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Biophysical characterization of the structural change of Nopp140, an intrinsically disordered protein, in the interaction with CK2 α

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Abstract

Nucleolar phosphoprotein 140 (Nopp140) is a nucleolar protein, more than 80% of which is disordered. Previous studies have shown that the C-terminal region of Nopp140 (residues 568–596) interacts with protein kinase CK2 α , and inhibits the catalytic activity of CK2. Although the region of Nopp140 responsible for the interaction with CK2 α was identified, the structural features and the effect of this interaction on the structure of Nopp140 have not been defined due to the difficulty of structural characterization of disordered protein. In this study, the disordered feature of Nopp140 and the effect of CK2 α on the structure of Nopp140 were examined using single-molecule fluorescence resonance energy transfer (smFRET) and electron paramagnetic resonance (EPR). The interaction with CK2 α was increased conformational rigidity of the CK2 α -interacting region of Nopp140 (Nopp140C), suggesting that the disordered and flexible conformation of Nopp140C became more rigid conformation as it binds to CK2 α . In addition, site specific spin labeling and EPR analysis confirmed that the residues 574–589 of Nopp140 are critical for binding to CK2 α . Similar technical approaches can be applied to analyze the conformational changes in other IDPs during their interactions with binding partners.

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Conflicts of interest

The authors declare no conflict of interest.

Keywords

Intrinsically disordered protein; Single-molecule FRET; Nopp140; Casein kinase 2; Electron paramagnetic resonance; Structural diversity

1. Introduction

Nucleolar phosphoprotein p140 (Nopp140) is a nucleolar protein of mammalian cells that contains 710 amino acids and is involved in nucleolus biogenesis during cell division. It interacts with a variety of proteins, such as protein kinase CK2 alpha subunit (CK2 α), RNA polymerase I, p80 coilin, NAP57 and snRNPs that are involved in the synthesis and assembly of ribosomes [1]. The cellular function of Nopp140 has been studied using gene silencing and the interaction partners have been identified, but efforts to determine the 3-dimensional structure of Nopp140 have not been extensively reported. Sequence analysis of Nopp140 suggest that Nopp140 has a distinct structural feature: more than 80% of the sequence was predicted to be composed of random coil [2]. Extensive sequence analysis of Nopp140 using several disordered region prediction algorithms indicated that Nopp140 is one of the intrinsically disordered proteins (IDPs) that consist mostly of disordered structure [1]. The high sensitivity of Nopp140 to proteases, random coil features found in circular dichroism (CD) spectra, and its hydrodynamic properties further confirmed that Nopp140 is a member of the IDPs [2].

Nopp140 has a unique characteristic among IDPs in which majority of the sequences are disordered and lacks a well-folded domain. It contains only a few short stretches of potential helix that are located at the N- and C-termini (Fig. 1A). So far, only a small number of IDPs that consist almost exclusively disordered regions have been reported. One of these, U1-70k, has the disordered regions that represent 60% of the whole sequence. Crystal structures of the protein complex containing U1-70k showed that U1-70k wraps around the core domain of U1 snRNP and interacts with several components of U1 snRNP particle [3]. The structure of this complex demonstrated how an IDP interacts with several protein components, stabilizes the complex structure, and its own disordered regions.

The interaction between Nopp140 and the catalytic subunit of CK2 (CK2 α) has been extensively studied. The kinase active of CK2 is suppressed by several cellular factor(s), and this suppression is relieved by IP₆ [4]. Nopp140 was shown to be one of the cellular suppressors of CK2 α , and the C-terminal region of Nopp140 (residues 568–596) contained the binding site for CK2 α [5]. Structural analysis of CK2 α and IP₆, together with mutational studies, further confirmed the competitive interactions between Nopp140 and IP₆ for CK2 α [5]. Although the interaction site of Nopp140 to CK2 α was identified, the structural features that were involved, and the effect of the interaction on the overall structure of Nopp140 still remain unknown.

