Induction of DNA Replication-mediated Double Strand Breaks by Psoralen DNA Interstrand Cross-links

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The effect of DNA interstrand cross-links (cross-links) on DNA replication was examined with a cell-free SV40 origin-dependent DNA replication system. A defined template DNA with a single psoralen cross-link and the SV40 origin of replication was replicated by HeLa cell-free extract in the presence of SV40 large T antigen. The psoralen cross-link inhibited DNA replication by terminating chain elongation at 1–50 nucleotides before the cross-linked sites. The termination of DNA replication by the cross-links mediated the generation of double strand breaks near the cross-linked sites. These results are the first biochemical evidence of the generation of double strand breaks by DNA replication.

DNA interstrand cross-links (cross-links) are unique DNA damage because the two complementary strands of duplex DNA are covalently linked through DNA lesions across the strands. DNA replication and transcription are strongly inhibited by cross-links because the two strands of duplex DNA cannot be separated, and thus cross-links are highly cytotoxic (1). Cross-links are induced by commonly used chemotherapeutic agents, such as melphalan and cisplatin; however, very little is known about how cross-links affect on DNA replication or transcription at molecular level (2).

Cross-links are also challenging to DNA repair, because most DNA repair systems use the complementary strand to restore the genetic information after removal of DNA lesions from one strand of the duplex (3). The molecular mechanism of cross-link repair in mammalian cells remains to be elucidated. Genetic data implicated the involvement of XPF-ERCC1 complex, Rad51 paralogs (XRCC2, XRCC3, Rad51B, C, and D), and other double strand break (DSB) repair proteins because mutant cells defective in these factors are highly sensitive to DNA cross-linking agents such as mitomycin C (1, 4). XRCC2 (5) and XRCC3 (6) are involved in DNA double strand break repair by recombination. Recent biochemical data demonstrated that the Rad51 paralogs modulate Rad51-catalyzed DNA strand exchange (7, 8). The XPF-ERCC1 complex is a cross-link-specific 3’ to 5’ exonuclease (9) and is required for targeted homologous recombination as well (10, 11). In the cross-link repair reaction dependent on the XPF-ERCC1 complex and recombination machinery, DNA strand breaks may be requisite for XRCC2 and XRCC3 to participate in the cross-link repair reaction, and a 3’ end is needed for the 3’ to 5’ exonuclease activity of the XPF-ERCC1 complex. However, how these DNA breaks are generated and what proteins are required for generation of the breaks are not understood. Interestingly, DNA replication-dependent double strand breaks have been detected in mammalian cells after treatment of the cells with DNA cross-linking agents (12). Thus double strand breaks could be an important intermediate in cross-link repair in mammalian cells, because these breaks provide a 3’ end near the cross-linked site for the processing of the cross-link by the 3’ to 5’ exonuclease activity of the XPF-ERCC1 complex to generate an appropriate substrate for homologous recombination. It is also reported that DNA replication is required to elicit a cell cycle delay response to cross-link in mammalian cells (13). These data strongly implicate a crucial role of DNA replication in cross-link repair in mammalian cells.

To study the effect of cross-links on DNA replication, a cell-free SV40 ori-dependent DNA replication system (14) was used. DNA replication was inhibited by the psoralen cross-links and mediated formation of double strand breaks near the cross-linked sites. These DNA replication-mediated double strand breaks are likely the intermediates in cross-link repair reactions in human cells.

MATERIALS AND METHODS

Enzymes and Oligonucleotide—Restriction enzymes, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs (Beverly, MA). T4 DNA polymerase and T4 DNA ligase to prepare substrate DNA were purchased from Roche Molecular Biochemicals. SV40 large T antigen was purchased from Molecular Biology Resources Inc. (Milwaukee, WI). A 13-mer oligonucleotide (5'-GCTCGGTACCCGG-3') containing a furan-side psoralen mono-adduct (MA) was a generous gift from Dr. John Hearst at Cerus Corporation (Concord, CA).

Preparation of Substrate DNA—A 350-bp fragment containing the SV40 origin of replication was isolated from pCDNA 3.1 (from Invitrogen) and subcloned at the EcoRV site of pIBI25 plasmid DNA to generate pEVE. Using a single-stranded pEVE DNA as template, a covalently closed circular-defined substrate DNA was prepared by second strand synthesis with T4 DNA polymerase and T4 DNA ligase using a 13-mer primer containing a single furan-side psoralen mono-adduct (15). The reaction products were then digested by KpnI restriction endonuclease to eliminate non-damaged DNA. The presence of a psoralen mono-adduct or cross-link at the 5’-TA-3’ site in the middle of the KpnI recognition site completely inhibits digestion by KpnI (16, 17). After KpnI digestion, the covalently closed circular DNA was purified by CaCl2 gradient centrifugation, and the mono-adduct-containing DNA was irradiated with UVA to convert the mono-adduct to cross-link (15). The psoralen cross-link is located at a 205 nt away from the SV40 origin of replication in the pEVE plasmid (Fig. 1). A substrate without SV40 origin of replication was prepared by the same method except using a single-stranded pIBI25 DNA as template.

