrinsically disordered regions), likely to facilitate promiscuous interactions with various target proteins. Unfortunately, however, its interaction with the N-terminal domain of PIAS1 (residues 1-70), which has been identified as one of the CP2b-binding sites, was not observed in the NMR-based titration experiments. Therefore, it could be postulated that the 251-309 region of CP2b would not contact with the PIAS1(1-70), but alternatively interact with another CP2b-binding region that encompasses residues 400-651 of PIAS1.

Keywords CP2, PIAS1, IDR, NMR

Introduction

The transcription factor, CP2 (CCAAT-binding protein 2) protein has been revealed to be ubiquitously expressed at diverse tissues and involved in many cellular events, such as hematopoiesis, immune response, cell cycle, and neural development, by regulating expression of specific target genes, including globins, interleukins, and thymidylate synthases.[1-4] Hence, the multifunctional aspects of CP2 are necessarily mediated by protein-protein interactions with many other proteins, including RNF2/Ring1b, YY1, Fe65, NF-E4, Sumo-1, TTRAP, REST, PKC, CKII, etc,[1-4] and associated with developments of several fatal human diseases, such as Alzheimer's disease, AIDS, drepanocytosis, and cancer.[1-4] The CP2 protein family consists of four isoforms (CP2a/NF2d9, CP2b, CP2c/CP2, and CRTR) in mouse and six isoforms (LBP-1a, -1b, -1c, -1d, -9, and -32) in human.[1-4] Among them, the CP2c isoform was originally identified as a transcription factor of the murine α-globin gene and its heteromeric complex with another isoform CP2b activates the α-globin expression.[3-6] Recently, it has been suggested that α-globin gene expression is regulated by molecular interactions of the CP2b/CP2c complex with another protein PIAS1 (protein inhibitor of activated STAT1)

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in nucleus, and the intermolecular interaction sites could be broadly mapped onto their amino acid sequences.[3] In particular, two different regions in CP2b encompassing residues 134-251 and 251-309, respectively, were identified as the PIAS1-interacting domains that are able to independently interact with the PIAS1. Likewise, in PIAS1, the N-terminal (residues 1-70) and C-terminal (403-651) regions were identified as independent, CP2b-binding sites. In this context, the present study aimed to initiate the first structural investigation of the CP2-family proteins and their molecular interactions with PIAS1. As such a preliminary attempt, one of the two PIAS1-binding domains of CP2b could be successfully prepared as a recombinant protein and its structural characterization is reported in this paper.

Experimental Methods

Protein preparation- DNA fragments encoding residues 251-309 of CP2b and the 1-70 of PIAS1 were PCR-amplified, respectively, from the previously constructed recombinant plasmids, pEGFPN1-CP2b and pcDNA3-FLAG-PIAS1,[3] as corresponding templates, using appropriate forward and reverse oligonucleotides primers that contained the *Nde*I and *Xho*I restriction sites, respectively. The PCR products were digested with the restriction enzymes and then ligated into the *Nde*I/*Xho*I-digested pET-15b vector for the CP2b(251-309) and pCold-I vector for the PIAS1(1-70), to produce N-terminally 6-histidine tagged proteins. The constructed plasmids, verified via DNA sequencing, was transformed into the E. coli BL21(DE3)pLysS strain. The transformed cells were grown in the M9 minimal medium at 37 °C and, when the A600 of cell growth reached about 0.6, protein expression was induced at 15 °C, by adding IPTG at a final concentration of 0.5 mM. In order to prepare the [^{15}N]-enriched CP2b(251-309) for NMR, the M9 minimal medium was supplemented with [^{15}N]NH^4Cl as a sole nitrogen source. The induction was prolonged for 12 hrs and the cells were harvested by centrifugation, followed by disruption by sonication. From the supernatants, the expressed target proteins were purified via sequential applications of nickel-affinity chromatography (HisTrap FF column, GE Healthcare), ion-exchange chromatography (HiTrap Q-Sepharose FF, GE Healthcare), and gel-permeation chromatography (HiLoad 16/60 Superdex 75, Pharmacia). The tagged histidines were then cleaved with corresponding proteases, thrombin for the CP2b(251-309) and factor Xa for the PIAS1(1-70), followed by the removal of the proteases and other impurities via the sequential application of nickel-affinity and gel-permeation chromatography again. Finally, the purified solution was concentrated and buffer-exchanged by ultrafiltration (Amicon). Protein concentration was estimated via typical Bradford and BCA assays.

