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Detection of Chromatin-bound PCNA in Mammalian Cells and Its Use to Study DNA Excision Repair

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Compelling evidence indicates that proliferating cell nuclear antigen (PCNA) is an indispensable factor not only in DNA replication but in nucleotide excision repair (NER), alternative pathway of base excision repair (BER), and mismatch repair. The common function of PCNA in each of these is to assist in the initiation of DNA synthesis by providing a scaffolding clamp as a trimer catalyzed by RF-C at the 3'-OH terminus of a nascent DNA strand, to which DNA polymerase δ or ε can bind. Interestingly, DNA synthesis is reported to be ingeniously inhibited in replication, but not in NER owing to the interaction with CDKN1A (formerly known as p21/WAF1/CIP1). Furthermore, several proteins, XPG, FEN1, and DNA ligase I, recently were shown to competitively bind to the same region of PCNA, the interdomain connector loop, to which DNA polymerase δ or ε also binds. PCNA therefore seems to have a regulatory role in these DNA transactions. The in vitro reconstituted experimental system has been a powerful tool to obtain these lines of evidence, but another approach, immunofluorescence studies, also has been a contributor. In fact, the involvement of PCNA in DNA replication, NER, and BER has for the first time been indicated by a unique method that makes visible only in vivo chromatin-bound PCNA. The usefulness of this method and the importance of cooperative studies done with in vitro and in vivo experimental systems is discussed in terms of DNA excision repair.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA), discovered by Miyachi et al. is recognized by certain autoantibodies from autoimmune disease patients[1]. Bravo et al. independently identified a 36 kD acidic nuclear protein, 'cyclin', whose expression was increased in the S-phase of cell cycle distribution[2,3]. PCNA and 'cyclin' were found to be identical[4,5]. Thereafter, immunofluorescence studies using autoantibodies contributed greatly to identifying the potential role of PCNA

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in DNA replication[6–8]. PCNA next was shown by Bravo et al.[9] and Prelich et al.[10] to be an auxiliary protein of DNA polymerase δ (pol δ), which stimulated the study of the specific mechanism of PCNA in an in vitro reconstituted DNA replication system. Finally, PCNA and pol δ were confirmed to be essential factors in DNA replication of the leading strand together with another factor, pol α, that is essential in lagging strand replication[11,12]. In contrast, most DNA excision repair studies focused on the recognition / incision steps of the repair process. The only available information concerning the DNA synthesis step was that pol α and β were involved respectively in nucleotide excision repair (NER) and base excision repair (BER), based on findings of experiments using aphidicolin, a specific inhibitor of pol α and δ[13]. Exactly the same approach used in DNA replication was applied to DNA repair studies and the first evidence of the involvement of PCNA in DNA repair was shown by immunofluorescence study results, described hereafter.

Immunofluorescence studies generally provide information on: i) the presence or absence of specific proteins in an individual cell[14]; ii) if present, the distribution of these proteins within the cell structure[15]; iii) the colocalization of different proteins when multi-immunofluorecence stainings are used[15,16]; and iv) the three dimensional distribution of these proteins when combined with confocal scanning techiques[17]. Some proteins are dynamically redistributed during certain events within the cell, and the change can be detected immunohistochemically between the accumulated proteins and unredistributed ones when the contrast is high enough. Conversely, the identification of that kind of phenomenon is a strong proof that the proteins are involved in the events. A recent successful use of the immnunofluorescence approach was the detection of redistributed and colocalized hMre11/hRad50 at the double strand break (DSB) sites following γ irradiation, which complex has been suggested to be involved in homologous recombination and non-homologous end joining[16]. Moreover, the hMre11/ hRad50 complex was reported to carry the Nijmegen breakage syndrome (NBS) gene product, and its cDNA has been cloned[18]. Interestingly, use of the conventional immunofluorescence method made the redistributed foci visible. In the case of PCNA, the situation was not so simple.

