Recruitment of mismatch repair proteins to the site of DNA damage in human cells

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Summary

Mismatch repair (MMR) proteins contribute to genome stability by excising DNA mismatches introduced by DNA polymerase. Although MMR proteins are also known to influence cellular responses to DNA damage, how MMR proteins respond to DNA damage within the cell remains unknown. Here, we show that MMR proteins are recruited immediately to the sites of various types of DNA damage in human cells. MMR proteins are recruited to single-strand breaks in a poly(ADP-ribose)-dependent manner as well as to double-strand breaks. Using mutant cells, RNA interference and expression of fluorescence-tagged proteins, we show that accumulation of MutSβ at the DNA damage site is solely dependent on the PCNA-binding domain of MSH3, and that of MutSα depends on a region near the PCNA-binding domain of MSH6. MSH2 is recruited to the DNA damage site through interactions with either MSH3 or MSH6, and is required for recruitment of MLH1 to the damage site. We found, furthermore, that MutSβ is also recruited to UV-irradiated sites in nucleotide-excision-repair- and PCNA-dependent manners. Thus, MMR and its proteins function not only in replication but also in DNA repair.

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Key words: DNA strand breaks, PCNA, UV damage, In situ analysis, Mismatch repair

Introduction

DNA mismatch repair (MMR) increases the fidelity of DNA replication and plays an important role in safeguarding genetic stability (Iyer et al., 2006; Jiricny, 2006). Its best understood function is the elimination of mismatches arising during DNA replication. In eukaryotes, mismatches produced during replication are recognized by the heterodimers MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3), the former of which recognizes mainly base-base mismatches and the latter insertion-deletion loops. The heterodimer MutLα (MLH1/PMS2) forms a ternary complex with one of the MutS complexes and promotes the repair process via its endonucleolytic activity, leading to an excision repair of the mismatch (Kadyrov et al., 2006). Defective MMR leads to an increased risk of cancer, because of an elevated rate of spontaneous mutation.

In addition to its role in correcting mismatches formed during replication, MMR has been implicated in the cellular DNA damage response (for reviews, see Iyer et al., 2006; Jiricny, 2006; Jun et al., 2006; Stojic et al., 2004). The roles played by MMR and MMR proteins in the DNA damage response include activating cell-cycle checkpoints and apoptosis. MMR-defective cell lines are more resistant to cell death induced by several DNA-damaging agents, including methylation agents, cisplatin and UV radiation, whereas they are more sensitive to cell death caused by interstrand crosslinking agents. The MMR status determines the sensitivity of cells to H_2O_2, suggesting that MMR and/or MMR protein complexes may be involved directly in DNA damage responses. Moreover, it has been suggested that MMR can play a role in repair of double-strand breaks (DSBs) (Evans et al., 2000; Sugawara et al., 1997), which is performed by homologous recombination and non-homologous end joining (NHEJ). It has been shown that, during homologous recombination, MMR proteins localize to recombination intermediates and suppress recombination between divergent sequences (Elliott and Jasin, 2001; Evans et al., 2000; Villemure et al., 2003). MMR proteins also influence NHEJ during the pairing of terminal DNA tails (Bannister et al., 2004; Smith et al., 2005).

Although these damage responses are believed to occur at replication fork, it is not known whether MMR proteins are recruited to the sites of DNA damage. In vitro studies showed that MutS complexes do not bind to DNA damage, unless it is a compound lesion containing both mismatch and damaged DNA (Mu et al., 1997). However, various types of DNA excision repair include repair synthesis, where misincorporation may frequently occur. We were therefore interested in whether MMR complexes are recruited to sites of DNA damage in living human cells. Our data, obtained by three different methods for production of local damage, show that MMR proteins are recruited immediately to single-strand breaks (SSBs), DSBs and UV-induced DNA damage in human cells. Recruitment is mediated not by direct binding of the MutS complex to DNA damage, but by protein-protein interaction. Our data indicate that MMR and MMR proteins play important roles in cellular responses and repair synthesis at the sites of various types of DNA damage in cells of human and other species.

Results

Recruitment of mismatch repair proteins to the sites of DNA damage

We previously established laser micro-irradiation systems that can induce various types of DNA damage locally in the nucleus of living cells and we investigated proteins responding to the DNA damage as real-time images (Hashiguchi et al., 2007; Lan et al., 2005; Lan et al., 2004b). We used this laser micro-irradiation system to analyze the response of MMR proteins to DNA damage.

Since laser light of 405 nm wavelength is hardly absorbed directly by DNA, it is reasonable to suppose that the laser light is absorbed by chromophores near to DNA within the cell and radicals produced by the photosensitization attack DNA. Therefore, oxidization of DNA is the product of the laser irradiation and the major damage type is DNA strand break and oxidative base damage. In the presence of the photosensitizer BrdU, DNA strand breaks, especially DSBs, are effectively produced at laser-irradiated sites, as determined by intensive accumulation of proteins involved in NHEJ and homologous recombination (Hong et al., 2008; Lan et al., 2005). Under the irradiation conditions used in this work, around 1000 DSBs were produced in a scanning path, which corresponds to the irradiation of a human cell with 30 Gy of X-rays (Hong et al., 2008) (see Materials and Methods). Using this system and antibodies against human MSH2, MSH3 and MSH6, we first analyzed whether or not these proteins are present at laser-irradiated sites in HeLa cells. Indeed, we found, by immunostaining immediately after irradiation, that endogenous MSH2, MSH3 and MSH6 accumulated at the irradiated site and colocalized with γH2AX (Fig. 1A, top, middle and bottom panels, respectively). We confirmed the specificity of the antibody reaction to MSH3 and MSH6 by immunostaining of the human cell line HCT116, which is MLH1-deficient and, in addition, does not express MSH3 and expresses MSH6 only weakly (Fig. 1B, upper and middle panels) (Chang et al., 2000). Specific recognition of MSH2 by the antibody was also confirmed using LoVo cells, which are defective in MSH2 expression (Fig. 1B, bottom panels). Thus, endogenous MSH proteins accumulate at the site of laser irradiation.