Gel-filtration analysis- Purified CP2b(251-309) and molecular weight standards were applied on a HiLoad 16/60 SuperdexTM 75 (GE Healthcare) gel-permeation column with a total bed volume (v_t) of 120 ml. The column was preequilibrated with the standard buffer (25 mM Tris-HCl containing 150 mM NaCl, at pH 7.5) and run at a 1 ml/min flow rate. The column void volume (v_o) was determined as the elution volume for Blue Dextran 2000. From the elution volume (v_e) of each sample, the gel-phase distribution coefficient (K_{av}) was calculated using the equation: $K_{av} = (v_e-v_o)/(v_t-v_o)$. The standard curve was generated by plotting K_{av} against the logarithm of molecular weight, for standard proteins.[7,8] By fitting the K_{av} of CP2b(251-309) into the standard curve, its apparent molecular weight was estimated.

Circular dichroism (CD) spectroscopy- CD experiments were performed using a Jasco J-710 spectropolarimeter equipped with a temperature-controlling unit. The standard far-UV CD spectra were measured with 20 μM of CP2b(251-309), dissolved in designated buffers at various pH, using a 0.2cm path-length cell, with a 1 nm bandwidth and a 4 sec response time. Three individual scans taken from 260 to 190nm at 20 ºC were summed and averaged, followed by the subtraction of solvent CD signals. Finally, the CD intensity was normalized as

the mean residue molar ellipticity (MRME). For the temperature scan experiment, the wavelength was fixed at either 222 or 216 nm and the signals were recorded at every 0.1 ºC from 20 ºC to 90 ºC, at an increasing rate of 1 ºC/min.

Nuclear magnetic resonance (NMR) spectroscopy- The conventional 2D-[^{1}H/^{15}N]-HSQC spectra of 0.6 mM [^{15}N]-enriched CP2b(251-309), dissolved in the aforementioned standard buffer containing 7% D_2O, were measured at 283 K, 298 K, and 313 K, on a Bruker Biospin Avance II 900 spectrometer equipped with a cryoprobe. To detect the molecular interaction with PIAS1(1-70), the isotopically non-labeled PIAS1(1-70) was added at 1:1 molar ratio to the [^{15}N]CP2b(251-309). Chemical shifts were directly referenced to DSS for the ^{1}H and indirectly for the ^{15}N atom, using the chemical shift ratio value suggested in the BMRB (http://www.bmrb.wisc.edu).

Results and Discussion

Based on the amino acid sequence, the molecular mass of the purified CP2(251-309) after cleavage of the attached His-tag was expected as approximately 7.1 kDa. However, in the gel-filtration analysis, the elution volume of the purified CP2(251-309) indicated an apparent molecular weight of 14.2 kDa, which was quite higher than the size detected in the SDS-PAGE (Fig. 1). This result could indicate a dimeric state of the purified CP2(251-309), if the protein adopted a well-folded, globular conformation. However, a dynamic light scattering (DLS) analysis, which is also dependent on the molecular shape in estimation of molecular weight, showed a different result that indicated approximately 33 kDa with the same protein sample (data not shown). This inconsistency between the gel-filtration and DLS analysis led us to assume a non-globular shape of the purified CP2(251-309). Then, the far-UV CD spectra clearly indicated a greatly disordered conformation of the protein, by showing a negative band centered at around 200 nm, commonly at different pH tested (Fig. 2A). Just modest contents of ordered conformation

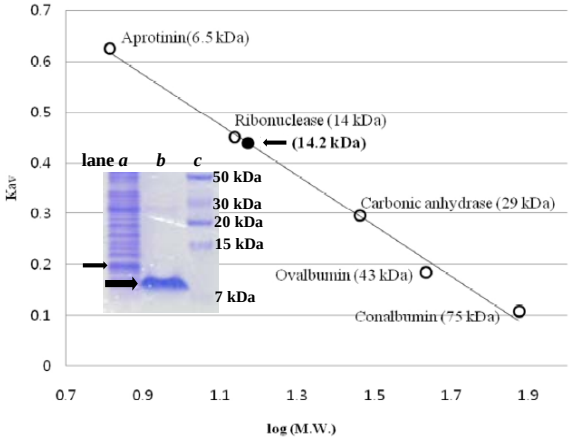

Figure 1. Apparent molecular weight of the recombinant CP2(251-309), as estimated by gel-filtration analysis. Open circles indicate the protein standards used, while the filled circle designates the purified CP2(251-309). In the inset, SDS-PAGE result shows molecular sizes (refer to markers in lane *c*) of the over-expressed CP2(251-309) with N-terminal His-tag (arrow in lane *a*) and the finally purified CP2(251-309) with cleavage of the His-tag.

could be estimated from the negative signals around 220 nm, which were slightly attenuated upon increasing salt concentration (Fig. 2B). Finally, the 2D-[^{1}H/^{15}N]-HSQC spectra of CP2(251-309) also corroborated the intrinsically disordered conformation of the protein, by showing a narrow spectral dispersion with sharp linewidths of individual peaks (Fig. 3). Collectively, all of the present results support that the 251-309 region of CP2b would resemble a typical IDR (intrinsically disordered region).[9-13] As represented by the TAD (transactivation domain) regions of the transcription factor P53, the IDRs are found in many proteins that function by promiscuous protein-protein interactions. Thus, the intrinsically disordered or unfolded properties of the CP2 proteins might be associated with their multifunctional aspects that are mediated by protein-protein interactions with various partners.

