Complexes of nucleosomal nanoparticles with proteins: spFRET microscopy study of olaparib and PARP-1 binding to core nucleosomes

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FRET microscopy of single fluorescently-labelled nucleosomal nanoparticles is an advanced and convenient approach to study structural re-organization of nucleosomal DNA induced by protein factors. Poly-(ADP-ribose)-polymerase 1 (PARP-1) interacts with nucleosomes during DNA repair and transcription, but the mechanism of this process is unknown. In the present work we study structural changes in the nucleosome-PARP-1 complex induced by anticancer compound olaparib, which binds to the active center of PARP-1 and inhibits its catalytic activity. Using single particle FRET microscopy we demonstrate that PARP-1 induces significant rearrangement of DNA in the core of a nucleosome after binding to a DNA end localized nearby. The PARP-1-induced structural changes in nucleosomal DNA can be reversed by NAD^+-dependent poly(ADP)-ribosylation. Olaparib does not prevent formation of nucleosome-PARP-1 complexes and does not affect the structure of nucleosomal DNA in these complexes. However, olaparib traps the inactive nucleosome-PARP-1 complex and abolishes NAD^+-dependent reversal of the nucleosome structure.

Keywords: FRET; microscopy; nanoparticle; nucleosome; chromatin; PARP-1; PARP-1 inhibition; olaparib; DNA repair

1. Introduction

Eukaryotic genome is organized into chromatin – compact DNA-protein complex. Minimal structural unit of the chromatin is a nucleosome. It consists of double stranded DNA segment (145-148 base pair length) wrapped in 1.65-1.7 gyres around the octamer of core histones [1, 2]. Realization and efficiency of nuclear processes such as transcription, replication and repair of DNA are regulated by DNA packing/unpacking in chromatin, and, in particular, by structural features of DNA folding in a nucleosome. Re-organization of DNA in nucleosomes is assisted by different protein factors. There are many nuclear proteins that bind to nucleosomes. To understand mechanisms of their functioning, it is important to know how these proteins affect a structure of nucleosomal DNA. Investigation of the structural features of nucleosome-protein complexes is a non-trivial task, which can be resolved using single particle Forster resonance energy transfer (spFRET) microscopy in combination with the experimental system based on fluorescently-labelled nucleosomal nanoparticles.

Nucleosomal nanoparticles assembled *in vitro* from an octamer of core histones and short DNA fragments with a strong nucleosome-positioning sequence were shown to be well applicable to reveal re-organization of nucleosomes under the influence of various proteins including chromatin remodelers, linker histones and RNA polymerases [3, 4]. To make these nucleosomes suitable for structural studies by spFRET microscopy, a pair of labels (donor, Cy3, and acceptor, Cy5) was introduced into neighboring gyres of nucleosomal DNA [5-7]. spFRET microscopy is an advanced technique enabling analysis of structurally different subpopulations of nucleosomes in heterogeneous samples [5-9]. In spFRET microscopy, diluted solutions of nucleosomes are used to measure fluorescent signals from single nanoparticles diffusing through the laser focus under microscope [5-7].

Poly-(ADP-ribose)-polymerase 1 (PARP-1) is a sensor of single- and double- strand DNA breaks. This multi-domain protein is located in large amount in cell nuclei of higher eukaryotes and performs different functions in DNA repair [10, 11], chromatin organization and transcription [12]. The enzyme is activated after binding with a substrate, namely, an available DNA end, which is recognized by zinc-finger domains of the protein [13]. PARP-1 binding to DNA end induces conformational changes in the protein [14, 15], which are accompanied by activation of the catalytic center of PARP-1 [16, 17]. Activated enzyme catalyses a reaction of poly(ADP)-ribosylation (PARylation) of target proteins (including self-PARylation [18]), using NAD^+ as a substrate providing ADP-ribose residues. Besides automodification, core histones [19] and linker histone H1 [20] are the targets for PARylation.