Laser micro-irradiation under the conditions used here produces mainly strand breaks, especially SSBs and DSBs. We, therefore, analyzed whether MMR proteins accumulate at the site of SSBs by using XPA-UVDE cells, which express UV damage endonuclease (UVDE) in nucleotide-excision repair (NER)-deficient xeroderma pigmentosum A (XPA) cells and converts UV lesions (cyclobutane pyrimidine dimers and 6-4 photoproducts) to SSBs by the action of UVDE at UVC-induced lesions (Okano et al., 2003). To produce SSBs locally in the nucleus of cell, cells were irradiated with UVC through a porous filter. As shown in Fig. 1C, all the MMR proteins were identified at the site where XRCC1 accumulated. (It will be shown later in this paper that the MMR proteins do not accumulate at UV lesions in NER-deficient cells.) Therefore, the result indicates that MMR proteins accumulate at UVDE-induced SSBs. Since the repair of SSB repair is initiated by the accumulation of XRCC1 at poly(ADP-ribose) synthesized at SSBs by poly(ADP-ribose) polymerase (Lan et al., 2004b; Okano et al., 2003), we pretreated XPA-UVDE cells with an inhibitor of poly(ADP-ribose) polymerase, 1,5-dihydroxyisoquinoline (DIQ) and analyzed the accumulation of MSH2 at the UV-irradiated site. As shown in the upper panels of Fig. 1D, MSH2 and XRCC1 did not make foci in the cells treated with DIQ, indicating that accumulation of MSH2 at SSBs is poly(ADP-ribose)-dependent. However, laser-induced accumulation of MSH2 could not be abrogated by DIQ (Fig. 1C, lower panels). Similar data were also obtained for MSH3 and MSH6 (not shown). This indicates that lesions at which MSH2 accumulated after laser irradiation include those other than SSBs. As explained below, the major target of the accumulation of MMR proteins after the laser irradiation is DSBs, although other lesions such as base damage might also contribute to the accumulation.

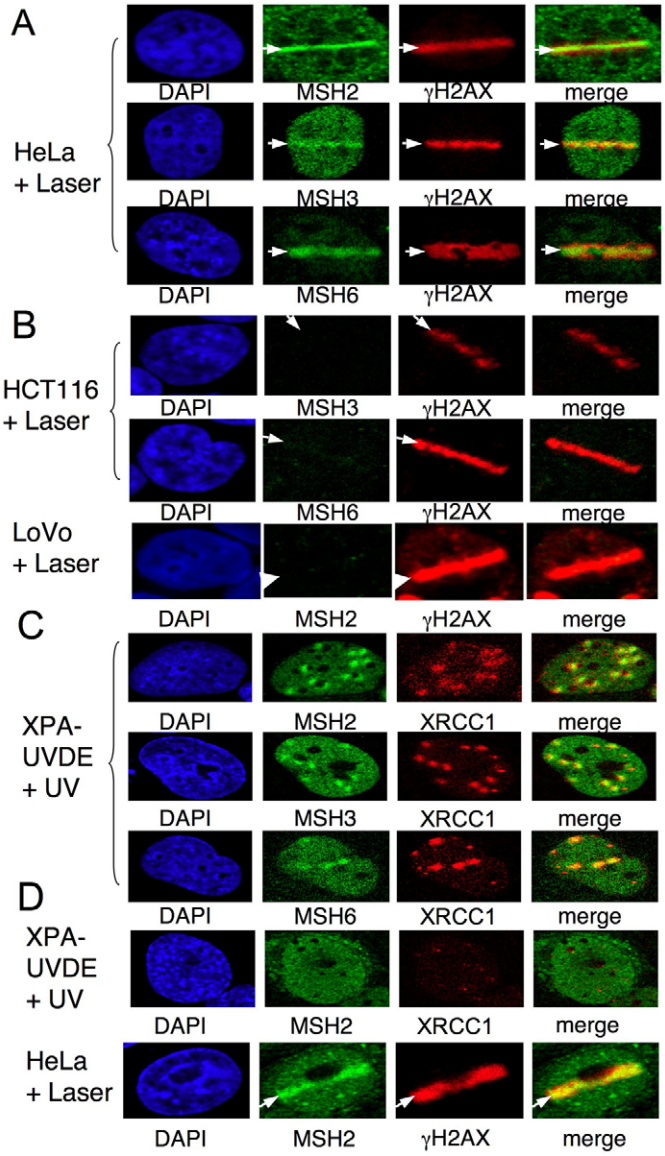

Fig. 1. Accumulation of MSH2, MSH3 and MSH6 at the sites of DNA strand breaks. Accumulation of MSH2, MSH3 and MSH6 at the laser-irradiated site. Immediately after laser irradiation cells were stained with antibodies against MSH2, MSH3 and MSH6 in HeLa cells (A), MSH3 and MSH6 in HCT116 cells (B, upper two lines of panels) and MSH2 in LoVo cells (B, bottom panels). Scanned site is indicated with a white arrow. γH2AX was stained and the images were merged. (C) Accumulation of MMR proteins at UVDE-induced SSBs and their colocalization with XRCC1 in XPA-UVDE cells after local UV irradiation (20 J/m^2). (D) Influence of the poly(ADP-ribose) polymerase inhibitor DIQ on the accumulation of MSH2 after UV irradiation in XPA-UVDE cells (upper panels) and after laser irradiation in HeLa cells (lower panels).

MSH3 and MSH6 accumulate at DNA damage sites independently of MSH2

In order to characterize the mechanisms of accumulation of MMR proteins at laser-irradiated sites, we attached the green fluorescent protein (EGFP) to MSH2, MSH3 and MSH6, introduced these into various cell lines and analyzed the response of the expressed fusion proteins to micro-irradiation in human cells (500 scans in the presence of BrdU). As shown in Fig. 2A, all of the GFP-tagged MMR proteins accumulated rapidly at laser-irradiated sites in HeLa

cells, MSH2 and MSH3 appearing within 1 minute, with MSH6 accumulating more slowly (see below). Accumulation of the GFP-tagged MMR proteins was observed in all cells transfected with a low or high level of expression and the proteins were still observed at the sites of irradiation after 1 hour (Fig. 2A, far right panels). Similar results were obtained by immunostaining of endogenous proteins (not shown). Repair proteins for SSBs and base damage dissociate very rapidly (Lan et al., 2004b), unlike proteins involved in DSB repair (NHEJ and homologous recombination), which can remain at the irradiated site for much longer than 1 hour (Kim et al., 2005). This suggests that DIQ-resistant accumulation of MMR proteins mainly targets DSBs after laser irradiation under the conditions used here.

MSH2, MSH3 and MSH6 are present in the heterodimers MutSα (MSH2,MSH6) and MutSβ (MSH2,MSH3). Having demonstrated that MMR proteins are recruited to laser-induced DNA damage sites, we analyzed, by using GFP-tagged MMR proteins and laser microirradiation, which proteins are responsible for organizing recruitment of the complexes to laser-irradiated sites. In HCT116 cells, we observed no accumulation of mDR-MSH2 (and GFP-MSH2, not shown) (Fig. 2B, left), following laser irradiation, whereas both GFP-MSH3 and GFP-MSH6 were recruited to laser-irradiated sites (Fig. 2B, middle and right panels). Interestingly, coexpression of either GFP-MSH3 or GFP-MSH6 with mDR-MSH2 restored recruitment of the MSH2 in the HCT116 cell line (Fig. 2C,D, left panels), suggesting that high expression of either MSH3 or MSH6 is required for the recruitment of MSH2. In order to see whether accumulation of MSH3 or MSH6 requires MSH2, we used the MSH2-defective cell line LoVo (Fig. 2E, left blot). As shown in Fig. 2E (right panels), both GFP-tagged MSH3 and MSH6 accumulate at laser-irradiated sites in LoVo cells. These data suggest that overexpressed MSH3 and MSH6 accumulate at laser-irradiated sites in the absence of MSH2.