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DNA. A psoralen interstrand cross-link placed 205 nt from the 3'-end is located at the middle of the SV40 ori site (80). The NcoI site is in the middle of the SV40 origin of replication (SV40 ori). The bold arrows represent the leading strand synthesis, and the dashed arrows represent the lagging strand synthesis. After the replication reaction, a termination site of DNA chain elongation can be detected on a sequencing gel after NcoI digestion. If a DSB occurs near a cross-link site, a short fragment with a similar length to the NcoI-XhoI fragment (~200 bp) will be detected on an agarose gel. B, restriction sites near the cross-link. Smal, BamHI, XhoI, Sall, PstI, and Xhol sites are located at 3, 10, 15, 22, 24, and 39 nt away from the cross-link site toward the SV40 ori, respectively. NcoI is located 10 nt from the cross-link away from the SV40 ori.

Preparation of Nuclear Extract—Exponentially growing HeLa S3 cells (10 liters) were purchased from National Cell Culture Center. Nuclear extract was prepared following the method described in Dignam et al. (18). The nuclear extract was dialyzed against hypotonic buffer (20 mM HEPES-KOH, pH 7.8, 5 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol (DTT), and 15% glycerol) using micro-dialyzer (Pierce) before DNA replication assay.

DNA Replication Assay—Template DNA (25–100 ng) was replicated with 40 μg of cell-free extract in the presence of purified SV40 large T antigen (1 μg) by incubating at 30 °C for the indicated times in 12.5 μL of reaction buffer containing 30 mM HEPES-KOH, pH 7.8, 7 mM MgCl2, 4 mM ATP, 200 μM of NTPs, 100 μM of dNTPs with [α-32P]dATP (6000 Ci/mmol; PerkinElmer Life Sciences), 40 mM creatine phosphate, and 1.0 μg of creatine phosphokinase. After the reaction, DNA was deproteinized with proteinase K/SDS, extracted with phenol/chloroform and then purified by ethanol precipitation. The product DNA was resuspended in 10 μL of TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) and used for the various assays described below.

Determination of Replication Termination Sites—The replicated DNA was digested with NcoI restriction enzyme at 37 °C for 1 h and analyzed on a 4% sequencing gel. The replicated non-damaged pEV DNA was digested with NcoI and KpnI and used as a marker. Leading strand synthesis generates a 103-nt fragment, and lagging strand synthesis generates a 99-nt fragment.

Detection of Double Strand Breaks—Replicated DNA was digested with NcoI restriction enzyme at 37 °C for 1 h and analyzed on a 2% agarose gel. After drying, the gel was exposed and analyzed by phosphorimaging (Molecular Dynamics). If DSBs occur, fragments shorter than full-length pEV DNA will be detected because NcoI digests pEV DNA once. Reaction products with a substrate DNA without the SV40 ori site are separated on a 2% agarose gel, and the fragment released was isolated from the agarose gel. One-half of the DNA was dephosphorylated and then labeled with 32P using T4 polynucleotide kinase. The replicated non-damaged pEV DNA was digested with NcoI with KpnI, SalI, BamHI, XhoI, Sall, PstI, or Xhol and used as markers. Each double-digestion gives size markers for lagging and leading strand synthesis.

RESULTS

Inhibition of DNA Replication by a Psoralen Cross-link in Vitro—A psoralen interstrand cross-link was placed 205 nt from the SV40 origin of replication (Fig. 1A) in the pEV plasmid. This template DNA was incubated with 40 μg of cell-free extract prepared from HeLa cells in the presence of [α-32P]dATP and 1 μg of SV40 large T antigen. The incorporation of [32P]dAMP by DNA replication was greatly diminished by the presence of the cross-link (80–90% inhibition of the incorporation of 32P in acid-insoluble DNA compared with the non-damaged template; data not shown). To determine the
DNA replication termination sites by the cross-links, DNA from the replication reaction was digested with NcoI restriction enzyme, which cuts the template DNA once in the middle of the SV40 ori (Fig. 1A), and then analyzed on a 4% sequencing gel (Fig. 2). Several fragments (lanes 3, bracket) appeared only after NcoI digestion (compare lanes 2 and 3). The size of the largest fragment was shorter than the NcoI/KpnI fragment of the leading strand synthesis (lane 1, upper arrow), and the others were shorter than the NcoI/KpnI fragment of the lagging strand synthesis (lane 1, lower arrow) from the replicated nondamaged pEV DNA used as a marker. Using a template with a furan-side psoralen mono-adduct at the same position as the cross-link (on the template strand for the leading strand synthesis) as control (lanes 4 and 5), the largest fragment could be the result of the termination at one nucleotide before the cross-link on the leading strand synthesis (lane 7), and the others were the results of terminations before the cross-link site both from leading and lagging strand synthesis. These data demonstrate that DNA replication was terminated at multiple sites before the cross-links.