Since the enzyme is up-regulated in some types of cancers, inhibitors of PARP-1 enzymatic activity are important anticancer compounds. Application of the inhibitor olaparib for cancer therapy is approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Olaparib interferes with essential nuclear processes in various tumors and causes cell death due to synthetic lethality [21, 22]. It is especially active against ovarian, breast, and prostate cancers with BRCA1 or BRCA2 mutations [23].

In the present study we are using spFRET microscopy, which is sensitive to changes in nucleosome structure caused by PARP-1, to investigate interactions of olaparib with nucleosome-PARP-1 complexes in the absence and presence of PARylation-inducing NAD^+.

2. Materials and Methods

2.1 Assembly of nucleosomes

Fluorescently-labelled DNA template of 167 bp length was obtained by polymerase chain reaction (PCR) using the modified nucleosome-positioning sequence s603-42 [24] as a template. The following oligonucleotides (Biotech Industry Ltd, RF) were used to introduce fluorescent labels in DNA template:
1 – 5'-d(CAAGCGACACCGGCACTGGGCCCGGTTCGCGCTCCCTCCTTCCGTGTGTTGTCG**T(-Cy3)**CTCT)-3'
2 – 5'-d(ACCCCAGGGACTTGAAGTAATAAGGACGGAGGGCC**T(-Cy5)**CTTTCAACATCGAT)-3'

PCR products were purified in 1% agarose gel and extracted by QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol.

Nucleosomes were assembled from fluorescently-labelled DNA and chicken donor chromatin without linker histone by salt dialysis as described earlier [25]. Quality of the nucleosome assembly was analyzed by the electrophoresis in native 4.5% PAGE (acrylamide:bisacrylamide 39:1; 0.5× TBE buffer). TBE buffer contained 90 mM Tris-borate, 2 mM EDTA (pH 8.3).

Recombinant human PARP-1 was expressed in *Escherichia coli* and purified as described previously [26]. Purified protein was provided by Dr. J. M. Pascal.

2.2 spFRET measurements

spFRET experiments were performed in a buffer containing 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 150 mM KCl and 1% dimethyl sulfoxide (DMSO). DMSO was present in all mixtures to provide an adequate comparison with those probes, where olaparib was added from a stock solution in DMSO. Fluorescently-labeled nucleosomes were incubated with PARP-1 for 45 min.

To induce PARylation, nucleosomes were incubated with PARP-1 for 10 min followed by additional incubation with NAD^+ for 35 min. Alternatively, PARP-1 was pre-incubated with NAD^+ for 35 min, and the mixture was incubated with nucleosomes for 10 min.

In experiments with olaparib, olaparib was always pre-incubated with PARP-1 for 10 min. Further, this mixture was: (1) added to nucleosomes for 10 min and post-incubated with NAD^+ for 35 min; (2) incubated with NAD^+ for 35 min and post-incubated with nucleosomes for 10 min; (3) incubated with nucleosomes for 45 min.
Final concentrations of components in reaction mixtures were 1.4 nM for nucleosomes, 500 nM for PARP-1, 100 μM for NAD^+ and 100 μM for olaparib. All the probes were incubated at +25°C in siliconized tubes.

spFRET measurements were performed as described previously [27]. Data from 1600-11000 single nucleosomes were analyzed. Each single nucleosome was characterized by FRET between Cy3 and Cy5 labels calculated as a proximity ratio (E_{PR}) in percent:

$$E_{PR} = (I_5 - 0.19 \times I_3) / (I_5 + 0.81 \times I_3) \times 100 \quad (1)$$

where I_3 and I_5 are fluorescence intensities of Cy3 and Cy5, respectively, and coefficients 0.19 and 0.81 provide correction for the spectral cross-talk between Cy3 and Cy5 detection channels. Frequency distributions of E_{PR} values were plotted and fitted by two Gaussian peaks. Goodness of fit (R^2) ranged from 0.89 to 0.98.