Since MMR-defective cell lines might have additional mutations, which could have influenced the DNA damage response, we used siRNA to suppress MMR gene expression in HeLa cells and analyzed the damage responses of MMR proteins. Fig. 2F depicts the suppression of MSH2 gene expression in HeLa cells (left) and accumulation of GFP-MSH3 and GFP-MSH6 at laser-irradiated sites in MSH2-depleted cells (right panels). MSH3 and MSH6 accumulated at the irradiated sites in MSH2-depleted cells. To see the influence of depleted MSH3 or MSH6 on the accumulation of MSH2 at DNA damage sites, each gene was suppressed separately by siRNA in HeLa cells (Fig. 2G, western blots). As shown in Fig. 2G (right panels), suppression of either MSH3 or MSH6 expression did not influence the accumulation of GFP-MSH2. However, if both genes were suppressed simultaneously (Fig. 2H), accumulation of GFP-MSH2 at the laser-irradiated site is abrogated (Fig. 2H, right panels). Thus, MSH2 is

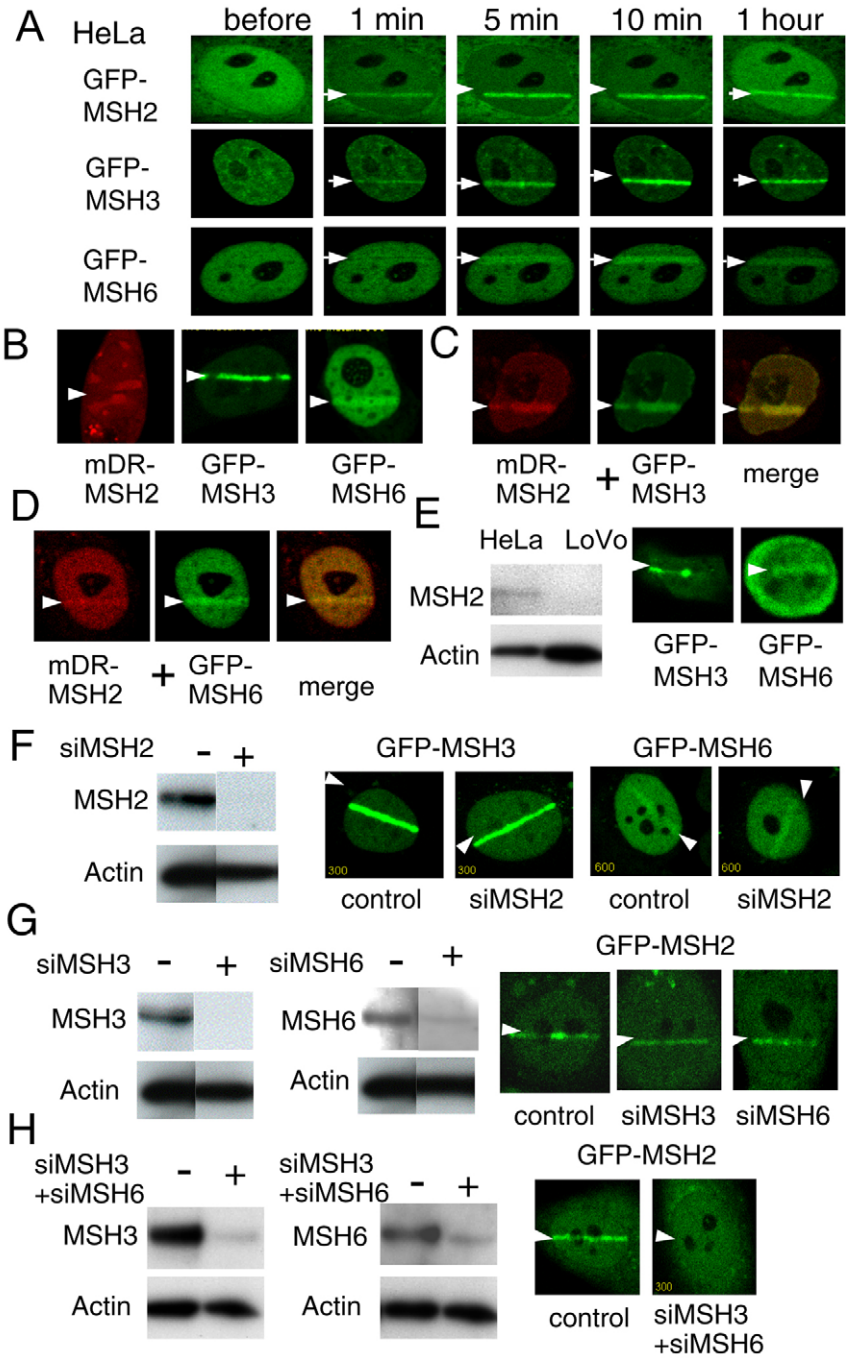

Fig. 2. Accumulation of GFP-tagged MSH2, MSH3 and MSH6 at the laser-irradiated site. (A) Accumulation kinetics of GFP-tagged MSH2, MSH3 and MSH6 at laser-irradiated site in HeLa cells. (B) GFP-MSH3 and MSH6 but not mDR-MSH2 accumulate at laser-irradiated site in HCT116. (C) mDR-MSH2 cotransfected with GFP-MSH3 or with GFP-MSH6 (D) accumulates at laser-irradiated site in HCT116 cells. (E) Western blot analysis of MSH2 in HeLa and LoVo cells (left). Accumulation of GFP-MSH3 and GFP-MSH6 at laser-irradiated site in LoVo cells (right). (F) Accumulation of GFP-MSH3 and GFP-MSH6 at laser-irradiated site after suppression of MSH2 expression in HeLa cells. (G) Influence of suppression of either MSXH3 or MSH6 on the accumulation of GFP-MSH2 at laser-irradiated site in HeLa cells. (H) Influence of suppression of both MSXH3 and MSH6 on the accumulation of GFP-MSH2 at laser-irradiated site in HeLa cells. More than 10 cells are analyzed for each condition and representative data are shown.

recruited to the laser-irradiated site through interaction with either MSH3 or MSH6.