The dynamic motion of IDP has been studied using spectroscopic methods such as nuclear magnetic resonance (NMR) and EPR. These methods can determine the overall flexibility of a region of interest [6–8]. Moreover, single-molecule fluorescence resonance energy transfer (smFRET) between two fluorescence probes labeled at different positions of α -synuclein

had been successfully applied to determine the conformational changes in the protein under different environments [9]. In this study, we examined the changes in conformation and flexibility of Nopp140 during its interaction with CK2 α using smFRET analysis, and examined the precise CK2 α binding region of Nopp140 using the EPR method.

2. Materials and methods

2.1. Preparation of proteins

For the preparation of the Nopp140 C-terminal regions expression vector, the DNA fragments expressing the C-terminus of Nopp140 (residues 352–704, Nopp140C) was amplified by polymerase chain reaction using primers containing restriction enzyme sites for *Bam*HI and *Hind*III. The amplified DNA fragments were inserted into pET-28a (Merck Biosciences, Germany), which was designed to express Nopp140Cs with an N-terminus His₆-tag sequence. Based on the Nopp140 vector, single (Nopp140C-S568C, -S589C and -S704C, respectively) and double cysteine mutants (Nopp140C-S352C/S467C, -S352C/S589C, -S352C/G660C, -S352C/S704C, respectively) were made using QuickChange® site-directed mutagenesis kit (Stratagene, USA). The Nopp140Cs were expressed and purified as described previously [1]. The CK2 α gene (encoding amino acids 1–335) was amplified by the PCR. The forward and reverse oligonucleotide primers were: 5'-G CTA TAT **CAT ATG** TCG GGA CCC GTG CCA AGC AG-3' and 5'-G CTA ATT **GGA TCC** TCA ACC CAT TCG AGC CTG GTC CTT CAC-3', respectively. The bases in bold represent the *Nde*I and *Bam*HI cleavage sites. The amplified DNA was digested with *Nde*I and *Bam*HI and was then inserted into the *Nde*I/*Bam*HI digested expression vector pET-21a(+) (Novagen, USA). The plasmid was transformed into *Escherichia coli* BL21(DE3) star cells for protein expression. The cells were grown at 37 °C to an OD_{600nm} of 0.5 in Luria–Bertani medium containing 100 µg/mL ampicillin, and protein expression was induced by 0.5 mM isopropyl- β -D-thiogalactopyranoside. The cells were grown at 18 °C for 12 h after isopropyl- β -D-thiogalactopyranoside induction and were harvested by centrifugation at $4611 \times g$ (V10004A rotor; Vision) for 30 min at 4 °C. The cell pellet was resuspended in ice-cold CK2 buffer (20 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM β -mercaptoethanol) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and was homogenized with an ultrasonic processor and a microfluidizer (M-110P, Microfluidics). The crude cell extract was centrifuged at $15,777 \times g$ (V506CA rotor; Vision) for 30 min at 4 °C. The supernatant was loaded onto a HiTrap 5-mL Heparin HP column (GE Healthcare, USA), which was previously equilibrated with CK2 buffer, and the protein was eluted using a linear gradient of 0.3–1 M NaCl in CK2 buffer. Further purification was performed by gel filtration on a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with CK2 buffer. The homogeneity of the purified protein was assessed by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% (wt/vol) SDS. The protein solution was concentrated to approximately 14.1 mg/mL using a centrifugal filter unit (Sartorius Stedim, USA). The protein concentration of CK2 α was estimated by measuring the absorbance at 280 nm, using the calculated molar extinction coefficient (ϵ) of $58,130 \text{ cm}^{-1} \text{ M}^{-1}$.

