DNA Repair 24 (2014) 46–56

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Differential binding kinetics of replication protein A during replication and the pre- and post-incision steps of nucleotide excision repair

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ARTICLE INFO

Article history:
Received 16 June 2014
Received in revised form
24 September 2014
Accepted 26 September 2014
Available online 16 October 2014

Keywords:
RPA
DNA replication
DNA repair
Replication stress
Nucleotide excision repair
DNA damage

ABSTRACT

The ability of replication protein A (RPA) to bind single-stranded DNA (ssDNA) underlines its crucial roles during DNA replication and repair. A combination of immunofluorescence and live cell imaging of GFP-tagged RPA70 revealed that RPA, in contrast to other replication factors, does not cluster into replication foci, which is explained by its short residence time at ssDNA. In addition to replication, RPA also plays a crucial role in both the pre- and post-incision steps of nucleotide excision repair (NER). Pre-incision factors like XPC and TFIIH accumulate rapidly at locally induced UV-damage and remain visible up to 4 h. However, RPA did not reach its maximum accumulation level until 3 h after DNA damage infliction and a chromatin-bound pool remained detectable up to 8 h, probably reflecting its role during the post-incision step of NER. During the pre-incision steps of NER, RPA could only be visualized at DNA lesions in incision deficient XP-F cells, however without a substantial increase in residence time at DNA damage. Together our data show that RPA is an intrinsically highly dynamic ssDNA-binding complex during both replication and distinct steps of NER.

1. Introduction

Replication protein A (RPA), the major eukaryotic single-stranded DNA binding protein, is required for several DNA metabolic processes including replication, repair, recombination and checkpoint activation. RPA is a heterotrimer consisting of 70, 32 and 14 kDa subunits and binds ssDNA with a 5′–3′ polarity [1,2]. RPA was initially identified as a crucial replication factor that, together with replication factor C (RFC) and proliferating cell nuclear antigen (PCNA), regulates the loading and processivity of different DNA polymerases onto the chromatin [3].

During replication in eukaryotes the trimeric sliding clamp PCNA, is loaded around the DNA at the 3′-OH end of the nascent DNA strand by the pentameric complex RFC in an RPA- and ATP-dependent manner in order to facilitate the tethering and processing of DNA polymerases δ and ε [4,5]. In eukaryotes, DNA replication is initiated and propagated from hundreds to thousands of replication sites that, together with associated replication factors, cluster into 'replication foci'. The location, number and size of these replication foci vary throughout S-phase. Three distinct replication patterns can be distinguished, that correspond to DNA synthesis in early S phase (small and discrete foci), mid S-phase (perinucleolar and perinuclear large foci) and late S-phase (large foci) [6].

Besides their function in replication RPA, PCNA and RFC are also essential for nucleotide excision repair (NER), a "cut-and-patch" mechanism that by the coordinated action of more than 30 different proteins, removes a wide variety of helix-distorting DNA lesions,

Abbreviations: ssDNA, single stranded DNA; DDR, DNA damage response; NER, nucleotide excision repair; UV, ultraviolet light; CPDs, cyclobutane pyrimidine dimers; 6-4PPs, 6-4 photoproducts; LUD, local UV-induced DNA damage; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; HU, hydroxyurea; AraC, cytosine-β-arabinofuranoside.

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http://dx.doi.org/10.1016/j.dnarep.2014.09.013
1568-7864/

including UV-induced DNA damages like cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts dimers (6-4-PP) [7]. NER can be sub-divided into two pathways which are activated by distinct recognition mechanisms. (1) Global genome NER (GG-NER) recognizes DNA damage throughout the genome via the concerted action of two damage recognizing complexes; XPC/HR23B/centrin complex and UV-DDB complex [7–9]. (2) Transcription coupled NER (TC-NER) is only active on the transcribed strand of active genes [10] and is initiated by the stalling of elongating RNA polymerase II on DNA lesions [11]. Following damage recognition, GG-NER and TC-NER converge into a common pathway by recruiting the ten-subunit transcription factor TFIIH that verifies the lesion and locally unwinds the DNA double helix around the lesion [12]. RPA then binds to the undamaged DNA strand and, together with XPA, stabilizes open complex formation, thereby stimulating and coordinating the incision by the endonucleases ERCC1-XPF and XPG [13,14,2]. A 24–32-nucleotide DNA fragment is excised and the undamaged strand is used as a template for DNA repair synthesis [15]. Finally the DNA is sealed by LigaseIII-XRCC1 or Ligase I [16]. *In vitro* and *in situ* experiments have revealed that, following dual incision, RPA remains bound to the DNA substrate where it initiates the recruitment of RFC, PCNA and either the DNA polymerase pol ε, or pol κ and δ [17–19].

In contrast to PCNA and RFC, which only play a role in the post-incision steps of NER, RPA is implicated in both the pre-incision and post-incision steps. In this paper, we compared the spatio-temporal distribution of RPA, RFC and PCNA, using immunofluorescence and live cell microscopy of the GFP-tagged versions of the three replication proteins, to uncover the dynamic interactions of these factors with the DNA template in different maintenance processes.