MSH3 accumulates at laser-irradiated sites via its PCNA-interacting domain

We examined which regions in MSH3 are necessary for its accumulation at the laser-induced DNA damage sites. Fig. 3A (top left) shows a schematic representation of MSH3 as well as the deletion constructs used in the experiments. MSH3 interacts with proliferating cell nuclear antigen (PCNA) via conserved PCNA-recognition motifs near the N-termini (Kleczkowska et al., 2001). Therefore, we analyzed whether or not PCNA is implicated in the accumulation of MSH3 at laser-induced DNA damage sites. We used GFP fusion proteins containing full-length or various deletions of MSH3. Although the MSH3 N-terminal region (residues 1-57) accumulated at laser-irradiated sites, a deletion mutant lacking the N-terminus (residues 75-1137) did not (Fig. 3A left and middle panels, respectively). Furthermore, a GFP-tagged full-length MSH3 fusion protein containing two amino acid substitutions in the putative PCNA-binding domain (F27A/F28A) did not accumulate at DNA damage (Fig. 3A, bottom, right panels). Thus, MSH3 accumulates at DNA damage in a manner dependent on the N-terminal PCNA-binding domain (Fig. 3A, top, right). Fragments harboring MSH2-binding domains (residues 75-1137) did not accumulate at DNA damage sites at all, as suggested by the results obtained using siRNA experiments in HeLa cells (Fig. 2).

Since PCNA can accumulate at laser-induced DNA damage in wild-type cells as well as in HCT116 cells (Fig. 3B), PCNA requires neither of these MSH proteins for its accumulation at DNA damage. An extremely high overexpression of a full-length PCNA containing the mutation D41A, which impairs loading of PCNA onto DNA (Zhang et al., 1999) and does not accumulate at laser-irradiated sites (Fig. 3C, lower panels middle), abrogates accumulation of the GFP-MSH3 at laser-irradiated sites in a dominant negative fashion (Fig. 3C, lower panels left). However, a similar overexpression of wild-type PCNA did not influence the accumulation of GFP-MSH3 (Fig. 3C, upper panels). The MSH3 N-terminus alone did not recruit MSH2 to laser-irradiated sites in HCT116 cells (not shown). Using HCT116 cells, if we expressed mDR-MSH2 and GFP-MSH3 with two mutations abolishing its PCNA binding ability (F27A and F28A), MSH2 did not accumulate at the irradiated site (Fig. 3D), although only the interaction of MSH3 with PCNA is lacking. These data confirmed that the MutSβ complex is recruited to the site of DNA damage by the interaction of MSH3 with PCNA.

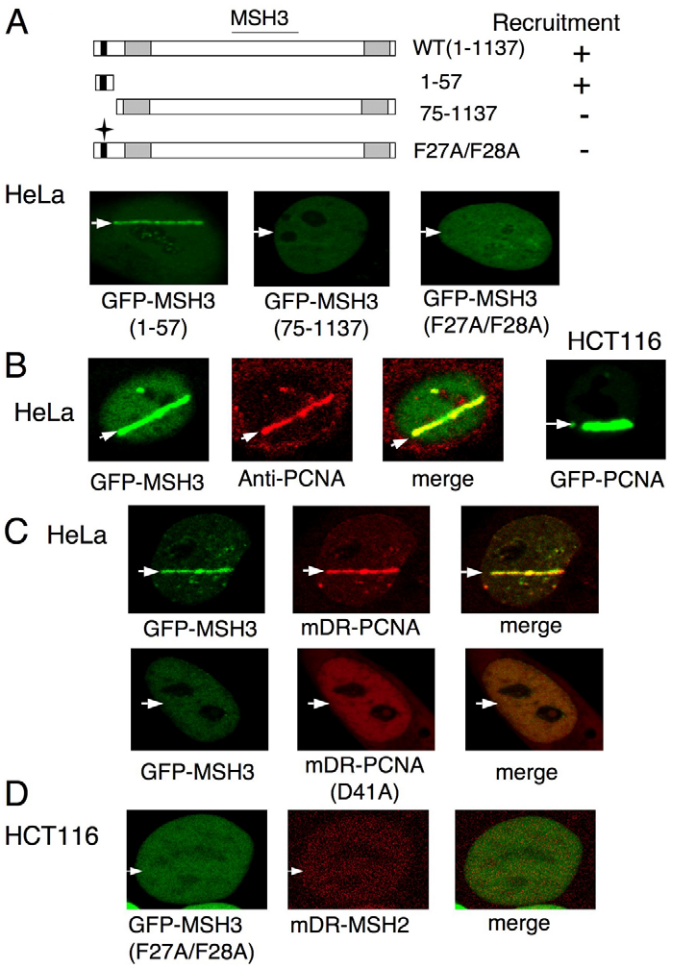

Fig. 3. Recruitment of MSH3 to laser-irradiated site is mediated by PCNA. (A) Schematic representation of MSH3 and its deletion mutants and the results of recruitment experiments. Black and gray boxes indicate PCNA-binding motif and MSH2-binding domains, respectively. The site of mutation (F27A/F28A) is indicated with a cross. (B) GFP-MSH3 colocalizes with endogenous PCNA at sites of laser-irradiation. GFP-PCNA is recruited to the site of laser-irradiation in HCT116 cells (right panel). (C) Coexpression of GFP-MSH3 with mDR-PCNA wild-type (upper panels) or with mDR-PCNA mutant (D41A) (lower panels) in HeLa cells. (D) mDR-MSH2 was not recruited to the site of laser-irradiation by coexpression of the GFP-tagged mutant MSH3 (F27A/F28A) in HCT116 cells. Arrows indicate the sites of irradiation.

MSH6 has two independent domains recruited to laser-induced DNA damage sites

Similarly to MSH3, MSH6 contains a functional PCNA-binding motif at the N-terminus (residues 1-77), as well as two MSH2-binding domains (Fig. 4 A, top left). A GFP-tagged fusion protein containing the N-terminus (residues 1-77) of MSH6 accumulated at laser-irradiated sites (Fig. 4B, top). However, the N-terminal deletion mutant of MSH6 (residues 78-1360), which is unable to interact with PCNA (Kleczkowska et al., 2001; Shell et al., 2007), still accumulated at laser-irradiated sites (Fig. 4B, the second panels from the top), suggesting that MSH6 contains another domain that responds to DNA damage. Previous work has shown that a yeast MSH6 mutant lacking the PCNA interaction activity, displayed only a modest increase in mutability in vivo (Flores-Rozas et al., 2000; Lau et al., 2002). More recently, the N-terminal region of MSH6 has been shown to contain two functional regions, either of which is sufficient for most MMR activities; the anterior region is involved in PCNA interactions, whereas the function of the posterior region is not yet known (Shell et al., 2007). We found that residues 78-368 of MSH6 responded to DNA damage and that it was recruited more rapidly and efficiently to laser-irradiated sites than the extreme N-terminus (residues 1-77) (Fig. 4B, second panels from the bottom; and Fig. 4C). By contrast, a deletion mutant lacking a large section of the N-terminus (residues 369-1360) was not recruited to the site of DNA damage (Fig. 4B, bottom panels). As shown in Fig. 2E and 2F, MSH6 accumulated at the site of DNA damage in MSH2-deficient or depleted cells. In contrast to the results obtained with MSH3 mutated in the PCNA-binding domain (Fig. 3D), MSH2 accumulated at DNA damage sites in HCT116 cells when coexpressed with MSH6 lacking the PCNA binding domain (Fig. 4D), indicating the