2.2. Labeling of Nopp140Cs for FRET

The purified double-cysteine mutants of Nopp140Cs were purified further by gel filtration in a Sephadex G-25 column (GE healthcare, USA) using dye labeling buffer (25 mM Tris-Cl, pH 7.4; 200 mM NaCl; 10 mM TCEP) and then concentrated to 100 μ M. A mixture of 1:2.5 (Cy3: Cy5, molar ratio) maleimide-conjugated Cy3/Cy5 pair (GE healthcare, USA) was added in a 20-fold excess. The reaction mixtures were incubated for 16 h at 4 °C with protection from light. The labeled proteins were isolated by gel filtration in a Sephadex G-25 column using FRET buffer (25 mM Tris-Cl, pH 7.4; 200 mM NaCl; 1 mM DTT). Labeling efficiencies were determined by measuring the absorbance of the sample at 280, 550, 647 nm and calculating the concentration of Nopp140 ($\epsilon = 6970 \text{ cm}^{-1} \text{ M}^{-1}$), Cy3 ($\epsilon = 150,000 \text{ cm}^{-1} \text{ M}^{-1}$) and Cy5 ($\epsilon = 250,000 \text{ cm}^{-1} \text{ M}^{-1}$) in the sample (1 cm light path) using the Beer-Lambert Law. The concentration of Nopp140 was corrected in the Beer-Lambert equation by subtracting the absorbance of Cy3 (8% of the absorbance at 550 nm) and Cy5 (5% of the absorbance at 647 nm). Overall labeling efficiency was around 90% and the ratio of Cy3 and Cy5 in Nopp140Cs were estimated as 1:0.8.

2.3. Ensemble FRET measurements and analysis

The emission spectra of 10 μ M samples of double-labeled Nopp140Cs (Nopp140C-S352C/S467C, -S352C/S589C, -S352C/G660C, and -S352C/S704C, respectively) were measured by FluoroMate FS-2 (Scinco, Korea) with excitation wavelength 550 nm, and emission within the range 560–700 nm. For CK2 α -treated samples, 100-fold molar excess of CK2 α was pre-incubated with Nopp140C. All measurements of ensemble FRET experiments were carried out in an ensemble FRET buffer (50 mM Tris-Cl, pH 7.4; 250 mM NaCl) at 25 °C.

2.4. smFRET measurements and analysis

The Nopp140C-S352C/S589C labeled with Cy3 and Cy5 in FRET buffer were immobilized onto a flow cell, in which a quartz slide was functionalized with PEG and PEG-biotin (Laysan Bio, 40:1). For immobilization of the His₆-tagged Nopp140C to the surface, the biotin-X-NTA (Anaspec, USA) in FRET buffer with Ni²⁺ was attached through streptavidin to the PEG surface for 10 min in advance and the free biotin-X-NTA was washed out using a 10-fold excess volume of the FRET buffer. After incubation of 50 nM His₆-tagged Nopp140C labeled with Cy3 and Cy5 to the surface for 10 min, free Nopp140 proteins were also washed out. For CK2 α -treated samples, 100-fold molar excess of CK2 α was pre-incubated with Nopp140C. All measurements of single molecule experiments were carried out in smFRET buffer (20 mM Tris-Cl, pH 7.4; 250 mM NaCl) containing an oxygen scavenging system (2 mM Trolox (Sigma, USA), 0.8% (w/v) D-glucose (Sigma, USA), 165 U/ml glucose oxidase (Sigma, USA), 2170 U/ml catalase (Sigma, USA) [10]. The single molecule signals of donor (Cy3) and acceptor (Cy5) were acquired in a prism-type total internal reflection fluorescence (TIRF) microscope (Nikon, Ti-U, water-type 60 $\times$ objective, NA = 1.2) by an electron multiplying charged-coupled device (Andor iXon EM +897) at 50 ms time resolution. The signals were filtered through 532 nm and 633 nm notch filters (NF03-532E-25 and NF01-633U-25; Semrock) and separated using DV2 (Photometrics, with T660lpxr, Chroma Technology). For collecting the signals from Nopp140 labeled with both Cy3 and Cy5, an alternative excitation of Cy3 and Cy5 was performed using a 532 and

633 nm laser, respectively, with a mechanical shutter (UniBlitz). The donor (I_D) and the acceptor (I_A) intensity were corrected for cross-talk between the donor and acceptor channels and the background, and then FRET efficiencies were calculated as the ratio of I_D to $I_D + I_A$ [10].