3. Results

3.1 Experimental approach

To study effects of olaparib on PARP-1-induced re-organization of chromatin near a double-strand break of DNA, nucleosomal nanoparticles labeled with a pair of Cy3 and Cy5 fluorophores were used (Fig. 1a). Nucleosomes were assembled with chicken histones on the previously characterized 603 nucleosome positioning sequence [24, 28] (147 bp) with a 20 bp linker imitating double-strand break of DNA. Positions of labels were selected in the neighboring gyres of DNA using a known nucleosome structure [29] to provide efficient FRET between donor (Cy3) and acceptor (Cy5) fluorophores without disturbance of histone-DNA contacts (Fig. 1a). Labels were positioned at +13 (Cy3) and +91 (Cy5) base pairs, relatively to the entry of linker DNA into a nucleosome core [30].

Quality of the nucleosome reconstitution was estimated by native PAGE (Fig. 1b). Nucleosome purity was >95%, with minor contaminating histone-free DNA. Nucleosome preparations contained some donor chromatin; therefore a high concentration of PARP-1 was used to guarantee efficient formation of complexes with labeled nucleosomes.

Different combinations of PARP-1, NAD^+ and olaparib were added to nucleosomes, and the resulted mixtures were studied with spFRET microscopy. Fluorescence of single nucleosomes was measured when they diffused freely

through the focus of a laser beam (514.5 nm wavelength) under microscope [27]. To characterize a proximity of Cy3 and Cy5 dyes in neighboring gyres, a proximity ratio (E_{PR}) was calculated for each measured nucleosome, and data were presented as a frequency distribution of E_{PR}. Limited data on an instrumental factor and quantum yields of Cy3 and Cy5 dyes did not allow one to estimate absolute distances between labels.

3.2 Olaparib does not affect PARP-1-induced re-organization of nucleosomal DNA

spFRET analysis of nucleosomes with an exposed DNA end and the donor-acceptor pair of dyes situated near the entry of the linker DNA fragment into the nucleosomal core (Fig. 1a) revealed two populations of nanoparticles (Fig. 1c): compact nucleosomes (major peak) and a small fraction (minor peak) of free DNA and nucleosomes with unwrapped DNA, likely a result of nucleosomal DNA "breathing" [31].

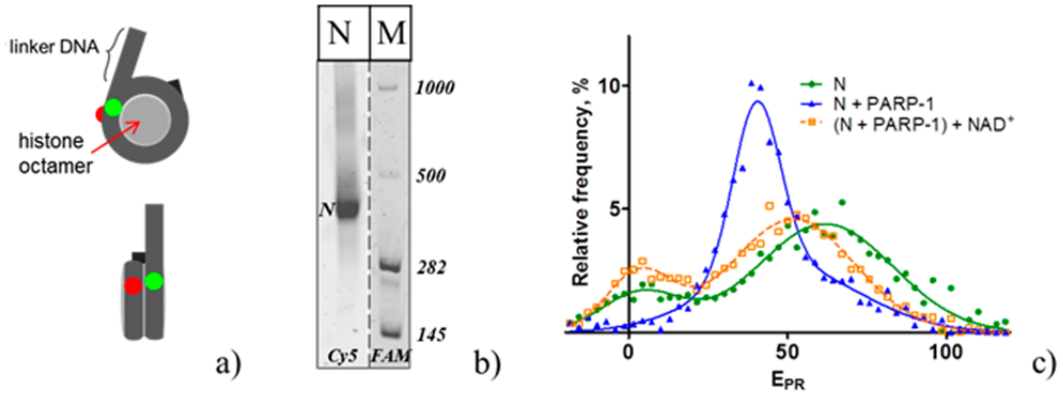

Fig. 1 The experimental approach. a) Mononucleosomal nanoparticles contain a pair of the dyes in positions +13 and +91 bp relative to the nucleosome boundary (Cy3 and Cy5 dyes are shown by green and red circles, respectively). b) Electrophoretic mobility of nucleosomes in native PAGE. N – nucleosomes. M – fluorescein-labeled DNA marker. Length in bp is shown**. c)** Frequency distributions of E_{PR} values for free nucleosomes (N), nucleosome-PARP-1 complexes and nucleosome-PARP-1 complexes in the presence of NAD^+. PARP-1 changes conformation of nucleosomal DNA in a core region near linker DNA. NAD^+-dependent automodification of nucleosome-PARP-1 complexes restores almost completely the initial structure of nucleosomes.

