Recruitment of RNA polymerase II cofactor PC4 to DNA damage sites

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Abbreviations used in this paper: Aph, aphidicolin; CK2, casein kinase II; CTD, C-terminal domain; HU, Hydroxyurea; MEF, mouse embryonic fibroblast; PAR, poly(ADP-ribose); PC4, positive cofactor 4; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; ssDNA, single-stranded DNA.

Introduction

The human positive cofactor 4 (PC4) is an abundant nuclear protein that plays an important role in various cellular processes including transcription, replication, and chromatin organization. Recently, PC4 has been identified as a suppressor of oxidative mutagenesis in Escherichia coli and Saccharomyces cerevisiae. To investigate a potential role of PC4 in mammalian DNA repair, we used a combination of live cell microscopy, microirradiation, and fluorescence recovery after photobleaching analysis. We found a clear accumulation of endogenous PC4 at DNA damage sites introduced by either chemical agents or laser microirradiation. Using fluorescent fusion proteins and specific mutants, we demonstrated that the rapid recruitment of PC4 to laser-induced DNA damage sites is independent of poly(ADP-ribosyl)ation and γH2AX but depends on its single strand binding capacity. Furthermore, PC4 showed a high turnover at DNA damage sites compared with the repair factors replication protein A and proliferating cell nuclear antigen. We propose that PC4 plays a role in the early response to DNA damage by recognizing single-stranded DNA and may thus initiate or facilitate the subsequent steps of DNA repair.

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types of DNA damage, including single strand breaks and double strand breaks, which are substrates for different DNA repair pathways. Immunofluorescence stainings with specific antibodies revealed that endogenous PC4 accumulates at sites of DNA damage as early as 5 min after microirradiation in both human and mouse cells (Fig. 2, A, B, and D). Furthermore, we observed colocalization of PC4 with the replication and repair protein A (RPA)–ssDNA cocrystal structure (Bochkarev et al., 1997), regulating the activity of PC4 in mammalian cells (Ge et al., 1994). In proliferating mammalian cells, ~95% of PC4 was shown to be phosphorylated, which affects its DNA-binding properties. Phosphorylated PC4 was shown to lose its coactivator and double stranded DNA-binding activities, but maintained its ability to bind to ssDNA mediating transcriptional repression (Ge et al., 1994; Werten et al., 1998).

Recently, it has been shown that the ssDNA-binding capacity of PC4 is required for resistance to hydrogen peroxide (H2O2) and prevents mutagenesis by oxidative DNA damage in *Escherichia coli* and *Saccharomyces cerevisiae* (Wang et al., 2004). Although these genetic studies argue for a role of PC4 in DNA repair, the direct involvement of PC4 in the DNA damage response of mammalian cells remains elusive. We used a combination of live cell microscopy, laser microirradiation, and FRAP analysis to study the recruitment of PC4 to DNA damage sites in vivo. We found a very rapid and transient accumulation of PC4 at DNA damage sites, which was independent of poly(ADP-ribose)ation and phosphorylation of H2AX but depended on its ability to bind ssDNA. These results argue for a role of this multifunctional cofactor in the very early steps of DNA repair.

**Results and discussion**

**PC4 accumulates at DNA damage sites**

To investigate the role of PC4 in DNA repair we examined the redistribution of PC4 in response to DNA damage in human and mouse cells. After treatment with different chemical agents, which induce different types of DNA lesions, cells were in situ extracted and subsequently stained for endogenous PC4 and specific DNA damage markers. In untreated cells we found a diffuse distribution of PC4 in the nucleus. Upon treatment with H2O2 or Hydroxyurea (HU) PC4 accumulated at discrete subnuclear foci colocalizing with sites of DNA damage visualized by antibodies against poly(ADP-ribose) (PAR) and γH2AX, respectively (Fig. 1, A and B). Replication arrest with HU or aphidicolin (Aph), resulting in extended stretches of ssDNA bound by the single strand binding protein RPA (Gorisch et al., 2008), as well as treatment with H2O2, also led to a redistribution of PC4 into foci colocalizing with RPA (Fig. 1 C).