2.5. EPR spectroscopy

To investigate the CK2 binding region of Nopp140, the site-directed spin labeling (SDSL) approach of EPR was used. The purified single-cysteine mutants pf Nopp140Cs were purified further by gel filtration in a Sephadex G-25 column (GE healthcare, USA) using EPR buffer (25 mM Tris-Cl, pH 7.4; 200 mM NaCl) and concentrated to 100 μ M. MTSSL (Toronto Research Chemical, Canada) was added in 20-fold molar excess and the reaction mixtures were incubated for 16 h at 4 °C. The labeled proteins were isolated by gel filtration in a Sephadex G-25 column using EPR buffer. The spin labeling efficiency was determined as described using 3-carboxyproxyl (Sigma, USA) as a standard [11]. Overall labeling efficiency was around 40%. All EPR spectra were collected using EMXplus (Bruker, Germany).

3. Results & discussion

The C-terminal half of Nopp140 (residues 352–704, Nopp140C) consists of mostly disordered structure and contains the CK2 α -binding site. Due to the absence of cysteine residues and a higher stability of this fragment as compared to the full-length Nopp140, Nopp140C was considered appropriate for site-specific labeling of fluorescence or spin probes. For spin-labeling purposes, a single cysteine residue was introduced at G568, S589 or S704 of Nopp140C (Nopp140C-G568C, -S589C, or -S704C, respectively). In addition, two cysteine residues were introduced at S352/S467, S352/S589, S352/G660, or S352/S704 of Nopp140C (Nopp140CS352C/S467C, -S352C/S589C, -S352C/G660C, and -S352C/S704C, respectively) for the preparation of Cy3 and Cy5-labeled versions of the proteins (Fig. 1A). The purity of Nopp140Cs labeled with spin or fluorescence probes was confirmed by SDS-PAGE (Fig. 1B and C). The double cysteine mutants were reacted with Cy3- and Cy5-maleimide, and the labeled proteins were recovered by size-exclusion chromatography. The proper labeling of Nopp140C with Cy3- and Cy5-probes was confirmed by fluorescence imaging as well as absorption spectrum analysis (Fig. 1C and D). Based on the intensity at 280 nm, 550 nm, and 647 nm, it can be confirmed that about 90% of the Nopp140Cs were double-labeled with these dyes, and the ratio of Cy3 and Cy5 in Nopp140C was estimated to be 1:0.8.

To examine the effect of CK2 α on the relative distance between the two dyes in Nopp140C, the fluorescence spectra of the double labeled Nopp140Cs were measured in the presence or absence of CK2 α . With excitation at 550 nm, a peak at 660 nm was detected using ensemble FRET in all tested proteins (Fig. 2). The FRET intensities of Nopp140Cs decreased in the order of Nopp140C-S532C/S589C, Nopp140C-S352C/S467C, Nopp140C-S352C/G660C, and Nopp140C-S352C/S704C, indicating that the putative distances between the labeled positions are not proportional to the number of amino acids between the labeled positions. When CK2 α bound to Nopp140Cs, the FRET intensity of Nopp140C-S532C/S589C

significantly decreased (Fig. 2B), whereas the FRET intensities from other positions of Nopp140C showed only marginal changes (Fig. 2A, C, D).