Addition of PARP-1 results in formation of complexes with nucleosomes that is recognized as a shift of the high-E_{PR} peak from 62 to 40% and disappearance of low-E_{PR} peak (Fig. 1c and Table 1). A shift of the high-E_{PR} peak to lower values indicates to structural changes in the nucleosomal DNA, which is accompanied by an increase in a distance between neighboring gyres in the region of H2A-H2B dimer (position of +13 bp) and H4-H2B interface (position of +91 bp). A width of E_{PR} peak becomes noticeably narrower after PARP-1 binding that can be explained by reduced mobility of DNA in the complex. This conclusion is supported by disappearance of low-E_{PR} peak corresponding to a fraction of nucleosomes with unwrapped DNA.

To evaluate the effect of olaparib on PARP-1-nucleosome interaction, the enzyme was preincubated with the inhibitor, with subsequent addition of nucleosomes. Appearance of the E_{PR} peak with a maximum at 40% indicated that complexes of nucleosomes with PARP-1 were formed in the presence of olaparib. At the same time a partial preservation of E_{PR} peak with a maximum at 62% (Fig. 2a, Table 1), which is a characteristic feature of free compact nucleosomes, allowed one to conclude that efficiency of the complex formation was slightly reduced. Therefore, olaparib binding to the catalytic site of PARP-1 neither prevents enzyme complexation with nucleosomes, nor changes a mode of nucleosome re-organization induced by PARP-1.

Table 1 Positions of peak maxima in frequency distributions of E_{PR} values for studied nucleosome specimens

Specimen	Peak 1	Peak 2
N	3±1	62 ± 1
N + PARP-1	40 ± 1	62± 1
(N + PARP-1) + NAD^+	3 ± 1	52 ± 1
(PARP-1 + olaparib) + N	40 ± 2	62 ± 3
((PARP-1 + olaparib) + N) + NAD^+	36 ± 1	52 ± 2
(PARP-1 + NAD^+) + N	4 ± 2	54 ± 2
((PARP-1 + olaparib) + NAD^+)+ N	35 ± 1	50 ± 4

3.3 Impact of PARP-1 autoPARylation on the nucleosome-PARP-1complexes

To recapitulate the ability of the system to detect activation and automodification of PARP-1 after binding to the nucleosome, NAD^+ was added to the nucleosome-PARP-1 complexes (Fig. 1c and Table 1). It resulted in the formation of two subpopulations of nucleosomes with E_{PR} peak maxima at 3 and 52%, indicating nearly complete reversal of the E_{PR}-profile of free nucleosomes in the presence of NAD^+.

A similar E_{PR} profile, which was slightly changed as compared to the profile of free nucleosomes, was observed when NAD^+ was mixed with PARP-1 before the addition to the nucleosomes (Fig. 2 b). It is known that NAD^+-induced PARylation (and autoPARylation) occurs only after activation of PARP-1 initiated by its binding to DNA [32]. Therefore, these small changes in the E_{PR} profile can be interpreted either as a NAD^+-induced inhibition of complexation between PARP-1 and nucleosomes or as a recovery of nucleosome structure after nucleosome-PARP-1 complex formation and consequent NAD^+-induced autoPARylation of PARP-1. The last is most probable, because NAD^+ and olaparib bind in the same site of PARP-1 [33], and olaparib alone does not inhibit enzyme interactions with nucleosomes (Fig. 2a).