The CD spectra of CP2b(251-309) showed a very intriguing fluctuation upon increasing temperature (Fig. 4). The spectral change upon increasing temperature is represented by the signal intensification around 220 nm and concomitant reduction near 200 nm (Fig. 4A), which is indicative

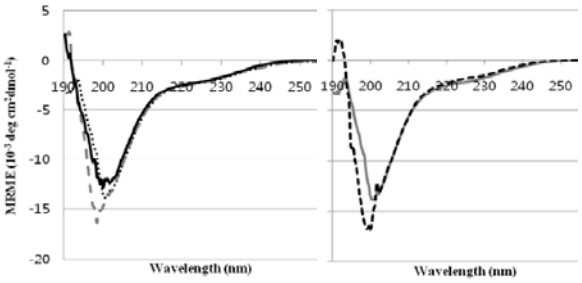

Figure 2. Far UV CD spectra of CP2(251-309) at various pH (left panel; solid line, pH 5; dashed line, pH 7; dotted line, pH 8) and in the presence of different salt concentrations (right panel; solid line, 50 mM NaCl; dashed line, 150 mM NaCl).

of increased extents of ordered conformation and decreased portion of unstructured region. Thus, this observation is generally unusual in folded proteins, as the changing pattern is interpretable as a heat-induced folding into an ordered conformation, which contradicts thermal denaturation of general proteins. However, it is a usually observed pattern for IUPs (intrinsically unfolded/unstructured proteins).[9-13] In addition, the CD signals gradually changed upon increasing temperature, without showing any midpoint of a cooperative transition (Fig. 4B). Thus, the observed spectral change could be regarded to reflect a certain dynamic equilibrium shift of conformational ensembles, rather than a conformational change. In this regard, comparison of NMR spectra between at different temperatures indicate a certain conformational exchange, of which rate is temperature-dependent. As shown in Fig. 3, the total number of observed peaks in the 2D-[^{1}H/^{15}N]-HSQC spectrum was increased upon decreasing temperature, even up to more signals than expected from the actual number of residues. For instance, the ^{15}N high-field region (around 110 ppm in the spectrum) is generally represented as a specific region where merely the glycine (intermittently including threonine) amide resonances appear.[14,15] In that region, the spectrum measured at a higher

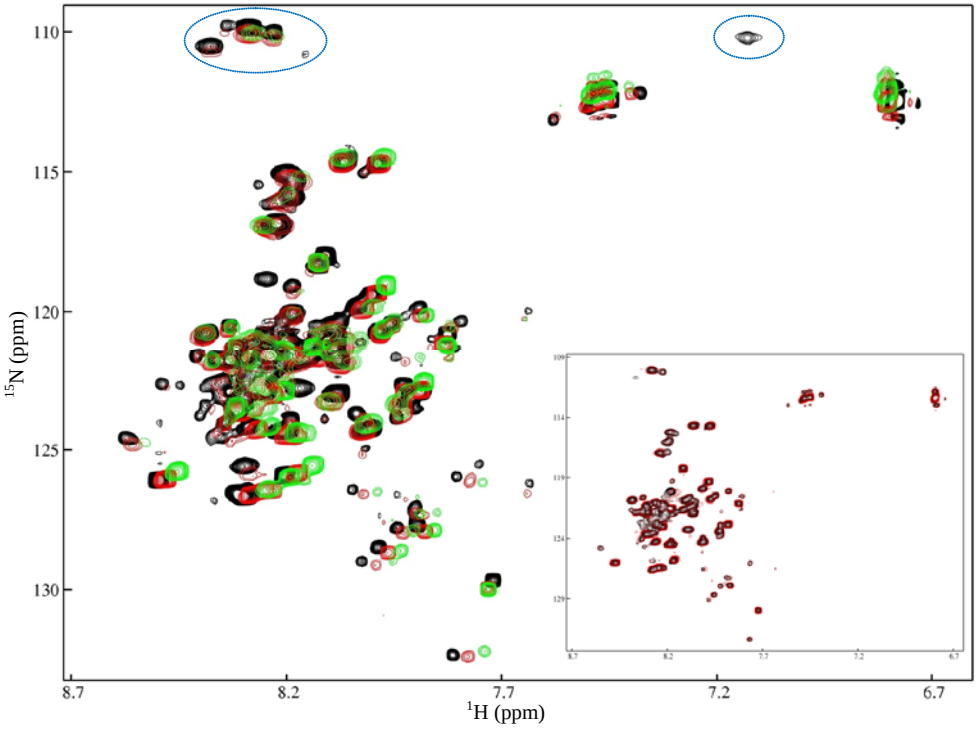

Figure 3. 2D-[^{1}H/^{15}N]-HSQC spectra of CP2b(251-309) at 283 K (black), 298 K (red), and 313 K (green). The resonances originating likely from glycine residues are indicated by blue circles. In the inset, the spectrum the spectra at 298 K, in the absence (black) and in the presence (red) of the equimolar PIAS1(1-70), were superimposed.

