Differential Requirement for Proliferating Cell Nuclear Antigen in 5’ and 3’ Nick-directed Excision in Human Mismatch Repair*

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Proliferating cell nuclear antigen (PCNA) is involved in mammalian mismatch repair at a step prior to or at mismatch excision, but the molecular mechanism of this process is not fully understood. To examine the role of PCNA in mismatch-provoked and nick-directed excision, orientation-specific mismatch removal of heteroduplexes with a pre-existing nick was monitored in human nuclear extracts supplemented with the PCNA inhibitor protein p21. We show here that, whereas 3’ nick-directed mismatch excision was completely inhibited by low concentrations of p21 or a p21 C-terminal fusion protein, 5’ nick-directed excision was only partially blocked under the same conditions. No further reduction of the 5’ excision was detected when a much higher concentration of p21 or a p21 C-terminal fusion protein was used. These results suggest the following: (i) There is a differential requirement for PCNA in 3’ and 5’ nick-directed excision; and (ii) 5’ nick-directed excision is conducted by a manner either dependent on or independent of PCNA. Our in vitro reconstitution experiments indeed identified a 5’ nick-directed excision pathway that is dependent on PCNA, hMutSα, and a partially purified fraction from a HeLa nuclear extract.

The DNA mismatch repair (MMR) system plays an important role in maintaining genomic stability in all living organisms, from bacteria to humans. The importance of the MMR system in humans is underscored by the fact that defects in this system are the pathological basis of hereditary non-polyposis colorectal cancer (HNPPC) and other sporadic cancers (1–3). At the present time, seven proteins (MutSα, MutLβ, MutLα, exonuclease 1, polymerase δ, PCNA, and replication protein A) are known to be required for human MMR, and completion of the repair involves the concerted action of these and other unidentified proteins. Initiation of MMR is thought to occur through the binding of either MutSα (the MSH2-MSH6 heterodimer) or MutLβ (the MSH2-MSH3 heterodimer) to a mismatch or a small insertion/deletion mispair, followed by the recruitment of MutLβ to form an initiation complex. Removal of the mispaired base(s) occurs by strand excision. In this poorly understood process, a strand break located either 5’ or 3’ to the mismatch can serve as a starting point for the unwinding of the DNA duplex to allow exonuclease digestion of the nicked strand. Recent studies have implicated exonuclease 1 (Exo1), a 5’ → 3’ exonuclease, in both 5’ and 3’ nick-directed excision (4, 5), but how this enzyme conducts excision for both orientations is not understood. Once the strand is degraded beyond the mismatch, DNA resynthesis occurs through the catalysis of the polymerase δ (6) in the presence of PCNA (7) and replication protein A (8, 9). The remaining nick is then sealed by an unidentified ligase, completing the repair process (for reviews, see Refs. 1–3).

Increasing evidence suggests that PCNA plays an important role in the MMR process. As a cofactor that greatly enhances the processivity of DNA polymerase δ, PCNA is required for DNA resynthesis in MMR (7). Work by Umar et al. (10) suggested a role for PCNA in MMR at a step prior to DNA resynthesis. Subsequently, PCNA was found to physically interact with MSH6 and MSH3 (11–14). More recent evidence indicates that PCNA may transfer MutSα to the mismatched site (15). Additionally, other studies suggest that PCNA also binds to many different kinds of DNA replication and repair proteins (for a review, see Ref. 16), including the flap endonuclease 1 (FEN-1). However, the involvement of PCNA in MMR initiation/excision is still not fully understood.

In this report, we show a differential requirement for PCNA in 3’ versus 5’ nick-directed excision in human MMR. Our data show that, whereas 3’ nick-directed excision is completely abolished by the PCNA-inhibitor p21, 5’ nick-directed excision is only partially sensitive to p21. These results suggest that, while 3’ nick-directed excision absolutely requires PCNA, 5’ nick-directed excision occurs in both PCNA-dependent and -independent manners. Therefore, we hypothesize that, as seen with Escherichia coli MMR, multiple exonucleases are involved in the human 5’ nick-directed pathway.