To locally introduce DNA lesions at preselected subnuclear sites we used microirradiation with a 405-nm diode laser as described previously (Mortusewicz et al., 2006, 2007). This treatment results in the generation of a mixture of different types of DNA damage, including single strand breaks and double strand breaks, which are substrates for different DNA repair pathways. Immunofluorescence stainings with specific antibodies revealed that endogenous PC4 accumulates at sites of DNA damage as early as 5 min after microirradiation in both human and mouse cells (Fig. 2, A, B, and D). Furthermore, we observed colocalization of PC4 with the replication and repair...
protein proliferating cell nuclear antigen (PCNA) at laser-induced DNA damage sites (Fig. 2, C and E). Collectively, these results show that PC4 accumulates at sites of DNA damage generated by chemical agents or laser microirradiation.

**Recruitment kinetics and mobility of PC4 at DNA repair sites**

Having shown that endogenous PC4 accumulates at DNA damage sites, we generated GFP- and RFP-tagged fusion proteins to study the recruitment of PC4 in living cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200808097/DC1). As a positive control we chose the processivity factor PCNA, which is involved in various DNA repair pathways including nucleotide excision repair (Shivji et al., 1992), base excision repair (Gary et al., 1999; Levin et al., 2000), mismatch repair (Johnson et al., 1996; Umar et al., 1996; Jiricny, 2006), and repair of double strand breaks. Using a combination of microirradiation and time-lapse analysis we followed the spatiotemporal accumulation of GFP-PC4 and RFP-PCNA in vivo. For quantification, the fluorescence intensity at the irradiated sites was measured, corrected for background and total nuclear loss of fluorescence over the time course, and normalized to the preirradiation value as described previously (Mortusewicz et al., 2006, 2007). We found that GFP-PC4 accumulated at DNA damage sites immediately after microirradiation, preceding recruitment of RFP-PCNA (Fig. 3 A). Although GFP-PC4 showed a slow and constant increase of accumulation at repair sites during the observation period of 5 min, fluorescence intensities of GFP-PC4 declined after reaching a maximum around 20–40 s after microirradiation (Fig. 3 B). To determine whether the recruitment of GFP-PC4 to DNA damage sites is cell cycle dependent, we microirradiated cells in different S phase stages using RFP-PCNA as a cell cycle marker. We found that GFP-PC4 accumulates at laser-induced DNA damage sites in early, mid, and late S phase cells (Fig. S2).

To determine the mobility of GFP-PC4 at laser-induced DNA damage sites, we performed FRAP analysis 5 min after microirradiation. The irradiated region was bleached with a high energy laser pulse for 300 ms and the fluorescence recovery was determined. After bleaching of the repair foci, we observed complete recovery of the GFP signal within 5 s, indicating a high mobility of PC4 at repair sites (Fig. 3 C and D). In contrast, no recovery of PCNA at repair sites could be observed within the observation period, which is in good agreement with previous studies where DNA damage was induced by chemical agents or irradiation with a UV lamp (Solomon et al., 2004; Essers et al., 2005). As the fluorescence intensity of GFP-PC4 already begins to decline during the observation period of 5 min, we also performed FRAP analysis 20 s after microirradiation to determine the mobility of PC4 at the peak of accumulation. We could not detect any differences in the mobility of GFP-PC4 20 s or 5 min after microirradiation (Fig. 3 E). The constant increase in RFP-PCNA fluorescence observed when FRAP analysis was performed 20 s after microirradiation can be explained by new RFP-PCNA molecules being recruited during the time course of the FRAP experiment. Collectively, these results show an early and transient binding of PC4 at DNA damage sites, suggesting a role for PC4 in the early steps of DNA repair, like damage recognition and/or signaling.

This raises the question of how PC4 gets recruited to DNA lesions. Given that the single strand binding capacity of PC4 is needed for resistance against H2O2 in repairing deficient E. coli (Wang et al., 2004), it was tempting to speculate that PC4 is recruited by binding to ssDNA generated at microirradiated sites. In addition, the crystal structure of PC4 shows high similarity to the single strand binding domains of RPA70 and RPA34 (Fig. S1 B; Bochkarev et al., 1997, 1999; Brandsen et al., 1997). Therefore, we directly compared the recruitment kinetics and the mobility of RFP-PC4 with GFP-RPA34. We found that both PC4 and RPA34 were recruited immediately after microirradiation, with PC4 accumulating slightly faster than RPA34 (Fig. 4 A). Like PCNA, RPA34 showed a slow and constant increase in fluorescence intensity at the irradiated site, whereas the intensity of PC4 gradually declined after reaching a maximum (Fig. 4 B). FRAP analysis revealed distinct recovery rates, indicating that PC4 exhibits a higher mobility at DNA damage sites than RPA34 (Fig. 4, C and D). Collectively, we could demonstrate that in comparison to the single strand binding protein RPA34, PC4 shows distinct recruitment and binding properties at laser-induced DNA damage sites.