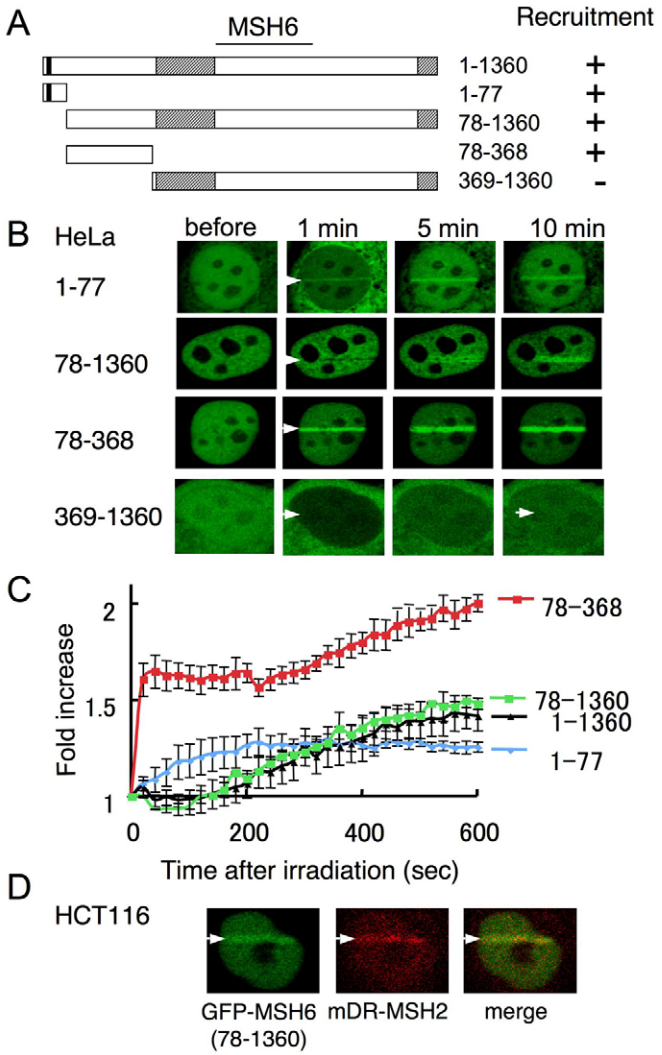

Fig. 4. MSH6 is recruited to the site of laser irradiation via its N-terminal region. (A) Schematic representation of the MSH6 and deletion mutants (left) and the results of recruitment experiments (right). Black and gray boxes indicate PCNA-binding motif and MSH2-binding domains, respectively. (B) Recruitment of MSH6 deletion mutants. Arrows indicate the sites of irradiation. (C) Accumulation kinetics of GFP-tagged MSH6 and its deletions. Data are means ± s.e.m. of 3-5 independent experiments. (D) Accumulation of mDR-MSH2 in HCT116 by coexpression of GFP-MSH6 (78-1360).

importance of residues 78-368 in the N-terminus of MSH6 for the recruitment of MutSα to DNA damage sites. We compared the accumulation kinetics of GFP-tagged deletions with that of the full-length MSH6 (Fig. 4C). Although the accumulation of the extreme N-terminus (residues 1-77, blue line) is rapid but weak, accumulation of the region of the N-terminus beyond this (residues 78-368) is very strong and occurs in two phases, a rapid and a slow accumulation phase (indicated by red line in Fig. 4C). The full-length MSH6 accumulates more slowly than either of the N-terminal regions and its kinetics was similar to that obtained for MSH6 without the PCNA-binding domain (residues 78-1360) and similar to the later and slower part of the two-phases kinetics of residues 78-368. These data suggest that the region encompassing residues 78-368 of the N-terminus determines the accumulation of MSH6 at DNA damage site, whereas the N-terminus may bind to PCNA after the protein has accumulated at the site of DNA damage.

Recruitment of MLH1 to DNA damage is dependent upon the MSH2-interacting domain

The presence of MutSα or MutSβ alone is not sufficient for MMR during replication. MLH1 and PMS2 form the complex MutLα, which further forms a ternary complex with either MutSα or MutSβ during MMR at replication. Therefore, we investigated whether MLH1 is also recruited to laser-induced DNA damage sites. Indeed, we observed the presence of endogenous MLH1 at the irradiated sites (Fig. 5A, upper panels), suggesting that MutLα also responds to DNA damage. MLH1 is not expressed in HCT116 cells and therefore, the antibody did not detect the protein at the laser-irradiation site (Fig. 5A, lower panels). GFP-tagged MLH1 accumulates rapidly at the site of laser irradiation within 5 minutes of irradiation and remains for more than 1 hour, as is the case for other MMR proteins (Fig. 5B). MutLα interacts with the MutS complex via the N-terminus of MLH1 (Plotz et al., 2003). In addition, MLH1 has recently also been shown to interact with PCNA via a PCNA-binding motif near its C-terminus (Lee and Alani, 2006). Therefore, we used GFP fusions of C- and N-terminal MLH1 deletion mutants (Δ1-389 and Δ389-756, respectively) to find out which MLH1 regions were required for accumulation at DNA damage sites. We observed that the N-terminal fragment was recruited to DNA damage, whereas the C-terminal fragment was not, suggesting that MLH1 is recruited to DNA damage not by interaction with PCNA, but rather with the MutS complex (Fig. 5C). These data strongly suggest that MutL complexes are also recruited to the sites of DNA damage in a sequential fashion involving protein-protein interaction, similarly to MMR at DNA replication.