EXPERIMENTAL PROCEDURES

Nuclear Extract Preparation and Protein Purification—HeLa S cells were either purchased from the National Cell Culture Center (Minneapolis, MN) or cultured in our own laboratory, and nuclear extract was prepared as described previously (17). Constructs for the bacterial overexpression of human N-terminal hexahistidine-tagged PCNA and human N-terminal hexahistidine-tagged p21Cyc/WAF1 (referred throughout as p21C) were generously provided by Jerard Hurwitz (Memorial Sloan-Kettering Cancer Center, New York, NY) and Yue Xiong (University of North Carolina, Chapel Hill, NC), respectively. These proteins were overexpressed in E. coli BL21 DE3 (pLysS) and BL21 DE3

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cells, respectively, and purified to homogeneity as described previously (16, 17). Constructs coding for the overexpression of the glutathione-S-transferase (GST) fusion protein containing the C- or N-terminal domain of p21 (referred to as p21C or p21N, respectively) were generously provided by Anindya Dutta (University of Virginia, Charlottesville, VA), and these proteins were overexpressed in E. coli BL21 DE3 (pLysS) cells and purified to homogeneity using anion exchange and gluthathione-Sepharose chromatography as essentially as described (20). As originally constructed (21), the amino acid compositions for the p21N and p21C fusion proteins were GST1–218-SDP-p211–90 and GST1–218-SDPM-p21, respectively. hMutSo was purified from Si9 insect cells that were co-infected with baculoviruses containing the hMSH2 and hMSH6 cDNAs (a gift from Joe Jiricny, University of Zurich, Switzerland) as described (22). Before use, all proteins were centrifuged at 16,000 × g for 15 min at 4 °C to remove aggregated material, and the concentrations of the protein in solution were determined from UV absorbance measurements of native samples using extinction coefficients calculated from the amino acid sequences of the protein. All proteins were judged to be >95% pure.

**Heteroduplex Preparation**—Heteroduplexes used in this study contained a single G-T mismatch and a strand break at either 125 bp 5′ to the mismatch (5′ G-T substrate) or 172 bp 3′ to the mismatch (3′ G-T substrate, see Fig. 1). The 5′ G-T substrate was constructed by hybridizing Sau96I-digested hM13mp18-UKY1 dsDNA with hM1R1 single-stranded DNA and purified essentially as described previously (23). The 3′ G-T substrate was constructed by hybridizing PstI-digested M13mp18-UKY1 dsDNA with M13mp18-UKY2 single-stranded DNA. First, double-stranded M13mp18 DNA was double-digested with BamBI and BstI, and the larger fragment was purified and ligated with the oligonucleotide duplex 5′-ctggTCGACATTAGCAGTACGGCATTTACCTACGTCAAG-3′, where the nucleotides in small case indicate overhangs and the 3′-end strand near the SspI restriction site. Reaction products were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography. The nicked strand was repaired by proteinase K digestion, and DNA samples were recovered by phenol extraction and ethanol precipitation. For the 5′ substrate, the restriction enzyme used to construct the UKY1 and UKY2 phages by replacing the BamH I-SphII sequence of the derivative with oligonucleotide duplex I (UKY1; 5′-GATCATGCCATCGAGACATG-3′ and 3′-TACGTTGAGCTCT-5′) and oligonucleotide duplex II (UKY2; 5′-GATCATGCCATCGAGACATG-3′ and 3′-TACGTTGAGCTCT-5′), respectively, where the bold typed base pairs are the only difference between these two duplexes. The resulting phages were purified by DNA sequencing.