Although the ensemble FRET spectra of Nopp140C-S532C/S589C showed a notable change upon binding to CK2 α , the results provided only limited information. The reduced FRET intensity may be caused by an increased distance between two dyes or by a change in FRET distribution. To further analyze the FRET changes of Nopp140C-S532C/S589C, the FRET signals from individual Nopp140C-S532C/S589C proteins were measured using a total internal reflection fluorescence (TIRF) method. About 3000 single-molecule fluorescence signals of Nopp140C-S532C/S589C were collected using a prism-type TIRF microscope with 50 ms time resolution in the presence of an oxygen scavenging system [10]. The signals of double-labeled Nopp140C-S532C/S589C proteins were obtained from traces of fluorescence intensity and FRET efficiency in the presence of surface immobilized double-labeled Nopp140C-532/589C (Fig. S1). The FRET histogram of double-labeled Nopp140C-S532C/589C plotted with counts versus FRET efficiency (E) showed two Gaussian distributions, with FRET-peaks centered on 0.03 and 0.45 E (Fig. 3A), suggesting that Nopp140C has at least 2 conformations in its native condition. In the presence of 6 M guanidine hydrochloride (GdmCl), the entire middle-FRET population shifted to the low-FRET efficiency population (Fig. 3E), indicating that the middle-FRET states of Nopp140C-S532C/S589C became extended into a conformation in which the distance between the dyes was larger than that in the native condition. In the presence of CK2 α , 33% of the middle-FRET population shifted to low-FRET (Fig. 3C), which was consistent with to the ensemble FRET pattern (Fig. 2B), suggesting that the Nopp140C of the middle-FRET population underwent a conformational change in which the distance between the labeled positions increased during interaction with CK2 α .

We further examined the flexibility of Nopp140C using a motion sensitive EPR spectrum of spin-labels. To label Nopp140Cs with a thiol reactive methanethiosulfonate spin label (MTSSL), three positions of each Nopp140Cs (aa568, 589, 704) were substituted with cysteine (Fig. 1A) and labeled with MTSSL. The homogeneity of the single-cysteine substituted Nopp140Cs and the spin-labeled Nopp140Cs was confirmed by SDS-PAGE (Fig. 1B). All of the EPR spectra of these spin-labeled Nopp140Cs showed narrow peaks that reflected very fast motion of the nitroxide groups (Fig. 4). These results implied that all three of the positions of Nopp140 were in disordered and flexible states, which is consistent with the bioinformatics data (Fig. 1A). In the presence of CK2 α , the EPR spectrum of Nopp140C-S589C (Fig. 4B, red line) became slightly broader than S589C without CK2 α (Fig. 4B, black line), indicating that the tumbling motion at amino acid 589 was noticeably reduced. In contrast, Nopp140C-G568C and -S704C (Fig. 4A, C) showed almost identical peak shapes in the absence or presence of CK2 α . Biochemical and structural studies of the interaction between CK2 α and Nopp140 showed that the 568–596 region of Nopp140 competed with IP₆ for binding to the substrate binding site of CK2 α [5]. Hence, the reduced motion at amino acid 589 of Nopp140C might be due to the interaction with CK2 α .

4. Conclusion

There are several examples of IDPs that have been analyzed by smFRET. Double-labeled prion protein, the adenovirus early region 1A (EA1), and α -synuclein [9,12–14] showed one Gaussian peak of FRET efficiency, suggesting that their structural fluctuations occurred faster than the detection timescale. In contrast, I κ B α and CTD2 showed several Gaussian peaks of FRET efficiency [15,16], indicating that these IDPs have locally constrained conformations, or their conformational fluctuation is slower than the detection timescale. The FRET efficiency of Nopp140 showed two Gaussian distributions with FRET-peaks centered on E_{FRET} of 0.03 and 0.45 E . Hence, the E_{FRET} population of Nopp140 indicates that structural fluctuations of Nopp140 are slower than the detection timescale.