MSH3 and MSH2 colocalize with PCNA at UV-irradiated sites in an NER-dependent manner

Since PCNA is involved in various repair processes, including NER of UV-induced DNA damage (Essers et al., 2005a; Essers et al., 2005b; Shivji et al., 1995), we asked whether MMR proteins are recruited to UV-irradiated sites. XPA-WT cells, in which XPA cDNA is expressed in the NER-deficient XPA cell line (XP12ROSV), shows an equivalent UV-resistance to wild-type human cells (Lan et al., 2004a; Satokata et al., 1992). In these cells, endogenous MSH2 was recruited to the locally UV-irradiated sites, where it colocalized with PCNA (Fig. 6A, upper panels). Similar data were obtained in HeLa cells (not shown). However, when the control XPA-C2 cell line, which is the XPA cell line transfected with vector plasmid, was used, neither MSH2 nor PCNA was recruited to UV-irradiated foci (Fig. 6A, lower panels). MSH3 was similarly recruited to locally UV-irradiated sites and colocalized with PCNA in XPA-WT cells (Fig. 6B, upper panels), whereas neither of these proteins was recruited in XPA-C2 cells (Fig. 6B, lower panels). Recruitment of MutSβ to UV-irradiated sites was independent of the cell cycle and could be observed 8 hours after irradiation. In XPA-WT cells, the GFP-tagged MSH3 N-terminal domain (residues 1-57) was recruited to UV-irradiated sites, where mDR-PCNA molecules also localized (Fig. 6C, top panels). However, GFP-MSH3 with a mutation in the PCNA-binding motif (F27A/F28A) and a deletion mutant without the PCNA-binding domain of MSH3 failed to accumulate at UV-irradiated sites (Fig. 6C, middle and bottom panels, respectively). Thus, our data indicate that MutSβ accumulates at UV-induced DNA damage in a NER- and PCNA-dependent manner. In contrast to

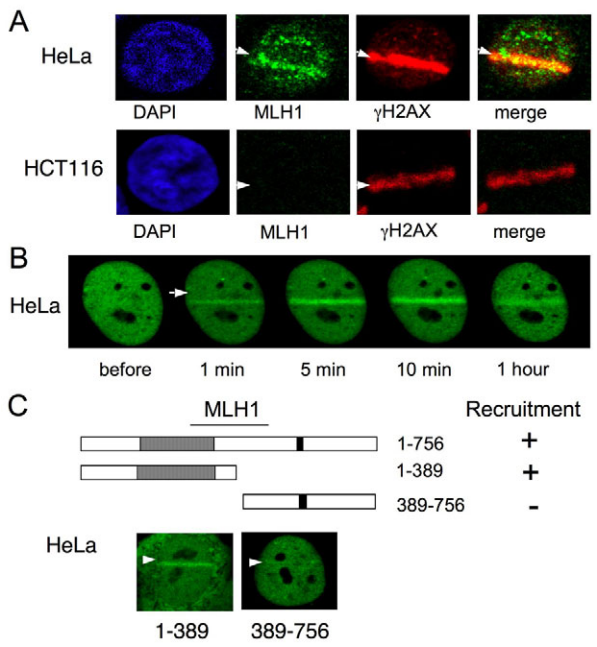

Fig. 5. MLH1 is recruited to the site of laser irradiation via its MutS-binding domain. (A) Colocalization of endogenous MLH1 with γH2AX after laser irradiation in HeLa cells (upper panels). There was no accumulation of MLH1 at laser-irradiated site in HCT116 cells (lower panels). (B) Recruitment of GFP-MLH1 to the site of laser irradiation in HeLa cells. (C) Schematic representation of the recruitment of MLH1 deletion mutants. Black and gray boxes indicate PCNA-binding motif and MSH2-binding domain, respectively. The MLH1 deletion mutant containing residues 1-389 is recruited to irradiation sites, whereas that containing residues 389-756, is not.

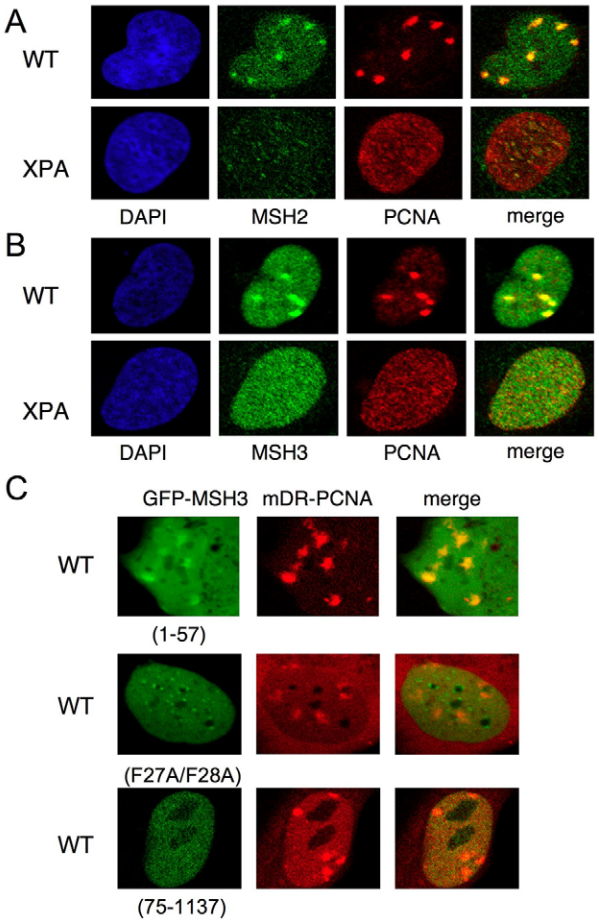

Fig. 6. MSH2 and MSH3 are recruited to UV-induced DNA damage in an NER-dependent manner. (A) Immunostaining of MSH2 and PCNA in locally UV-irradiated (100 J/m^2) NER proficient (XPA-WT, labelled WT) and deficient cells (XPA-C2, labelled XPA). (B) Immunostaining of MSH3 and PCNA in locally UV-irradiated (100 J/m^2) NER proficient (upper panels) and deficient cells (lower panels). (C) MSH3 is recruited to UV-induced DNA damage via its PCNA-binding motif in XPA-WT cells. GFP-MSH3 (1-57) harboring PCNA-binding domain accumulates at UV-irradiated sites (upper panels), whereas mutation in the PCNA-binding motif and MSH3 without the PCNA-binding domain abrogates the accumulation (middle and bottom panels, respectively).

MSH3, we did not observe MSH6 accumulating at UV-irradiated sites under the experimental conditions we used. Thus, MutSβ but not MutSα is engaged in the UV-induced DNA damage response in human cells.