UNIQUE CHARACTERISTICS OF PCNA IMMUNOFLUORESCENCE STAINING

PCNA is an abundant, stable protein (half life ~ 20h) within the nucleus[8], and its expression is growth-regulated with a peak at the S-phase in the cell cycle distribution[1–8]. Therefore, using the conventional approach, it should have been difficult to clearly detect only the chromatin-bound PCNA in S-phase cells due to the presence of high concentrations of unredistributed PCNA. PCNA immunostaining, however, was observed only in S-phase cells, and its patterns during the S-phase changed markedly in accord with the incorporation sites of ^{3}H-thymidine detected by autoradiography and 5-bromodeoxyuridine (BrdU) detected by immunostaining[6–8]. Bravo and Macdonald-Bravo reported that methanol fixation somehow had a key role in detecting chromatin-bound PCNA[8]. Indeed, when cells were fixed in formalin, the nuclei were almost all homogeneously stained[8]. Later it was reported that the methanol fixation method was successful only with autoantibodies, not monoclonal antibodies[19]. Anti-PCNA autoantibodies therefore have

Table 1. Characteristics of PCNA immunostaining

Fixation method	Antibody	
	Monoclonal antibody (PC10)	Autoantibody (AK)
4% formaldehyde	B + U[a]	B + U
Detergent extraction[c] + 4% formaldehyde	B[b]	B
Methanol	B + U	B
Detergent extraction + methanol	B	B

[a]Both chromatin-bound and -unbound PCNA are visible.
[b]Only chromatin-bound PCNA is visible.
[c]Detergent treatment with Triton X-100 done prior to fixation.

unique characteristics that allow them to recognize chromatin-bound PCNA under methanol-fixed conditions. These autoantibodies recognized conformation-dependent epitopes of PCNA[19], which may be one reason for the differential recognition of PCNA status. Another method for detecting chromatin-bound PCNA uses detergent treatment prior to fixation. Chromatin-unbound PCNA was successfully extracted by detergent treatment, e.g. Triton X-100, leaving only tightly chromatin-bound PCNA and was made visible by both monoclonal antibodies and autoantibodies[8,20]. The unique characteristics of PCNA immunostaining are summarized in Table 1. These findings obtained using the unique method strongly suggest that PCNA migrates to the DNA synthesis sites on the chromatin and therefore should be directly involved in the DNA replication process.

DETECTION OF CHROMATIN-BOUND PCNA IN THE NER PROCESS

NER is a very complicated repair system that removes bulky DNA damage, such as pyrimidine dimers induced by UV-irradiation. Repair is initiated by recognition of the DNA lesion and incision on both sides of the lesion by proteins, including the XP proteins deficient in the hereditary disease xeroderma pigmentosum. About 30 oligonucleotides including the lesion are removed, and then the gap is filled by DNA polymerase[21]. This method of autoantibodies and methanol fixation was used to study the role of PCNA in the DNA synthesis step of NER in UV-irradiated cells. Celis and Madsen reported that UV-irradiation induced PCNA staining in non S-phase cells 30 min after irradiaiton and that the staining was not abolished by cycloheximide treatment[22]. These findings were the first to suggest the migration of pre-existing PCNA to DNA damage sites and its involvement in the NER process. In the authors' discussion, cells derived from a xeroderma pigmentosum group A (XP-A) patient (XP25RO) were also described as showing UV-induced PCNA staining. They concluded that not all the steps of the NER process are inhibited in XP-A cells. Toschi and Bravo characterized the properties of UV-induced PCNA staining in more detail[23]. PCNA staining using the autoantibody AK, a different antibody than