**Nick-directed Excision Assay**—Nick-directed excision was assayed essentially as described previously (17) in a 15-μl reaction mixture containing 100 ng of heteroduplex DNA, 50 μg of nucleotide extract, 10 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 110 mM KCl, and 1.5 mM ATP. No exogenous dNTPs were added to the mixture to minimize DNA resynthesis. After incubation at 37 °C for various times, reactions were terminated by proteinase K digestion, and DNA samples were recovered. To score the amount of excision beyond the mismatch, the 3′ substrate was digested with the restriction enzymes BstIRI and HindIII (the scoring enzyme; see Fig. 1), and the 5′ substrate with BgDI and NheI (the scoring enzyme; see Fig. 1). The products were electrophoresed on 1% agarose gels, and DNA bands were visualized by UV illumination in the presence of ethidium bromide. For the 5′ substrate, the band located at 6.4 kb corresponds to excision products (loss of NheI site). For the 3′ substrate, the band located at 7.1 kb corresponds to excision products (loss of HindIII site). When p21 or the p21C was exposed to a32P end-labeled oligonucleotide probe (5′-AATTTA-3′), which is complementary to the sequence on the nicked strand near the SspI restriction site, reaction products were visualized by autoradiography.

**RESULTS**

**p21 Inhibits 3′ but Not 5′ Nick-directed Mismatch Excision**—To more closely examine the role of PCNA in MMR prior to the DNA resynthesis step, we monitored the time course of mismatch-provoked, strand-specific excision in HeLa nuclear extract in the presence or absence of p21Cip1/WAF1 (p21), a protein that tightly binds to PCNA and blocks MMR (7, 10) and other DNA metabolic pathways that require PCNA (21, 25). A characteristic of MMR-associated excision is that the process starts at the strand break, proceeds to the mismatch along the shorter path between the two sites, and stops at a point ~150 nucleotides (nt) beyond the mismatch, thereby generating a single stranded gap on the substrate (26). Taking advantage of this property, we constructed nicked circular heteroduplex substrates that contain a restriction endonuclease site immediately 3′ or 5′ (depending on the excision orientations) to the mismatch (see Fig. 1). Thus, under conditions of limited DNA synthesis synthesis (i.e. in the absence of exogenous dNTPs), orientation-specific excision by MMR can be scored by conversion of the restriction sequence (NheI for the 5′ substrate and HindIII for the 3′ substrate) from double-stranded DNA to single-stranded DNA, rendering the DNA resistant to the enzyme (4, 27).

Fig. 2 shows a time course of mismatch-provoked excision by this analysis. At time zero, almost all of the DNA substrate (both 5′ and 3′ substrates) could be digested into two smaller fragments, indicative of no generation of a single-stranded DNA gap (i.e. no excision). As incubation time increased, reactions without p21 accumulated a larger molecular weight DNA species for both the 5′ and 3′ substrates, suggesting that a single strand gap extended (from the pre-existing strand break) beyond the HindIII (3′ substrate) or NheI (5′ substrate) site, because the excision products were resistant to digestion by the corresponding scoring enzymes (Fig. 2, A and B). At 25 min, the accumulation of the larger species of DNA reached a plateau, with 40 and 32% for 3′ and 5′ substrates, respectively. These numbers are essentially equivalent to the percentage of heteroduplexes repaired by HeLa nuclear extracts (data not shown; Ref. 17). The great majority of the excision apparently occurred in a manner dependent on a functional MMR system and on the presence of a mismatch, because nuclear extracts derived from hMLH1-deficient HCT116 (H6) cells (28, 29) could generate no more than 10% of HindIII- or NheI-resistant products (Fig. 2E) and data not shown) after 25 min of incubation. Additionally, much less excision was also observed in reactions containing a homoduplex (Fig. 2E).