Discussion

MutSα and MutSβ bind to the base mismatches and mismatched loops, respectively, formed during DNA replication and perform excision repair in the newly synthesized strand. Unless a lesion contains a mismatch, these protein complexes do not bind specifically to DNA damage, such as DNA strand breaks or UV-induced lesions in vitro (Mu et al., 1997). Therefore, MMR has not been considered to function in DNA damage repair. However, various genetic studies have shown that, although MMR does not increase the survival, it influences mutation frequencies in response to various types of DNA damage (Iyer et al., 2006; Jiricny, 2006). In the early studies of *E. coli* mutagenesis, NER-dependent increase of UV-induced mutation frequencies was reported (Bridges and Mottershead, 1971; Bridges and Sharif, 1986). Since DNA repair includes repair synthesis, it is reasonable to suppose that MMR may function during DNA repair, provided that MMR proteins are recruited to the sites of repair. We found here that MSH2, MSH3 and MSH6 accumulate at UVDE-induced SSBs and that PARP inhibitor completely abolishes this accumulation (Fig. 1C,D). Poly(ADP-ribose) recruits XRCC1, the scaffold protein for SSB repair, which recruits additional repair proteins including PCNA, Polβ and Ligase IIIα (Hashiguchi et al., 2007; Lan et al., 2004b). MutS complexes are probably recruited by the PCNA, which organizes a long-patch-repair synthesis of SSBs, where MMR may function. Recruitment of MutS complex to SSB repair sites might also occur via a possible interaction of MSH6 with poly(ADP-ribose) (Pleschke et al., 2000). Intensive accumulation of MSH2 at laser-irradiated sites in the presence of DIQ (Fig. 1D, lower panels) suggests that not only SSBs but also other types of DNA lesions, including DSBs and base damage, recruit MMR proteins. Judging from the irradiation conditions, and the accumulation and retention kinetics of MMR proteins, we conclude that DSBs are the major targets of the accumulation after laser irradiation.

Using HCT116 cells and siRNA in HeLa cells, we showed that MSH2 is recruited to DNA damage sites through interactions with either MSH3 or MSH6. We tested another human cell line, HCT15, which is known to be defective in MSH6 but not in MSH3 (Chang et al., 2000). However, GFP-MSH2 did not accumulate at laser-irradiated sites, and coexpression of either MSH3 or MSH6 rescued the accumulation of MSH2 (supplementary material Fig. S1A),

suggesting that both gene products of HCT15 cells are deficient in the damage response. Although MSH3 was expressed in the cells (supplementary material Fig. S1B), neither cDNA nor genomic DNA of the N-terminal region of MSH3 could not be obtained from the cells (not shown), suggesting the presence of a mutation in the PCNA-binding domain of the gene encoding MSH3. Thus, the recruitment of MSH2 to damage sites may be used as an assay to analyze the functions of MSH3 and MSH6.

Data presented in Figs 2-4 show that MSH3 and MSH6 recruit the heterodimeric MutSβ and MutSα complexes, respectively, to the site of DNA damage via protein-protein interactions. The accumulation of MutS complexes at the site of DNA damage is not due to the recognition of the damage by MSH3 or MSAH6 but rather to the binding of the proteins to PCNA or an unidentified target at the damage site. In contrast to MSH3, which binds efficiently to PCNA accumulated at DNA damage sites, a region beyond the extreme N-terminus of MSH6 plays a more important role in the damage response than the N-terminal PCNA-binding domain (Fig. 4). Judging from the very rapid accumulation kinetics (within 30 seconds) of the posterior region alone (Fig. 4B,C), it may bind directly to DNA damage itself, a suggestion proposed by recent crystal structure data on the MSH2-MSH6 complex and in vitro binding assays for the N-terminal region of MSH6 (Clark et al., 2007; Warren et al., 2007). However, we do not see this rapid accumulation in the property of full-length MSH6. Although the mechanisms underlying these observations remain to be determined, it appears that the slow accumulation of the posterior part determines the recruitment of MSH6 to the site of DNA damage, which is independent of both PCNA binding and the direct DNA binding. The rapid accumulation property is masked in the full-length protein and might have a role later in the repair process.

Our results suggest that PCNA and MMR complexes are recruited to DSBs. Indeed, involvement of PCNA in NHEJ of DSBs has been shown by the fact that depletion of cell extracts for PCNA resulted in a reduction in end-joining activity (Pospiech et al., 2001). Both PCNA and replication factor C are necessary for the recruitment of each protein to the sites of DNA damage (Hashiguchi et al., 2007). Therefore, MutSβ is recruited to PCNA, which has been loaded on DNA by replication factor C. MutS-dependent rapid recruitment of MLH1 to the site of DNA damage, shown in Fig. 5A,B, suggests that a ternary complex is immediately built with a MutS complex at the site of loaded PCNA. The accumulation kinetics of MMR proteins (shown in supplementary material Fig. S2) reflects the order of recruitment to the damage site within the cell.

What possible roles do the MMR proteins play at the site of DNA damage? In NHEJ, mismatched DNA generated by the annealing of non-cohesive DNA ends can be detected by MutSα or MutSβ, and MMR may initiate another round of end-processing (Bannister et al., 2004; Smith et al., 2005). It was reported recently that MutSβ in yeast acts in the repair of base-base mismatches and in the suppression of homology-mediated duplication and deletion mutations (Harrington and Kolodner, 2007). It is, therefore, tempting to suppose that the MMR complex competes in NHEJ with XLF, which promotes the ligation of mismatched and non-cohesive DNA ends during NHEJ (Tsai et al., 2007). In addition to NHEJ, previous genetic experiments suggest that MMR functions in homologous recombination of DSBs (Elliott and Jasin, 2001; Evans et al., 2000; Villemure et al., 2003). The MMR system may either abort the strand-exchange process and provide another chance to identify a better homology in homologous recombination, or perform mismatch repair leading to gene conversion (Jiricny, 2006). Indeed, homologous recombination between divergent sequences is suppressed by MMR (Elliott and Jasin, 2001). We think that the accumulation mechanisms of MMR proteins at DNA damage might play important roles, not only in excision repair of DNA damage, but also in homologous recombination of DSBs. Since MMR only controls in-sequence diverged pairings in NHEJ and homologous recombination, MMR deficiency does not influence cell survival in response to DSBs, as previously reported (Papouli et al., 2004; Yan et al., 2001).

PCNA-dependent recruitment of the MMR complex is also found in response to UV-induced DNA damage (Fig. 6). Although this is the first report to demonstrate the presence of the MMR complex in NER, its presence is not unexpected. Although we reported that MMR reduces the mutagenicity of UV lesions in NER-deficient cells (Nara et al., 2001), avoidance of mutation during NER synthesis by MMR has never been reported. However, as mentioned before, NER-dependent mutagenesis has been reported before (Bridges and Mottershead, 1971; Bridges and Sharif, 1986) and the involvement of MMR in NER remains to be demonstrated.