DNA Replication-mediated Generation of Double Strand Breaks Near Cross-linked Sites—Having established that DNA replication is inhibited by the psoralen-crosslinks, I next examined whether DSBs are induced during DNA replication reaction at the cross-link sites. After the replication reaction, the DNA was digested with NcoI and analyzed on a 2% agarose gel. About 2 and 0.1% of the total 32P incorporated were found in the short fragments in lanes 1 and 2, respectively, and these values were detected as a graph next to the gel. The short fragment in lane 2 was detected only after a longer exposure (data not shown). A non-damaged DNA was replicated with the same way and digested with NcoI and KpnI, and a part of the reaction was used as a marker (lane 3, NcoI/KpnI). It is noted that if the generation of DSB is not associated with DNA synthesis, the DSB cannot be detected under the assay conditions used. B, SV40 origin of replication and SV40 large T-antigen are required for generation of DSB at the cross-link site. A cross-link containing DNA (100 ng) was incubated in the presence (lanes 5 and 7) and absence of SV40 large T-antigen (lanes 4 and 6) and analyzed as described above. In lanes 4 and 5 the substrate with the SV40 ori was incubated for 2 h, and the DNA was digested with NcoI. In lanes 6 and 7, the substrate without the SV40 ori was incubated for 4 h, and the DNA was digested with EcoRI to detect DSBs. The induction of DSB required both SV40 ori and SV40 large T-antigen. No fragment was detected after a 2 day-exposure to phosphorimaging screen in lanes 4, 6, and 7. C, saturation of the generation of DSBs. A cross-link containing DNA (100 ng) was replicated, and the aliquots were withdrawn from the reaction mixture at the indicated time points and resolved on 2% agarose gels. The efficiency of the generation of DSB was quantitated as described above, and average values of three independent experiments were plotted as a graph next to the gel. Bars represent the standard error. Lanes 8 and 9, a cross-link containing DNA was incubated for 6 h under replication conditions in the absence of SV40 Tag. Lane 9 is a longer exposure of lane 8. Lanes 10–13, time course experiments. The arrow shows the DSB product. Nearly all of the products were replicated two rounds after 8 h incubation when non-damaged pEV was used as template under the same conditions (data not shown).

Omission of rNTPs and/or dNTPs also abolishes generation of DSBs (data not shown). Interestingly, a much lower level of DSB was detected with a template DNA containing a psoralen mono-adduct (MA) (Fig. 3A, lane 2; see the graph next to the gel), even though the mono-adduct was just as effective as the cross-link in the inhibition of replication (Fig. 2). The generation of DSBs reached a plateau after 4 h (Fig. 3C), presumably because of the competition between the ongoing DNA replication and cross-link repair.

I also determined the incision sites of DSBs on the template strand and whether the incision occurs before or beyond the cross-linked site relative to the direction of the movement of a replication fork. The short fragments generated by NcoI-digestion (indicated by arrows in Fig. 3) were isolated from the agarose gel, and the fragments were labeled by T4 polynucleotide kinase after dephosphorylation. Because the template strands are not labeled in the replication reaction, a new fragment after the kinase reaction most likely represents an incised fragment from the template strand. I confirmed that DSBs were generated near the cross-link because only the same sizes of the fragments as the replication termination fragments (see Fig. 2B, lane 3) were found in the isolated short fragment (Fig. 4, lane 1). A new fragment was not detected after the kinase reaction (Fig. 4, lane 2), indicating that the incision site(s) on the template strand to generate DSBs were similar in size to the termination fragments. These data suggest that incisions on the template strands during the generation of DSBs occur right across the replication termination sites, i.e. before the cross-link relative to the direction of the movement of a replication fork. The products from a reaction conducted under the same conditions except using non-radio-
FIG. 4. Mapping of the sites of DSB induced by replication. The substrate with a XL was replicated for 4 h as described in the legend to Fig. 2. After digestion with NcoI, the reaction products were separated on a 2% agarose gel, and the short fragment released was isolated from the gel. One-half of the isolated DNA was dephosphorylated and then labeled with 32P by T4 polynucleotide kinase. The isolated DNA before (lane 1) and after labeling (lane 2) was analyzed on a 4% sequencing gel. The bracket indicates the mixture of the termination sites of DNA replication (newly replicated strands, lane 1) and the incision sites on the template strands (lane 2). Replicated non-damaged DNA was digested by NcoI plus KpnI (K), SmaI (S), BumH1 (B), XhoI (X), SalI (SalI), PsI (P), or XhoI and used as markers. Each double-digestion gives size markers for lagging and leading strand synthesis.