Interestingly, when p21 was added to the reaction to inhibit
endogenous PCNA, there was a large difference in nick-directed, mismatch-provoked excision between reactions containing 5' and 3' substrates. For the 3' substrate, p21 at a concentration of 1.5 μM reduced gapped molecules by ~75% (compare A and C, Fig. 2). However, little reduction in gapped molecules was detected in reactions containing the 5' substrate under the same conditions (compare B and D, Fig. 2). These results strongly suggest a differential requirement for PCNA in 5' and 3' nick-directed, mismatch-provoked excision. To determine whether a higher concentration of p21 could completely block the 5' nick-directed excision, titration experiments were performed. We found that >95% of 3' nick-directed excision was inhibited by p21 at a concentration of 3 μM, but p21 had no significant effect on 5'-directed excision at concentrations approaching 10 μM (data not shown). Because of limitations in the solubility of recombinant p21, the highest useable concentration of this protein in the in vitro assay was at 10 μM. To confirm the results obtained with the full-length p21 protein, a fusion protein containing the C-terminal portion of p21 (p21C), a domain that is known to bind to PCNA with very high affinity (20), was used in this analysis. Fig. 3 shows the dependence of the level of MMR-dependent excision for both the 5' and 3' substrates on the concentration of p21C protein used over a two-order of magnitude range. Whereas p21C at a concentration of 0.3 μM completely blocked 3' nick-directed excision, the majority (63%) of 5' nick-directed excision was insensitive to p21C at much higher concentrations (from 0.3–3 μM). For a negative control, we also carried out similar experiments using a fusion protein containing the N-terminal domain of p21, which does not bind to PCNA (20). No obvious inhibition in excision was detected for either substrate at any of the concentrations of this protein used (data not shown). The results obtained using p21C largely confirm those that were obtained using the recombinant full-length p21 protein described above, i.e. there is a different utilization of PCNA in 3' and 5' nick-directed excision in human MMR. It is also noteworthy that, although the majority of the 5' nick-directed excision was tolerant to high concentrations of p21C, a significant fraction (37%) of the excision was blocked by the fusion protein at lower concentrations (Fig. 3). As demonstrated below in our in vitro reconstitution experiments, the p21C-inhibited 5' nick-directed mismatch excision is dependent on both a mismatch and hMutSα. Therefore, these experiments suggest that the 5' nick-directed excision may contain both the PCNA-dependent and -independent components.

To confirm the observation made using the restriction enzyme assay, Southern blot analysis was performed to directly visualize excision intermediates under conditions of limited DNA synthesis (24). DNA products were digested with SspI (in the case of 5' substrate) or SspI-DraIII (in the case of 3' substrate), and excision intermediates were mapped using a probe that hybridizes at a site on the nicked strand well beyond the mismatch (see the position of the probes in Fig. 1). As shown in Fig. 4, nicked strands that undergo mismatch-provoked excision are indicated by the appearance of shortened fragments on the gel as compared with the nicked strand of the un-reacted substrate (Fig. 4, lane 1 for 3' G-T and lane 5 for 5' G-T). As
expected, shortened fragments (<669 nt for the 3' substrate and <544 nt for the 5' substrate) were generated in the nicked strand when either the 3' G-T (lane 2) or the 5' G-T (lane 6) was incubated with HeLa nuclear extracts under conditions of limited DNA synthesis, indicative of occurrence of excision. For the 3' G-T substrate, this fragment pattern was found to largely disappear upon the addition of p21C to the reaction (Fig. 4, lane 3), suggesting that excision of the 3' substrate was inhibited by p21C in these conditions. The p21C inhibition on 3' substrate excision was apparently reversed when excess exogenous PCNA was added to the reaction mixture, as evidenced by the fact that excision intermediates reappeared in the reaction (Fig. 4, lane 4). In contrast to the results seen for the 3' substrate, the 5' substrate was found to undergo excision in the presence of p21C at levels similar to that seen in the absence of the fusion protein (compare lanes 6 and 7, Fig. 4). These observations further confirm that there is a differential requirement for PCNA in 3' and 5' nick-directed excision in human MMR.