**Figure 2.** PC4 accumulates at laser-induced DNA damage sites. Wide-field fluorescence images of mouse C2C12 and human HeLa cells are shown. Fixation and immunostaining was performed 5 min after laser microirradiation. Arrowheads mark the sites of irradiation. Laser microirradiation results in local generation of DNA damage (A, B, and D) detected by antibodies against γH2AX and PAR, respectively. Endogenous PC4 accumulates at DNA damage sites in mouse (A and B) and human (D) cells and colocalizes with PCNA (C and E). Bar, 5 μm.
The C-terminal single strand binding domain of PC4 mediates recruitment to DNA damage sites

The fact that PC4 and RPA show different recruitment kinetics and turnover rates at DNA repair sites raises the question of whether PC4 indeed recognizes ssDNA generated after microirradiation. Earlier studies revealed a bipartite structure of PC4 comprising an N-terminal regulatory domain (amino acids 1–62) and a C-terminal ssDNA binding and dimerization domain (amino acids 63–127; Kretzschmar et al., 1994). It has also been shown that the ssDNA binding activity is required for transcription repression but is not needed for the activator-dependent stimulatory activity of PC4 (Werten et al., 1998). To investigate the mechanisms mediating the recruitment of PC4 to DNA damage sites we generated GFP fusion constructs comprising either the N-terminal regulatory domain (GFP-PC4 1–61) or the CTD (GFP-PC4 62–127). For direct comparison, we cotransfected the N-terminal domain and the CTD together with the full-length PC4. We found only a minor accumulation of GFP-PC4 1–61 at microirradiated sites (Fig. 5A). In contrast, GFP-PC4 62–127 showed the same recruitment kinetics as the full-length protein (Fig. 5B). In addition, we analyzed the recruitment of a fusion protein lacking the SEAC motif within the first 22 amino acids of PC4. This serine and acidic amino acids–rich motif is phosphorylated by CK2 (Kretzschmar et al., 1994), which has recently been implicated in the DNA damage response (Ayoub et al., 2008; Spycher et al., 2008). However, deleting this N-terminal domain did not significantly affect the recruitment of PC4 to DNA damage sites.
sis of the PC4 mutants are summarized in Fig. S1A and shown in detail in Fig. 5. Both mutations led to a reduced accumulation of PC4 at microirradiated sites in the context of the full length and the CTD of PC4 (Fig. 5, C–F), indicating that the single strand binding capacity of PC4 is needed for efficient recruitment of PC4 to DNA repair sites in living cells.

The fast and transient binding of the transcriptional cofactor PC4 at DNA damage sites identified in this study raises several interesting questions concerning potential roles in DNA repair and connections to transcriptional regulation. The observation that the recruitment of PC4 depends on its single strand binding capacity suggests that PC4 might fulfill similar roles in DNA repair as RPA. However, the different binding kinetics and mobility of PC4 and RPA at DNA damage sites would argue for distinct functions in DNA repair.

As PC4 has been implicated in the regulation of DNA replication (Pan et al., 1996), it could stop DNA replication near DNA lesions. Similarly, PC4 might also stop transcription as a response to DNA damage, which is supported by the fact that PC4 is a potent repressor of transcription at specific DNA structures such as ssDNA, DNA ends, and heteroduplex DNA, which are generated during DNA repair (Werten et al., 1998). Moreover, PC4 could have a helicase-like function (Werten et al., 1998; Werten and Moras, 2006), which through binding and multimerization along ssDNA is predicted to enable ATP-independent unwinding of duplex DNA. In this regard, one could also envision a protective role of PC4 in preventing degradation of ssDNA by nucleases.

DNA damage sites (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200808097/DC1). We conclude that the CTD of PC4 is necessary and sufficient for recruitment to DNA damage sites and that PC4 recruitment does not depend on its N-terminal CK2 phosphorylation sites.