that used by Celis and Madsen, was not induced after methanol fixation when ATP was depleted or when cells were irradiated at 0°C. In contrast, under both conditions PCNA staining was clearly present after detergent treatment plus formalin fixation. On the basis of these findings, they proposed three different PCNA populations following UV-irradiation: i) chromatin-unbound, free PCNA in the nucleoplasm; ii) chromatin-bound PCNA that does not consume ATP ('pre-synthetic complex'), which is not recognized by AK under methanol-fixed conditions; and iii) chromatin-bound PCNA that consumes ATP ('replicative complex') and which is recognized by AK under methanol-fixed conditions. On the basis of our current biochemical knowledge of PCNA, the PCNA forming 'replicative complex' should be an anchor clamp loaded onto the DNA catalized by RF-C in an ATP-dependent manner[24,25]. We therefore re-examined UV-induced PCNA staining using AK and XP-A cells. In contradiction to the results reported by Celis and Madsen, strong PCNA staining was present in normal cells, but none in XP-A cells 30 min after irradiation[26]. The same results were obtained when cisplatin was used to induce DNA lesions that are believed to be repaired by NER[27]. Furthermore, XP-A cells fused with normal cells restored UV- or cisplatin-induced PCNA staining[27]. We also showed that there was PCNA staining in BrdU-incorporated XP-A cells following UV-irradiation, where spontaneous DNA strand breaks take place[26]. These results confirmed the involvement of PCNA in the NER process and indicated that ATP-consuming chromatin-bound PCNA is formed after the incision steps in the NER process. The discrepancy in results of UV-induced PCNA staining in XP-A cells may be attributed to the types of mutation harbored in the XPA genes of the patients or to different properties of the autoantibodies. The former possibility is remote because both types of mutation lead to undetectable levels of XPA protein expression[28,29]. Conceivably some autoantibodies recognize PCNA other than tightly chromatin-bound type consuming ATP even under methanol-fixed conditions. Prosperi et al. developed a detergent treatment method under hypotonic conditions to use following UV-irradiation in which only ATP-dependent chromatin-bound PCNA is detected by the monoclonal antibody PC10[20]. Aboussekhra and Wood reported no PCNA staining in XP25RO cells using severe detergent treatment and PC10 following UV-irradiation[29]. Although the 'pre-synthetic complex' has yet to be confirmed, the tightly chromatin-bound PCNA consuming ATP detected by the immunofluorescence method well reflects the biochemical properties of PCNA clamp formation in vitro. The ATP-consuming type hereafter is referred to as chromatin-bound PCNA. After PCNA involvement in NER was shown by immunofluorescence [22,23,26], Shivji et al.[30], and Nichols and Sancar[31] proved that PCNA is an essential factor in the DNA synthesis step in the reconstituted NER system in vitro and that pol δ or ε, not pol α, is involved in it.

To confirm the involvement of PCNA in the NER process, the relationship between the function of the mutant XPA protein and UV-induced PCNA staining was examined in quiescent XP-A cells harboring various mutations in the XPA gene. XPA protein has a pivotal role in the recognition of DNA damage and is an essential factor in NER[28]. The structure of the mutant XPA protein and results of PCNA stainings are presented in Fig. 1. XP35OS is homozygous for mutation of a G→C transversion at the 3' splice acceptor site of intron 3 leading to the expression of undetectable levels of XPA protein, which is most common in Japanese XP-A patients with severe clinical symptoms[32]. GM2009 is heterozygous for a G→T transversion at nucleotide 323

altering the Cys-108 codon to the Phe codon, and for 5-bp deletion at nucleotide 349–353 producing severe clinical symptoms[33]. The cells express only a significant level of normal-size XPA mRNA derived from the allele harboring the missense mutation in the C4-type zinc finger domain which plays an essential role in the XPA protein function[33]. These two types of cells showed no PCNA staining 30 min after UV-irradiation as was the case with XP67TO cells described above (Fig. 1B, C). XP39OS and XP11TU are homozygous for a C→T transversion altering the Arg-228 codon to a nonsense codon[34,35]. These cells express 3–5 times less the amount of XPA protein lacking the C-terminal 46 amino acids, including the H2C2-type zinc finger motif[34,35]. They showed very faint, but reproducible staining (Fig. 1D, E). XP8LO is heterozoygous for a G→ C transversion at the last nucleotide of exon 4 and for an A→G transversion altering the His-244 to Arg in exon 6, which gives very mild symptoms[36]. The cells express only normal-size mRNA derived from the allele harboring the missense mutation in exon 6[36]. They showed strong PCNA-staining very similar to that seen in normal cells (Fig. 1F). In previ-