\textbf{p21 Inhibits MMR of PCNA-independent 5' Nick-directed Excision at the DNA Resynthesis Step—}Previous studies have demonstrated that p21 completely inhibits MMR for both the 5' and 3' substrates (10). Our data show here that the inhibition for 3' substrates is at a step prior to or at excision, but this is largely not the case for the 5' substrate. To determine whether p21 blocks 5' nick-directed MMR at the DNA resynthesis step, repair assays were carried out in the presence of exogenous dNTPs, and repair products were assayed for the formation of a homoduplex at the mismatch site by virtue of their sensitivity to NsiI (for 3' G-T), or HindIII (for 5' G-T) (see Fig. 1), followed by Southern blot analysis. As expected, a band (497 nt in length for 3' G-T and 414 nt for 5' G-T; see Fig. 5, lanes 2 and 6, respectively) representing the repaired products was detected under normal conditions. When p21C was added to these reactions, the band representing the repair products was not detected for either substrate (Fig. 5, lanes 3 and 7 for 3' and 5' G-T, respectively). For the 3' substrate, two major NsiI-resistant bands were observed; the top band (964 nt in size) was the directly ligated substrate, and the lower band was the unreacted, nicked substrate. In the case of the 5' substrate, many bands smaller than 544 nt (Fig. 5, lane 7) were evident, indicative of the formation of excision intermediates. This result suggests that the excision in the reaction occurred, but the resynthesis was inhibited. However, the defect in DNA resynthesis was restored by the addition of excess amount of exogenous PCNA to the reaction (lane 8) as judged by the disappearance of the excision intermediates and the concomitant appearance of the repair product, i.e. the 414-nt band. These observations indicate that p21 inhibits 5' nick-directed MMR largely at the step of DNA resynthesis.

\textbf{Partial Reconstitution of the PCNA-dependent 5' Excision—}Although the 5' nick-directed mismatch excision is much less sensitive to p21 or p21C as compared with the 3' nick-directed excision, the addition of p21C at a relatively low concentration (0.6 µM) reduced the 5' nick-directed excision by 37%, suggesting an involvement of PCNA in the reaction. To explore this possibility, we utilized a partially purified phosphocellulose fraction (PF) that is required for mismatch-provoked, nick-directed excision in \textit{in vitro} MMR.\textsuperscript{7} We incubated this fraction along with a 5' G-T substrate in the presence or absence of exogenous hMutSα or PCNA. As shown in Fig. 6A, either PF alone or PF with either exogenous hMutSα or PCNA catalyzed minimal amounts of excision activity on the 5' G-T substrate (lanes 2–4). However, when all three components were mixed together (lane 5), 5' nick-directed excision was significantly stimulated compared with the levels found for the two component mixtures (PF-PCNA or PF-hMutSα). Notably, the level of excision using this partially purified extract and purified proteins reached a level that was even higher than that found for the whole cell nuclear extract. The stimulation of excision in

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\textsuperscript{7}F. Yuan, Y. Zhang, and G.-M. Li, unpublished results.
When present, p21C concentration was 0.06, 1.6, or 4.8 μM. In the presence of hMutSα or PF-hMutSα (5′ substrate mixtures) for the heteroduplex, excision was not stimulated by the addition of PCNA (5′ substrate) or HindIII (3′ substrate). DNA products were digested with NheI and HindIII, and the digested products were separated on a 6% polyacrylamide gel.

**TABLE**

|   | 5′ G-T | 5′ A-T | 3′ G-T |
|---|---|---|---|
| PF | + + + + + + | + + + + + + | + + + + + + |
| hMutSα | + + + + + + | + + + + + + | + + + + + + |
| PCNA | + + + + + + | + + + + + + | + + + + + + |
| p21C | - - - - - - | - - - - - - | - - - - - - |
| BSA | - - - - - - | - - - - - - | - - - - - - |

**Excision (%)**

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| PF | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| hMutSα | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| PCNA | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Fig. 6. Involvement of PCNA in 5′ nick-directed excision. DNA substrates were incubated with reaction mixtures containing different concentrations of 1.5 μg of a PF, 28 nM hMutSα, and 67 nM of PCNA, as indicated, for 20 min at 37 °C. DNA products were digested with NheI and HindIII, and the digested products were separated on a 6% polyacrylamide gel. The gel was stained with ethidium bromide.