3.4 Olaparib stabilizes the nucleosome-PARP-1 complex and prevents NAD^+-dependent reversal of the nucleosome structure

We have found that the presence of olaparib eliminates the NAD^+-induced recovery of the nucleosome structure in the nucleosome-PARP-1 complexes (Fig. 2a, Table 1). It is well consistent with the published data that olaparib inhibits enzymatic activity of PARP-1 [34] by competing with NAD^+ for the binding to the catalytic site of the enzyme [33]. This effect of olaparib was equally observed when complexes between PARP-1, olaparib and nucleosomes were formed before addition of NAD^+ (Fig. 2a) or free nucleosomes were added to the mixture of PARP-1, olaparib and NAD^+ (Fig. 2b). In the last case PARP-1 was able to form complexes with nucleosomes and re-organize their structure rather similar as in the absence of NAD^+ and olaparib (Fig. 1c), but these complexes were not sensitive to a conventional NAD^+ action. More precisely, the nucleosome-PARP-1 complexes were slightly changed in the presence of both olaparib and NAD^+. Although olaparib alone did not affect the position of E_{PR} peak maximum of the PARP-1-nucleosome complex, presence of olaparib with NAD^+ resulted in a shift of the E_{PR} peak maximum from ~40% to ~35%. It means that inhibition of PARylation with olaparib does not prevent some NAD^+ interaction with nucleosome-olaparib-PARP-1 complex.

Taken together, the data suggest that olaparib inhibits PARP-1 automodification and thus abolishes a release of PARP-1 from the PARP-1-nucleosome complex in the presence of NAD^+.

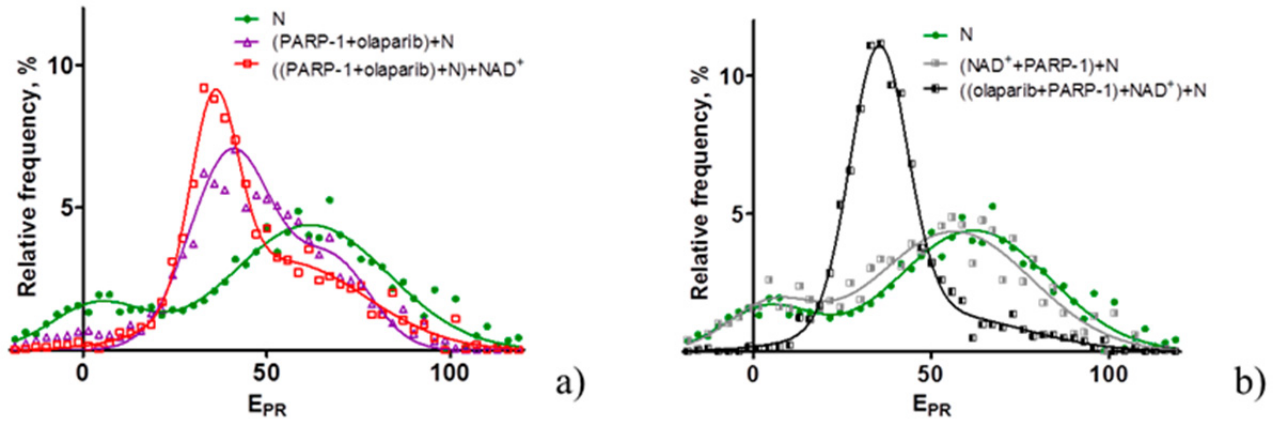

Fig. 2 Olaparib reduces slightly the efficiency of formation of nucleosome-PARP-1 complexes and prevents NAD+-induced recovery of the initial nucleosome structure. a) PARP-1 was pre-incubated with olabarib, and nucleosomes were then added to the inhibited enzyme in the presence or absence of NAD^+. In the presence of olabarib PARP-1 still changes conformation of nucleosomal DNA in an area near linker DNA (purple graph). However, NAD^+-induced recovery of the initial nucleosome structure is prevented (red graph, compare with Fig. 1c). b) PARP-1 was preincubated with NAD^+ before the addition to nucleosomes. In this case formation of nucleosome-PARP-1 complex occurs only in the presence of olaparib. Frequency distributions of E_{PR} values are shown.

4. Discussion

Our spFRET experiments of the fluorescently-labelled nucleosomal nanoparticles allowed detection of PARP-1-induced re-organization of the nucleosomal DNA in the presence of double-strand DNA break nearby (Figs. 1-2). Mononucleosome with one DNA linker available for PARP-1 binding is a convenient model of an exposed DNA double-strand break in the chromatin environment [32]. This type of substrate is shown to bind one molecule of PARP-

1 [32]. We identified that the enzyme bound to the DNA end induces disturbance of the nearby nucleosomal DNA.