active dNTPs, which were isolated from a 2% agarose gel after digestion with NcoI and labeled by T4 PNK after dephosphorylation, gave similar results (data not shown). I conclude that DSBs are generated near the cross-link site during DNA replication. This is the first demonstration using a defined substrate that DNA replication generates a DSB at or near a cross-link.

DISCUSSION

Little is known about the molecular mechanism of repair of DNA interstrand cross-links in humans. Based on the genetic data with DNA cross-linking agent-hypersensitive mutant cell lines, it has been proposed that a structure-specific endonuclease XPF-ERCC1 complex (4) and DSB repair proteins, Rad51 paralogs (1), and BRCA2 (19) play important roles in cross-link repair in mammalian cells. In addition, two recent findings suggest a crucial role of DNA replication in cross-link repair in mammalian cells. Hartley and co-workers (12) showed the induction of DSBs in dividing nitrogen-mustard-treated Chinese hamster ovary cells, but not in confluent cells, using pulsed-field gel electrophoresis. Grompe and co-workers (13) demonstrated that psoralen cross-links are not repaired in G1 or G2 phase of the cell cycle until the subsequent S phase. However, there is no direct evidence that DNA replication mediates formation of DSBs at or near cross-link sites. This report is the first biochemical demonstration of the link between DNA replication and cross-link repair. Based on the data presented here, I propose a molecular mechanism of cross-link repair in humans (Fig. 5). Three major steps will be considered in cross-link repair in humans: (1) generation of a DSB at or near a cross-link site, (2) unhooking of the cross-link, and (3) process-

ing of the DSB and the unhooked gapped DNA. DNA replication is inhibited near the cross-link sites probably for two reasons: one is due to the inability of the two strands to be unwound and another is because of the physical blockage to polymerase progression by the DNA lesions on each of the strands. A stalled DNA polymerase generates a unique DNA structure, which is a substrate for a structure-specific endonuclease to incise one of the template strands. The result is generation of a double strand break at one side of the growing fork and a gap on the other side. If an incision occurs on a template for leading strand synthesis, a 3’ end to the cross-link will be generated. The 3’ to 5’ exonuclease activity of XPF-ERCC1 complex will unhook the cross-link. After unhooking of the cross-link, the DSB and the gap are repaired by DSB repair. A lesion left on one strand after unhooking of the cross-link will be repaired by nucleotide excision repair.

A major role of DNA replication in cross-link repair will be formation of a “Y-shape” DNA structure at a stalled site. The Y-shape structure contains two junctures with different polarity. One in lagging strand synthesis is suitable for the FEN-1 family of nucleases (20) and another in leading strand synthesis is a good substrate for XPF-ERCC1 complex (21–24) and Mus81 nuclease (25). An incision in either juncture results in a formation of a DSB; however, the latter juncture will be preferable because an incision at this juncture will provide a 3’ nick to a cross-link, which is further processed by the cross-link-unhooking exonuclease activity of XPF-ERCC1 complex (9).
Identification of the corresponding nuclease in the generation of DSBs during DNA replication-mediated cross-link repair is under way. It will also be very interesting to examine whether a stalled DNA polymerase plays a direct role in recruiting an endonuclease to a cross-linked site.

Using a cell-free system without DNA replication, it has been shown that psoralen cross-links induce an XPF-ERCC1- and XRCC3-dependent DNA synthesis both in damaged and undamaged DNA (homologous or heterologous DNA), and hMutSβ and XPF-ERCC1 complex are required to unhook psoralen cross-links during the DNA synthesis in the absence of DNA replication (26, 27). Both reports demonstrated “DNA repair synthesis” in damaged DNA in the presence of exogenous DNA; however, it is not clear that the observed DNA synthesis is the complete repaired product or a futile DNA repair synthesis (9) because fully repaired fragments were not examined. It is very interesting to examine the effect of DNA replication on cross-link repair using the cell-free DNA replication system reported here.

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