2. Materials and methods

2.1. Cell culture and transfection

All cell lines were cultured under standard conditions at 37 °C and 5% CO_2 in a humidified incubator. U2OS cells and SV40-immortilized MRC5 cells were grown in a 1:1 mixture of Ham's F10 and DMEM (Lonza), supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (PS). Human primary wild-type control fibroblasts (C5RO) and XPF deficient fibroblasts (XP51RO) were cultured in Ham's F10 supplemented with 15% FCS and 1% PS. C5RO and XP51RO cells were grown to confluence and then incubated for 5 days with medium containing 0.5% FCS to induce quiescence.

A retroviral plasmid encoding RPA70-GFP was stably expressed in U2OS, SV40-immortilized MRC5, C5RO and XP51RO cells. Additionally, a cDNA encoding GFP-PCNA was stably expressed either by retroviral infection in C5RO hTERT [20] or by transfection [21] in SV40 immortalized MRC5. Cell lines expressing other fluorescent tagged proteins were used as described previously: RFC140-GFP [22], XPC-GFP [23] and GFP-XPA [24].

30 min before irradiation cells were treated with 100 mM hydroxyurea and 10 μM cytosine-β-arabinofuranoside to inhibit DNA synthesis. For global and local UV irradiation cells were washed with PBS and subsequently exposed to a UV-C germicidal lamp (254 nm, Philips) at the indicated dose [25]. To apply local UV damage cells were UV irradiated through an isopore membrane filter (Millipore), containing 5 μm pores.

2.2. Western blotting

For western blotting, primary mouse antibodies against RPA (B-6/sc-28304, Santa Cruz Biotechnology, Inc.) and GFP (11 814 460 001, Roche) were used in combination with Alexa Fluor 795 donkey anti-mouse antibodies (LI-COR). Antibody complexes were visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences).

2.3. Immunofluorescence

Cells were fixed using 2% paraformaldehyde supplemented with 0.1% Triton X-100. Samples were processed as described previously [24]. For GFP staining, cells were permeabilized with 0.5% Triton X-100 for 30 s prior to fixation. The following primary antibodies were used: anti-Ki67 (Ab833, Abcam), anti-XPC [26], anti-TFIIH p89 (s-19, Santa Cruz Biotechnology), anti-6-4pp (64M-2, Cosmo Bio), anti-RPA32 (ab2175, Abcam), anti-GFP (ab290, Abcam) and combined with secondary antibodies labeled with ALEXA fluorochromes 488 or 594 (Invitrogen; The Jackson Laboratory) for visualization. Samples were finally embedded in DAPI vectashield (Vector Laboratories). Anti-XPA (FL-273, Santa Cruz Biotechnology) or mouse anti-CPD (TDM-2, MBL International) was used as marker of local UV damage, depending on the species in which the other antibody was raised. Colocalization was defined as an >2 fold increase in fluorescent intensity at the LUD and quantified by counting at least 40 cells. Edu (5-ethynyl-2′-deoxyuridine) incorporation was visualized using Click-iT Alexa Fluor 647 according to the manufacturer's protocol (Invitrogen). Optical images were obtained using a Zeiss LSM 510 META confocal microscope equipped with 63 × oil Plan-Apochromat 1.4 NA oil immersion lens (Carl Zeiss Inc.) and a pinhole aperture setting of 2.0 airy units.

2.4. Live cell confocal laser-scanning microscopy

Confocal laser scanning microscopy images were obtained using a Zeiss LSM 510 microscope equipped with a 25 mW Argon laser (488 and 561 nm) and 63 × oil Plan-Apochromat 1.4 NA oil immersion lens (Carl Zeiss Inc.).

Kinetic studies of GFP-tagged RPA, PCNA, RFC, XPC and XPA accumulation were executed as described previously [27]. Cells were grown in glass bottom dishes (MatTek, Ashland, MA, USA) and irradiated with a UV-C source containing four UV lamps (Philips TUV 9W PL-S) above the microscope stage. For induction of local damage, cells were UV-irradiated through a polycarbonate mask (Millipore Billerica, Massachusetts, USA) with pores of 5 μm and subsequently irradiated for 39 s (100 J/m^2) [28,29] and monitored for up to 5 h. Fluorescence intensity was normalized between 0 and 100%. Assembly kinetics were measured on a Zeiss Axiovert 200 M wide field fluorescence microscope, equipped with a 100x Plan-Apochromat (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany), a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, U.K.) and an objective heater and climate chamber. Images were recorded with a cooled CCD camera (Coolsnap HQ, Roper Scientific, USA) using Metamorph 6.1 imaging software (Molecular devices, Downingtown, PA, USA). Cells were examined in microscopy medium (137 mM NaCl, 5.4 mM KCl, 1,8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM D-glucose and 20 mM HEPES) at 37 °C.

To determine protein mobility FRAP was performed as described [30]. Indicated areas were photobleached by two iterations using 100% 488 nm laser power. The recovery of fluorescence in the photobleached area was monitored for the indicated times. Data was normalized to the overall fluorescence of the cell before bleaching.

Half nucleus bleaching combined with FLIP-FRAP was performed as described previously [22]. Half of the nucleus was bleached and subsequently the fluorescence recovery in the bleached area and the loss of fluorescence in the non-bleached area was measured for up to 4 min. For data analysis the difference in fluorescence signal between FLIP and FRAP before bleaching was set at 0 and the difference between FLIP and FRAP after bleaching