Detection and repair of a double-strand break in the cell nuclei require remodeling of the chromatin [35]. In particular, accumulation of PARP-1 in the region of damaged DNA results in eviction of linker and core histones from the surrounding chromatin [36]. The effect largely depends on the enzyme activity of the protein, but in presence of PARP-1 inhibitors some loss of histones still occurs. Our data suggest that PARP-1 induces partial uncoiling of nucleosomal DNA and therefore could facilitate exchange and/or displacement of the core histones *in vivo*, before PARP-1 leaves the DNA break after automodification. This process likely requires other factors that are currently missing in our *in vitro* system.

Our results reveal NAD^+-dependent reversal of PARP-1-induced re-organization of the nucleosomal DNA (Fig. 1b and Fig. 2b). Rearrangement in the structure of PARP-1 after binding to a DNA double-strand break [7] destabilizes HD subdomain and results in activation of the catalytic center of the enzyme [15, 37, 38]. Automodified PARP-1 becomes negatively charged and dissociates from the DNA [39]. According to our data PARP-1 auto-PARylation leads to a nearly complete recovery of the initial structure of the nucleosome (Fig. 1b, 2b and Table 1) that is presumably associated with eviction of PARP-1 from the complex.

The results also suggest that spFRET microscopy is a sensitive assay that could be used for further analysis of PARP-1 inhibitors. Olaparib is the inhibitor that is structurally related to PARylation substrate NAD^+ [40]. Therefore the action of other compounds containing nicotinamide pharmacophore group and interacting with the active center of PARP-1 (rucaparib, veliparib, niraparib and others [41]) should also be successfully detected in our spFRET assays. Therefore the system could be used for further screening and analysis of the PARP-1 inhibitors.

Well-established PARP-1 inhibitor olaparib does not prevent formation of the complex between nucleosomes and PARP-1 and has no impact on the structure of the complex (Fig. 2). However, the inhibitor strongly represses NAD+-dependent changes in nucleosome structure (Fig. 2 and Table 1) and likely traps the protein on the DNA double-strand break in the vicinity of the nucleosome. In the living cell it can trap the inhibited PARP-1 in non-productive complexes with nucleosomes and thus cause down-regulation of the protein activity. As the enzymatic activity of the protein is a factor in cancer survival, PARP-1 trapping could be involved in the olaparib-induced synthetic lethality of tumor cells [21, 22]. Stabilization of the non-productive PARP-1-nucleosome complexes by olaparib might also interfere with histone displacement, which could impair DSB repair by NHEJ and/or HR pathways [42]. It is also possible that the stable PARP-1-nucleosome complexes could be formed at the transcription start sites [12].

5. Conclusions

The nucleosomal nanoparticles are fluorescent sensors that enable detection of minor changes in conformation of nucleosomal DNA. In combination with a single particle FRET microscopy it is a powerful and precise experimental system to study chromatin structure.

Using fluorescently-labelled nucleosomal nanoparticles in combination with spFRET microscopy, PARP-1-induced re-organization of the nucleosomal DNA and NAD^+-dependent reversal of the effect were found (Fig. 1b). It is shown that well-known PARP-1 inhibitor olaparib that is structurally related to PARylation substrate NAD^+ [40] does not affect the structure of the PARP-1-nucleosome complex (Fig. 2a and Table 1). However, olaparib strongly represses PARP-1 enzymatic activity (Fig. 2 and Table 1) and traps the protein on the DNA double-strand break in the vicinity of the nucleosome. Surprisingly, NAD^+ can interact with olaparib-inhibited nucleosome-PARP-1 complex but cannot induce PARylation-dependent recovery of initial nucleosome structure.

The results suggest that spFRET microscopy is well applicable to study PARP-1 interaction with chromatin, its enzymatic activity and PARP-1 inhibitors.

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