In addition to phosphorylation of DNA repair factors by CK2, two other posttranslational modifications, poly(ADP-ribosylation) and phosphorylation of H2AX, have been shown to play a central role in the recruitment and/or retention of factors involved in later stages of the DNA repair process. We determined the recruitment kinetics of PC4 to laser-induced DNA damage sites in wild-type mouse embryonic fibroblasts (MEFs) in comparison with PARP-1−/− and H2AX−/− MEFs, which are largely devoid of DNA damage–induced poly(ADP-ribosylation) and phosphorylation of H2AX, respectively (Trucco et al., 1998; Celeste et al., 2002). Interestingly, recruitment of PC4 did not depend on any of these modifications (Fig. S3, C and D), which is another indication for the involvement of PC4 in the very early steps of the DNA damage response.

To further characterize the recruitment of PC4 to DNA damage sites, we generated mutants in the context of the full length and the CTD of PC4. We introduced a point mutation at position 89 replacing Trp by Ala (GFP-PC4W89A and GFP-PC4CTDW89A) and a triple mutation at positions 77, 78, and 80 (GFP-PC4Δ2β3 and GFP-PC4CTDΔ2β3), which were previously described to be essential for ssDNA binding of PC4 (Werten et al., 1998). The results of the microirradiation analysis of the PC4 mutants are summarized in Fig. S1A and shown in detail in Fig. 5. Both mutations led to a reduced accumulation of PC4 at microirradiated sites in the context of the full length and the CTD of PC4 (Fig. 5, C–F), indicating that the single strand binding capacity of PC4 is needed for efficient recruitment of PC4 to DNA repair sites in living cells.

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The crystallization of PC4 in complex with ssDNA revealed that the subunits of the PC4 homodimer cooperate in the sequence-independent binding (Ballard et al., 1988) of two opposing DNA backbones, exposing the DNA bases to the surrounding environment (Werten and Moras, 2006). These observations, together with the rapid recruitment of PC4 to DNA damage sites, argue for a role of PC4 in the detection and/or exposure of DNA damages. During the subsequent repair process PC4 may be displaced, as suggested by the observed transient binding at damaged sites.

As PC4 is a cofactor of RNA polymerase II and also interacts with p53 (Banerjee et al., 2004), a central regulator of the cellular DNA damage response, it is tempting to speculate that binding to ssDNA may lead to a transient depletion of nuclear levels of free PC4 that affects the stoichiometry and/or activity of regulatory complexes and thereby contributes to the sensing and signaling of DNA damage.

**Materials and methods**

**Cell culture and transfection**

Human HeLa, wild-type MEFs, PARP1−/− MEFs (Trucco et al., 1998), H2AX−/− MEFs (Celeste et al., 2002), and mouse C2C12 cells were cultured in DME containing 50 μg/ml gentamicin supplemented with 10 and 20% FCS, respectively. PARP1−/− and H2AX−/− MEFs were provided by V. Schreiber (Ecole Superieure de Biotechnologie de Strasbourg, Strasbourg, France) and A. Nussenzweig (National Cancer Institute, National Institutes of Health, Bethesda, MD), respectively.

Cells grown on μ-slides (Ibidi) or on gridded coverslips were cotransfected with jetPEI (PolyPlus Transfection) according to the manufacturer’s instructions. For microirradiation experiments, cells were sensitized by incubation in medium containing 10 μg/ml BrdU for 24–48 h. HU, H2O2, and Aph were obtained from Sigma-Aldrich.

**Expression plasmids**

The generation of PC4 deletion and point mutants was previously described (Kretzschmar et al., 1994; Werten et al., 1998). Corresponding GFP-PC4 fusion constructs were constructed by ligation of either restriction fragments (NdeI–ClaI for GFP-PC4; EcoRI–ClaI for the constructs GFP-PC4 1–61, GFP-PC4 22–127, GFP-PC4 CTD p2j3, and GFP-PC4 CTD W89A) or PCR products (forward primer, 5′ GGAATTCTCAATCATCTCTG 3′; reverse primer, 5′ TGGAAATCTCATTGATATGCC 3′; BglII–EcoRI cloning for GFP-PC4 1–61) into matching restriction sites of pEGFP-C1 (Clontech Laboratories, Inc.). GFP-PC4 fusion constructs were verified by sequencing and tested by expression in HeLa cells followed by Western blot analysis. A red variant of PC4 was generated by replacing GFP with RFP (Campbell et al., 2002) and termed RFP-PC4. The mRFP1 expression vector was supplied by R. Tsien (University of California, San Diego, La Jolla, CA).