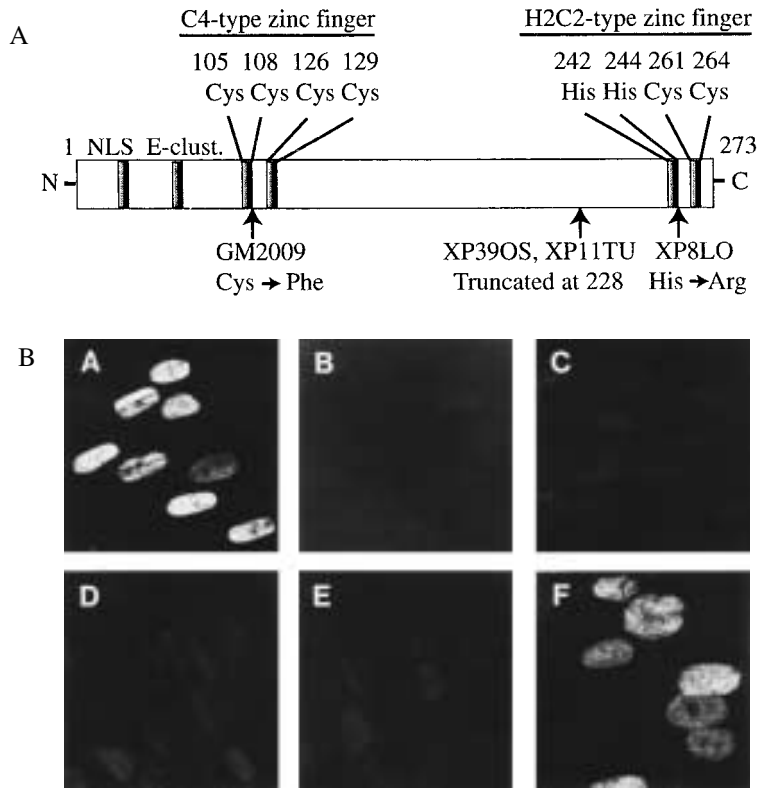

Fig. 1 Structure of the mutant XPA protein and its effect on UV-induced PCNA staining. A: Structures of XPA protein and mutant XPA protein expressed in GM2009, XP39OS, XP11TU, and XP8LO cells. Numbers give the amino acid sequence of the XPA protein. NLS, nuclear localization signal; E-cluster, glutamic acid cluster. B: PCNA staining 30 min after 10 J/m^2 of UV-irradiation: A, SFN4 (normal); B, XP35OS; C, GM2009; D, XP39OS; E, XP11TU; F, XP8LO.

ous studies, UV-survival rates were correlated with the functions of mutant XP proteins[33–36], indicative that the function of mutant XPA protein, UV-survivals, and PCNA staining 30 min after UV-irradiation are well correlated and that the initial recognition step and PCNA-dependent DNA synthesis step are closely linked in the NER process.

We next pinpointed at which stages chromatin-bound PCNA is formed in the NER process. Because UV-induced PCNA staining was restored in BrdU-incorporated XP-A cells, the incision step may be important for PCNA clamp formation. XPG and XPF/ERCC1 are reported to have roles in the dual incisions in the NER process respectively at the 3' and 5' side of the DNA lesion [21,37,38]. XP-G, mouse ERCC group 5 (equivalent to human XP-G), and XP-F cells were used to test this possibility, and UV-induced PCNA-staining was examined 30 min after irradiation. No PCNA staining was detected in XP-G (Sps-1) or ERCC group 5 (XL216–7) cells, but it was present when a plasmid carrying a full coding region of the wild type ERCC 5 cDNA was tranfected to ERCC group 5 cells[39]. XP-F (XP2YO) cells, which are considered to harbor non-leaky mutations that result in the expression of undetectable levels of XPF and ERCC1, there was faint PCNA staining[39]. These results suggest the following possibilities: i) the PCNA clamp is not formed without a 3' side incision; ii) the PCNA clamp is formed, but at low efficiency, even if a 5' incision is deficient; and iii) therefore, an incision step, in particular a 3' side incision, is essential for PCNA clamp formation. Aboussekhra and Wood made a comprehensive study of quantitative UV-induced PCNA staining using all types of XP cell strains[29]. They found: i) no PCNA staining in XP-A or XP-G cells; ii) ~50 % of the staining intensity in normal cells was detected in XP-B, XP-C, XP-D, and XP-F cells; and iii) there was normal staining in the XP-E and variant cells. They concluded that NER proceeds except in XP-A and XP-G cells, and that dual incisions take place at the 3' side first. It seems likely that no incision occurs in XP-G cells, but that 3' side incision occurs at low efficiency in XP-F cells and the PCNA clamp is formed at the incision site in the NER process. The findings on the dual incisions were confirmed in a reconsituted NER system in vitro by Evans et al.[40]. Quantitative detection of chromatin-bound PCNA therefore reflects incision activities in NER in vivo.