In previous experiments [5], we showed that the 568–596 region of Nopp140 competes with IP₆ for binding to CK2 α , and that phosphor-Ser574 of Nopp140 was critical for binding to CK2 α . The smFRET study reported here showed that the middle-FRET population shifted to low FRETs, suggesting an increased distance between residues 352 and 589 of Nopp140C upon binding to CK2 α , and the EPR study indicated that the region near amino acid 589, rather than amino acid 568, was involved in direct contact with CK2 α . Molecular docking stimulation of the 568e589 region of Nopp140 suggested that the interaction with CK2 α increases the distance between amino acids 568 and 589 of Nopp140C (Fig. S2). A model of the complex of the 568–596 region of Nopp140 with CK2 α suggests that the amino acid 574 of Nopp140 is located near the IP₆ binding site of CK2 α , and a region around amino acid 589 more closely interacts with CK2 α than amino acid 568 (Fig. S3). These results suggest that the 574–589 region of Nopp140 is critical for binding to CK2 α . However, it has not been determined whether the FRET population shift is caused from, such as, conformational selection or coupled folding and binding [17]. A single molecule kinetic study of the interaction between Nopp140 and CK2 α would provide more detailed information.

It is difficult to study the structural features of IDPs using conventional methods for structural determination such as X-ray crystallography, small-angle X-ray scattering (SAXS), NMR and CD, owing to the multiple and disordered structural characteristics of the proteins. In contrast, the smFRET and EPR methods can provide useful information about IDPs, such as the distributions of structural regions in the proteins in various conditions, and the effects on structure of their interactions with binding partners. Since most IDPs perform their biological functions through interactions with their binding partners [18], the approaches presented here could substantially contribute to the determination of modes of interaction of many other IDPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2016.06.040>.

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Fig. 1.

(A) Schematic representation of Nopp140 and Nopp140Cs. Secondary structure regions of Nopp140 were predicted with the Quick2D bioinformatics toolkit (http://toolkit.tuebingen.mpg.de/quick2_d). Fluorescent dye labeled positions (S352C/S467C, S352C/S589C, S352C/G660C, and S352C/S704C) are indicated with green asterisk symbol (*). EPR spins labeled positions (G568C, S589C, and S704C) are indicated with red arrows (↓). (B) SDS-PAGE analysis of MTSSL labeled Nopp140C (lane 1: before labeling, lane 2: after labeling) (C) SDS-PAGE analysis of Cy3- and Cy5-labeled Nopp140C. Images of lane 3 and lane 4 were obtained by fluorescence gel documentation. (lane 1: before labeling, lane

2: after labeling, lane 3: green laser excited Nopp140 C-terminus fragment, lane 4: red laser excited Nopp140 C-terminus fragment). (D) Representative absorbance spectrum of Cy3 and Cy5 labeled Nopp140C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Bulk fluorescence spectra of Cy3 and Cy5 labeled Nopp140Cs in the presence or absence of CK2 α . With excitation at 550 nm, the spectra were obtained from 560 nm to 700 nm by with a fluorometer in the presence of 10 μ M double-labeled Nopp140C (S352C/S467C) (A), Nopp140C(S352/S589C) (B), Nopp140C (S352C/G660C) (C), and Nopp140C (S352C/S704C) (D). Each spectrum of Nopp140C and Nopp140C with CK2 α is presented with black and red dotted-lines, respectively. The molar ratio of Nopp140C-to-CK2 α was 1:100. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. smFRET histograms and representative time series of double-labeled Nopp140C-S352C/S589C only (A, B), with CK2α (C, D), and in 6 M GdmCl (E, F). Histograms were fitted with up to two Gaussian peaks distributions having a FRET-peak centered at 0.03 (blue) and 0.45 *E* (red) each. Percentages of the areas of each Gaussian peak are indicated above each graph. The molar ratio of Nopp140C-to-CK2α was 1:100. For collecting the signals from Nopp140 labeled with both Cy3 and Cy5, an alternative excitation of Cy3 and Cy5 was performed using a 532 and 633 nm laser, respectively. Magenta arrows indicated intensity of

Cy5 which was excited by 633 nm laser. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Fig. 4.

EPR spectra of spin-labeled Nopp140Cs (S568C, S589C, and S704C). The EPR spectra of spin labeled Nopp140Cs in the absence or presence of CK2α are presented as black and red dotted-lines, respectively. The spectra are normalized to the same integral for superimposition of data. The molar ratio of Nopp140C-to-CK2α was 1:100. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)