**DISCUSSION**

In this report, we demonstrate a differential requirement for PCNA for 3′ versus 5′ nick-directed mismatch removal, suggesting that distinct mechanisms are used for mismatch excision at different orientations. This is demonstrated in Figs. 2 and 3, where p21 completely blocks 3′ but not 5′ nick-mediated excision. Our work supports previous observations that PCNA is required for MMR at a step prior to or at mismatch-provoked excision (10), in addition to participating in the step of DNA resynthesis (7). We also find, however, using a partially purified nuclear extract, that 5′ nick-directed excision is likely to be carried out using both PCNA-dependent and -independent manners.

It is known that mismatch excision requires an assembly of MMR initiation factors at the mismatch site, followed by recruitment of exonuclease(s) to execute excision. PCNA has been shown to interact with human MutS and MutL homologs (7, 10-14) and may function to recruit these proteins to the mismatch site (15), indicating an involvement of PCNA in the assembly of the MMR initiation complex. On the other hand, PCNA may help to recruit or activate exonuclease(s). This assumption is based on the fact that PCNA is known to interact with and greatly enhance the activity of FEN-1 (reviewed in Ref. 16), a nuclease that may play a role in MMR (30), and that PCNA may be capable of interacting with other nucleases. However, it is not known whether the PCNA differentiation for mismatch excision in different orientations is made at the repair initiation level or at the excision level, or both.

Using the p21 C-terminal fusion protein, we show that even though the majority (63%) of 5′ nick-directed mismatch excision seemed to be insensitive to p21C (or independent of PCNA), more than one-third of excision was inhibited by the fusion protein (Fig. 3). This phenomenon suggests that 5′ nick-directed excision can occur in both PCNA-dependent and -independent manners. Available evidence appears to support this hypothesis. In our in vitro reconstitution experiments, we show that PCNA-hMutSα greatly stimulated 5′ nick-directed excision, suggesting the presence of a PCNA-dependent excision pathway (Fig. 6). Although Exo1 (a 5′ → 3′ exonuclease) has been shown to be involved in 5′ nick-directed MMR (4, 5), the PCNA-dependent excision we observed may not be conducted by Exo1. During the preparation of this manuscript, a study was published that demonstrated that human Exo1 catalyzed 5′ nick-directed excision in a manner independent of PCNA (31). Based on the fact that PF-catalyzed 5′ excision depends on PCNA and that HeLa extract-catalyzed 5′ excision is largely insensitive to p21C, we hypothesize that there are at least two types of nucleases that are involved in 5′ nick-directed excision in human MMR, i.e. one whose activities are not enhanced by PCNA (i.e. Exo1), and another whose activities are enhanced by PCNA (and whose identities remain to be identi-
The involvement of multiple exonucleases for the 5′ → 3′ orientation excision in the human cells would be homologous to the E. coli MMR system, as at least four exonucleases (two for each orientation) are implicated in mismatch excision in E. coli (32). Evidence from recent Exo1 knockout studies also supports this hypothesis (5). First, even though Exo1−/− mice display increased Hprt mutability, the mutation rate is 5-fold lower than that in Msh2−/− mice. Second, Exo1−/− cells still possess residual activity (~20% compared with wild type) to repair a 1-nt insertion/deletion mispair, a heteroduplex that is believed to be processed only by the MMR pathway. Finally, whereas all Msh2−/− animals were dead of cancer by 12 months of age, only 50% of Exo1−/− mice died at 17 months (5). The weaker mutator phenotype and reduced tumorigenicity in Exo1−/− mice suggest that MMR activity is not completely blocked in Exo1−/− mice, which is consistent with a notion that Exo1 is not the only nuclease involved in mammalian MMR. Additionally, evidence from yeast studies has suggested that, in addition to Exo1, additional nucleases, such as Rad27 (also called Rth1, a FEN-1 homolog) and the exonuclease activities associated with DNA polymerases δ and ε, may also function at the excision step of MMR (30, 33, 34).

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Differential Requirement for Proliferating Cell Nuclear Antigen in 5′ and 3′
Nick-directed Excision in Human Mismatch Repair
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