DETECTION OF CHROMATIN-BOUND PCNA IN THE UNIDENTIFIED PROCESS FOLLOWING UV-IRRADIATION OF XP CELLS

During the study of PCNA staining in NER, we noticed a different time course than that of normal cells in UV-induced PCNA staining in XP-A cells[41]. It began to appear at a later time, about 2–3 h after irradiation. Strong staining was detected even 24 h after irradiaiton, the staining in normal cells having almost disappeared at that time (Fig. 2). Interestingly, the staining pattern differed completely from that for normal cells reflecting NER, the fluorescent foci being larger in size but smaller in number (Fig. 2D). When XP-A cells were fused with normal cells, the very same time course as the normal staining pattern was observed, not the peculiar one[41]. This phenomenon was first seen in XP67TO cells but also is common in the other XP-A cell strains XP96TO, XP35OS, GM2009, XP39OS, XP11TU, and in XP8LO cells[42]. Li et al. also observed the same phenomenon in XP-A, XP-C, and XP-G cells and showed that CDKN1A

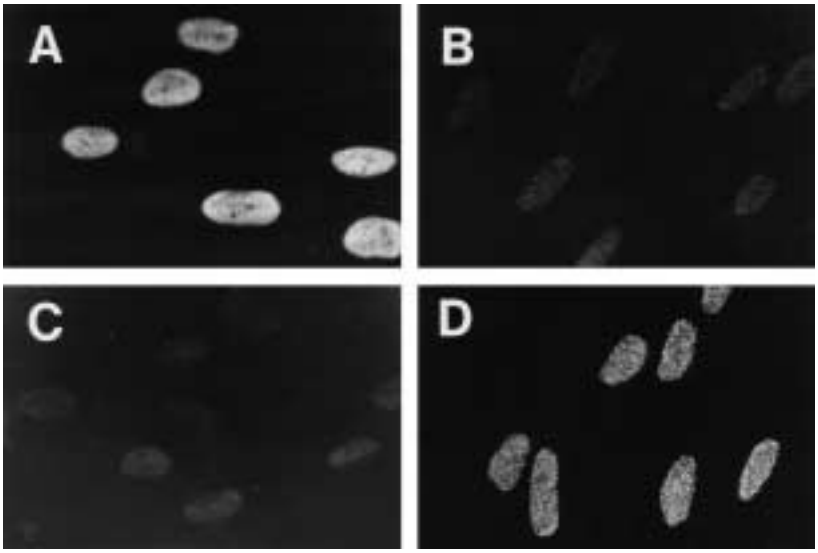

Fig. 2 Unique PCNA staining in XP-A cells at later times after UV-irradiation. PCNA staining for SFN4 cells (A, 3 h; C, 24 h) and XP67TO cells (B, 3 h; D, 24 h) following 10 J/m^2 of UV-irradiation.

(formerly p21/WAF1/CIP1) colocalized at the foci[43]. PCNA together with CDKN1A therefore was speculated to have a role in NER prior to the incision step, presumably by contributing to the initial sensing of DNA damage[43]. This hypothesis does not, however, explain the time course and staining pattern. Interestingly, peculiar staining was not seen in cisplatin-treated XP-A cells (unpublished data, Miura et al.), suggesting that it reflects an NER-unrelated repair process specific to certain kinds of UV-induced damage. The damage must normally be repaired by NER because normal cells proficient in NER do not show that kind of pattern. This interesting phenomenon was detected by an immunofluoresce study, and clarification of its nature awaits further study.