MutS complex recruited to the site of NER might recruit further proteins involved in DNA repair. We found previously that MSH2 interacts directly with DNA polymerase η (Wilson et al., 2005) and, thus, translesion synthesis might occur where DNA damage is also present on the template strand. This suggests a model in which a recruited MutS complex performs either MMR and/or translesion synthesis depending on the presence of mismatch or additional DNA lesion(s) on the newly synthesized strand or the template strand, respectively. We have recently shown that polymerase η is recruited to UV-irradiated and laser-irradiated sites independently of RAD18 (Nakajima et al., 2006). Other studies suggest a direct role for MMR proteins in the signaling of DNA damage for checkpoints and apoptosis that is independent of the MMR catalytic repair process (O'Brien and Brown, 2006). The data presented here show the mechanistic backgrounds for the involvement of MMR proteins in various aspects of DNA damage responses. Further analysis is necessary for a more detailed understanding of the roles of MMR in DNA damage and repair.

Materials and Methods

Construction of plasmids for expression of various genes

Plasmids expressing human genes encoding MSH2, MSH3, MSH6, MLH1 and PCNA were constructed by cloning cDNA amplified from HeLa cells. We modified the multiple cloning sites of the vectors EGFP-C1 and monomeric DsRed-C1 (mDR-C1; Clontech) to introduce various cDNAs attached with an in-frame *Xho*I or *Sal*I site at the start and *Not*I site at the stop codons. Deletion fragments of MSH3, MSH6 and MLH1 were generated using appropriate restriction endonucleases or PCR amplification, and then cloned into the modified EGFP-C1. The mutant GFP-MSH3 (F27A/F28A) and mDR-PCNA (D41A) constructs were generated using QuikChange® site-directed mutagenesis (Stratagene). All constructs were verified by sequencing.

Cell lines, culture and transfection

The following cell lines were used in this study: HeLa, HCT116 (MLH1-deficient colorectal carcinoma), LoVo (MSH2-deficient colorectal carcinoma), XPA-C2 (XP12ROSV cells expressing blank vector control. XP12ROSV is an SV40-transformed cell line derived from an XP-A patient without detectable NER activity) and XPA-WT (XP12ROSV cells expressing wild-type XPA cDNA with wild-type UV-resistance) (Lan et al., 2004a; Okano et al., 2003; Satokata et al., 1992). For UV-induced SSB production, XP12ROSV cells stably transfected with the UVDE gene from *Neurospora crassa* (XPA-UVDE) was used (Okano et al., 2003). Absence of the expression of MLH1 and MSH3 as well as a low expression of MSH6 in HCT116, and absence of expression of MSH2 in LoVo cells were confirmed by western blot analysis (not shown). All cell lines were propagated in Dulbecco's modified Eagle's medium (Nissui), supplemented with 1 mM L-glutamine and 10% fetal bovine serum (20% for LoVo cells) at 37°C and 5% CO_2. For UVA-laser irradiation, cells were plated in glass-bottomed dishes (Matsunami Glass) and transfected with expression vectors using FuGene6 (Roche), according to the manufacturer's protocol. More than

20 cells were analyzed for each experiment and a typical response found in most of the irradiated cells is shown.

Microscopy and UVA-laser irradiation

Fluorescence images were obtained and processed using a FV-500 confocal scanning laser microscopy system (Olympus). UVA-laser irradiation was used to induce DSBs in cultured cells, as described previously (Hashiguchi et al., 2007; Lan et al., 2005; Lan et al., 2004b). Briefly, cells in glass-bottomed dishes were micro-irradiated with a 405 nm pulse laser (Olympus) along a user-defined path. The laser was focused through a 40× objective lens. A single laser scan at full power delivers ~1600 nW. Cells were treated with 10 nM 5-bromo-2-deoxyuridine (BrdU) during the 24 hours before irradiation. Given the low treatment doses and the wavelength used, the influence of the laser light on DNA is indirect via photosensitization of natural or added (BrdU) chromophores near the DNA within the cell. The irradiation dose was fixed in the experiments as 500 scans in the presence of BrdU. The fluorescence intensity at an irradiated site was measured and divided with the intensity outside the irradiated site. The number of DSBs was determined by comparison of the number of γH2AX produced by laser micro-irradiation with that produced by X-ray irradiation, which is similar to the method reported previously (Bekker-Jensen et al., 2006). To examine the effect of inhibitor of PARP, cells were incubated in a medium supplemented with 1,5-dihydroxyisoquinoline (DIQ; 0.5 mM; Sigma) for 1 hour before irradiation.

Local UV irradiation

Local UV irradiation was performed as described previously (Okano et al., 2003). UV irradiation was delivered using a germicidal lamp (GL-10; Toshiba; predominantly 254 nm) at a dose rate of 1.25 J/m^2/second. Before UV irradiation, cells were washed once with Hank's buffer and gently covered with a polycarbonate isopore membrane filter containing 3 μm diameter pores (Millipore). Cells were irradiated locally with UV through the pores.

Immunofluorescence and western blotting

Cells were fixed in cold methanol/acetone (1:1) for 10 minutes at –20°C and then probed with anti-MSH2 (N-20, Santa Cruz), anti-MSH3 (a kind gift from Miyoko Ikejima, Nippon Medical School, Kawasaki, Japan), anti-MSH6 (sc-1243, Santa Cruz), anti-MLH1 (sc-582, Santa Cruz), anti-PCNA (Ab-1, Oncogene), anti-XRCC1 (ab1838, Abcam) and anti-γH2AX (jbw103, Upstate). Alexa Fluor 488 anti-rabbit IgG and 594 anti-mouse IgG (Molecular Probes) were used as secondary antibodies. Fluorescence microscopy observations were performed using a FV-500 confocal scanning laser microscopy system (Olympus). For western blot analysis, 20 μg protein was applied on each lane.

Suppression of MMR gene expression by siRNA

The following short 21-mer RNA (siRNA) sequences (sense strand sequences are shown) with high target specificity were designed based on published guidelines (Ui-Tei et al., 2004) and used to suppress the expression of MSH3, MSH6 and MSH2. MSH3, 5′-gCAAggAgUUAUggAUUAATT; MSH6, 5′-gAAUACgAgUUgAA-AUCUATT; MSH2, 5′-gAUUggUAUUUggCAUAUAAg. Purified nucleotides were fused with complementary strands and transfected into HeLa cells plated on 3.5 cm dishes at 20-30% confluence at a final concentration of 200 nM for the suppression of a single gene and 100 nM each for the suppression of two genes using Oligofectamine (Life Technology) as previously reported (Lan et al., 2004a). As a negative control, siRNA with scrambled or unrelated sequences were used for each knockdown experiment. Plasmid harboring GFP-tagged genes was introduced using FuGene6 and, 2 days later, cells were irradiated with the laser. Cell extracts were prepared and used for western blotting.

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