Mammalian expression constructs encoding translational fusions of human RPA34 and PCNA with either GFP or RFP were previously described (Spada et al., 2007). In all cases, expression was under the control of the cytomegalovirus promoter and correct expression of fusion proteins was verified by Western blot analysis.

**Immunofluorescence and detergent extraction**

Cells were fixed in 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 or ice-cold methanol for 5 min. The following primary antibodies (diluted in PBS containing 4% BSA) were used: anti-rH2AX (Ser139) mouse monoclonal antibodies (Millipore), anti-PAR mouse monoclonal antibodies (Freytag), anti-RPA34 mouse monoclonal antibodies (EMD), anti-PC4 rabbit polyclonal antibodies (SA2249; generated by standard techniques; Eurogentech), and anti-PCNA rat monoclonal antibodies (Spada et al., 2007). Primary antibodies were detected using secondary antibodies (diluted 1:200 in PBS containing 4% BSA) conjugated to AlexaFluor 488 or 555 (Invitrogen). Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories). For in situ extraction, cells were permeabilized for 30 s with 0.5% Triton X-100 in PBS before fixation.
Live cell microscopy, microirradiation, and photobleaching experiments were performed with a confocal laser scanning microscope (TCS SP2/AOBS or SP5/AOBS; Leica), each equipped with a U-V transmitting HCD PL 63x/1.4 objective. GFP and RFP were excited with a 488-nm Ar laser line and a 561-nm diode pumped solid state laser line (Leica, respectively. The microscopes were equipped with a heated environmental chamber set to 37°C. Confocal image series were typically recorded with a frame size of 256 x 256 pixels and a pixel size of 90 nm.

Microirradiation was performed as previously described (Mortusewicz et al., 2006, 2007). In brief, a preselected spot ~1 μm in diameter within the nucleus was microirradiated for 1 s with a 405-nm diode laser (Leica) set to 50–80 μW. The laser power was measured after passing through the objective lens with a laser power meter (Coherent). Before and after microirradiation, confocal image series of one mid z section were recorded at 2-s time intervals (typically 6 pre- and 150 postirradiation frames). For evaluation of the migration kinetics, fluorescence intensities at the irradiated region were corrected for background and for total nuclear loss of fluorescence over the time course and normalized to the pre-irradiation value.

For FRAP analysis, a region of interest was selected and photobleached for 300 ms with all laser lines of the Ar laser and the 561-nm diode pumped solid state laser set to maximum power at 100% transmission. Before and after bleaching, confocal image series were recorded at 150-ms time intervals (typically 10 pre- and 200 postbleach frames). Mean fluorescence intensities of the bleached region were corrected for background and for total nuclear loss of fluorescence over the time course and normalized to the median of the last four prebleach values.

For the quantitative evaluation of microirradiation and photobleaching experiments, data of at least nine nuclei were averaged and the mean curve and the standard error of the mean calculated and displayed using Excel software (Microsoft).

Images of fixed cells were taken with a widefield epifluorescence microscope (Axioskop 2; Carl Zeiss, Inc.) using a Plan Apochromat 63x/1.40 oil objective (Carl Zeiss, Inc.) and a cooled charge-coupled device camera (VisiView System).

We are indebted to Dr. R. Tsien for providing the mRFP1 expression vector and at http://www.jcb.org/cgi/content/full/jcb.200808097/DC1.

Online supplemental material

Fig. S1 shows a schematic outline of fusion proteins used in this study and a comparison of the crystal structure of PC4 (Brandsen et al., 1999) with RPA70 (Bochkarev et al., 1997) and RPA34 (Bochkarev et al., 1999). Fig. S2 shows that recruitment of PC4 to laser-induced DNA damage sites occurs in all S phase stages. Fig. S3 shows that the recruitment of PC4 is independent of its N-terminal CK2 phosphorylation sites, poly(ADP-ribose)ylation, and phosphorylation of H2AX. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200808097/DC1.

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