DETECTION OF CHROMATIN-BOUND PCNA IN THE BER PROCESS

Not only artificial base damage caused by agents, such as ionizing radiation (IR) or alkylating agents, but metabolically produced ones are considered to be repaired by the BER system. Repair is initiated by the removal of the damaged base by DNA glycosylase, and the 5' side of the apurinic / apyrimidinic (AP) sites is nicked by AP-endonuclease, after which the dRP or nucleotides are removed, and the small gap (1~several nucletides) is filled by DNA polymerase[21]. We found that IR also induces PCNA staining in normal and XP-A cells[26]. Confocal scanning of the PCNA staining showed innumerable small foci distributed all over the nucleus, indicative that the yield of causative DNA damage is high, presumably base damage or single strand breaks. This shows, for the first time the involvement of PCNA in short patch-type DNA repair, which is

surprising because IR-induced base damage has been considered to be repaired exclusively by pol β. This finding was confirmed by Stivala et al.[44] who used quantitative PCNA staining by flow cytometry. We further showed that there was no staining when ATP was depleted or when cells were irradiated at 0°C[45]. The staining diminished gradually with the increase in incubation time, eventually, disappearing almost completely about 12–15 h after irradiation[45]. Aphidicolin treatment did not inhibit staining, but did significantly inhibit its disappearance, which suggests that repair depends on pol δ /ε[45]. Unfortunately, as cells completely deficient in BER were not available, further specific study was impossible.

Matsumoto et al. developed an in vitro reconstituted BER system using extracts from xenopus laevis oocytes and showed that there are two pathways in BER which are pol β- and PCNA-dependent[46]. Similar results were obtained by Frosina et al. with extracts from hamster and human cells[47]. In contrast, Sobol et al., who used extracts from mouse embryo cells derived from pol β knockout and wild type littermates, reported that only the pol β-dependent pathway exists in BER[48]. Reduced and oxidized apurinic/apyrimidinic (AP) sites also have been reported to be repaired mainly by the pol β-dependent pathway in cooperation with DNaseIV/FEN1 stimulated by PCNA[49], which conclusion depended on the fact that the addition of anti-pol β antibody to the reaction mixture inhibited about 95% of BER activity[49]. Biade et al.[50], however, clarified the situation: i) the former two research groups used circular plasmids whereas the latter group used linear oligonucleotides as the DNA substrate; ii) PCNA can load and form the DNA clamp on circular plasmids but not on linear oligonucleotides[24], whereas pol β can bind to both and carry out DNA synthesis; and iii) in fact, extracts from pol β knockout cells did not repair AP sites on the oligonucleotides but did on the circular plasmids. Accordingly, when oligonucleotides were used as substrates the function of PCNA was underestimated under conditions in which it could not load onto DNA. Existence of the PCNA-dependent pathway was thus confirmed in vitro. The difference in the use of the two pathways in vivo is an interesting phenomenon that need to be clarified.

PERSPECTIVES

In this review, the role of PCNA in DNA excision repair is described mainly in terms of immunohistochemistry. The approach has been very useful in showing the potential role of PCNA but cooperative studies done with an in vitro reconstituted experimental system were essential to confirm that role. I believe that the immunofluorescence method also can be used to verify in vitro findings on the incision step because PCNA staining reflects in vivo incision activities in NER[29,39]. Several proteins, including XPG, are recently reported to bind to the specific site of PCNA[51–56]. Gary et al. identified a 29-amino acid region of human XPG that contains the PCNA binding activity[51]. Interestingly, the region shares key hydrophobic residues with the PCNA-binding domains of CDKN1A and FEN1[51]. It may be possible to examine the effect of this binding on incision activities in vivo if mutant proteins deficient in the PCNA binding site are expressed in the cells. The availability of cells with null mutation of specific genes by targeted disruption will extend the usefulness of the immunofluorescence study of PCNA. Pol β knockout

cells should provide useful information concerning the two different pathways in BER. Key information on transcription-coupled repair (TCR) mechanism, which is still behind that on global genome repair, may be given by immunofluorescence studies in combination with such genetic engineering. I strongly believe that these techniques will shed additional light on the true DNA excision repair mechanism.

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