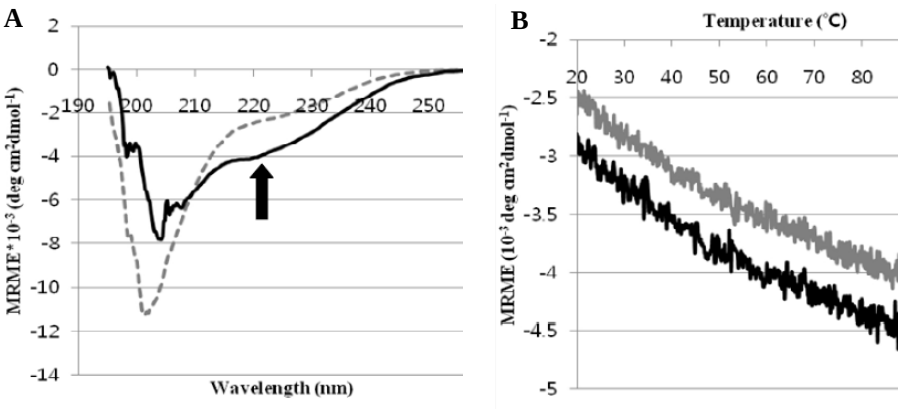

Figure 4. CD spectral change of CP2b(251-309) upon increasing temperature. (A) Comparison of far-UV CD spectra measured at 20°C (dashed line) and 90°C (solid line). (B) Signal intensification at 222 nm (gray) and 216 nm (black) upon increasing temperature.

temperature (313 K; green in Fig. 3) showed up just two resonances, which is consistent with the number of glycine residues contained in the CP2b(251-309). However, the number of glycine resonances was increased upon lowering temperature, and finally at 283K (black in Fig. 3), at least four (up to six) signals were obviously observed. In addition, the intensities of the multiple glycine resonances were distinctively varied between one another and dependent on temperature. All those observations clearly support that the CP2b(251-309) undergoes a conformational exchange, thereby showing multiple resonances at lower temperatures, due to the slowly exchanging rate at an NMR time scale, whereas less peaks at higher temperate, due to the accelerated exchanging rate. Conclusively, the present results verify that the 251-309 region of CP2b could be appreciated as a typical IDR by adopting a mostly unfolded conformation that is also undergoing a conformational exchange. Such a peculiar conformation and molecular dynamics would be thermodynamically favorable for its dynamic interactions with various target partners, by reducing energy barriers to adopt or to select an appropriate conformational ensemble.

Finally, we attempted to monitor the molecular interaction of the CP2b(251-309), by NMR. It has been previously detected by a pull-down assay that the isolated fragments of CP2b encompassing residues 134-251 and 251-309, respectively, directly interact with the full-length PIAS1.[3] Conversely, the isolated 1-70 and 400-651 fragments of PIAS1 could bind the intact CP2b. Thus, it was reasonable to expect an *in vitro* direct interaction of the CP2b(251-309) and/or CP2b(134-251) with the PIAS1(1-70) and/or PIAS1(400-651). Unfortunately, however, obtaining recombinant proteins for the CP2b(134-251) and PIAS1(400-651) was not successful, despite a massive trials to express enough amount of soluble protein samples (data not shown). Thus, we monitored the NMR spectral change of the prepared CP2b(251-309) in the presence of the PIAS1(1-70). Finally, as shown in the inset of Fig. 3, no significant interaction was detected between the two constructs. This result indicates that the 251-309 region of CP2 does not interact with the N-terminal region of PIAS1, but may bind to the C-terminal region of PIAS1. Conversely, the PIAS1(1-70) might be able to interact with the 134-251 region of CP2b, rather than the 251-309 region. Alternatively, the intermolecular interaction between CP2b and PIAS1 may require an intact structure of at least one of them.

In conclusion, the present results provide the first detailed structural characterization of the CP2-family proteins and revealed that the 251-309 region of

CP2b constitutes a typical IDR, probably to facilitate promiscuous interactions with other proteins. Particularly, its known interaction with the PIAS1 is expected to be mediated by the C-terminal region of PIAS1, rather than the N-terminal region. However, convincing evidences for those interactions and structural mode of the interaction remain to be further investigated.

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