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PCNA–MutSα-mediated binding of MutLα to replicative DNA with mismatched bases to induce apoptosis in human cells

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ABSTRACT

Modified bases, such as *O*^{6}-methylguanines, are produced in cells exposed to alkylating agents and cause apoptosis. In human cells treated with *N*-methyl-*N*-nitrosourea, we detected a protein complex composed of MutSα, MutLα and PCNA on damaged DNA by immunoprecipitation method using chromatin extracts, in which protein–protein interactions were stabilized by chemical crosslinking. Time course experiments revealed that MutSα, consisting of MSH2 and MSH6 proteins, and PCNA bind to DNA to form an initial complex, and MutLα, composed of MLH1 and PMS2, binds to the complex when the DNA is damaged. This sequential mode of binding was further confirmed by the findings that the association of PCNA–MutSα complex on chromatin was observed even in the cells that lack MLH1, whereas in the absence of MSH2 no association of MutLα with the chromatin was achieved. Moreover, reduction in the PCNA content by small-interfering RNA or inhibition of DNA replication by aphidicolin, an inhibitor of DNA polymerase, significantly reduced the levels of the PCNA–MutSα–MutLα complex and also suppressed an increase in the caspase-3 activity, a hallmark for the induction of apoptosis. These observations imply that the induction of apoptosis is coupled with the progression of DNA replication through the action of PCNA.

INTRODUCTION

Base mismatches are constantly produced at low levels during the normal process of DNA replication and cause base substitution mutations if they are not repaired. The occurrence of such events is accelerated when the cells are exposed to certain agents that modify DNA bases. O^6-methylguanine, produced by the action of alkylating agents, is one such lesion; it can pair with thymine as well as cytosine during DNA replication, leading to a G·C to A·T transition (1,2). To prevent such outcomes, organisms from bacteria to human are equipped with a specific DNA repair enzyme, O^6-methylguanine-DNA methyltransferase, which transfers a methyl group from the O^6-methylguanine moiety of the alkylated DNA to its own molecule, thereby repairing the DNA lesion in a single-step reaction (3,4). *MGMT*$^{-/-}$ mice, which are defective in the methyltransferase gene, are hypersensitive to the killing effect of alkylating agents and, when administered sublethal doses of the agents, produce a large number of tumors in their organs (5,6). In these mice, the damage by the alkylating agents is confined to tissues with rapidly growing cells, such as those in the bone marrow and intestinal mucosa, indicating that cell proliferation is required for killing the cells containing O^6-methylguanine in the DNA. Studies with *MGMT*$^{-/-}$ cells further revealed that the O^6-methylguanine-induced cell death occurs by apoptosis, which requires at least one round of DNA replication for its induction (7–9).

Another notable feature of O^6-methylguanine-induced apoptosis is the involvement of mismatch repair proteins in the process. Mismatch repair has been defined as a mechanism to correct replication errors, including mismatched bases and small insertions/deletions, by excising the error-containing region of the newly replicated strand, followed by repair synthesis and strand rejoining. The molecular mechanism of mismatch repair has been well characterized in *Escherichia coli*, in which at least 11 enzymes or protein components are involved (10). Among them, MutS and MutL play important roles in recognizing mismatched regions and initiating the repair reactions. Many mammalian counterparts of MutS and MutL have been found, among which MutSα, a heterodimer composed of the MutS homologs MSH2 and MSH6, and MutLα, a dimer composed of the MutL homologs MLH1

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and PMS2, are regarded as being essential for the recognition of base mismatches (11–14). Early studies with cell lines derived from human tumors revealed that the lack of some of these gene functions rendered O^6-methylguanine-DNA methyltransferase-deficient cells resistant to alkylating agents (15,16). Although tumor-derived cells would possess mutations and exhibit transcriptional silencing of some other genes, recent studies with a more defined system, namely *MGMT*$^{-/-}$ *MLH1*$^{-/-}$ cells derived from the gene-targeted mice, have clearly shown that the mismatch repair-related function is indeed required for the execution of apoptosis triggered by O^6-methylguanine (17,18).

In eukaryotic cells, the mismatch repair proteins appear to be involved in at least two different processes, one to repair replication-associated errors in a strand-specific manner and the other to signal for inducing apoptosis in cells with mismatched DNA bases. This was clearly shown by recent findings that certain MSH2 or MSH6 missense mutations can cause a deficiency in mismatch repair, whereas retaining the signaling functions that confer sensitivity to chemotherapeutic agents (19,20). The molecular mechanism of the former process, mismatch repair, has been elucidated by analyzing the interactions and reconstructing the protein complex *in vitro* (21,22). However, studies on the mechanism of the latter process, modified base-induced apoptosis, have been hampered mainly by the lack of appropriate systems for investigating the process occurring in cells. In this regard, human cell lines defective in the *MGMT* gene would be noteworthy, since the exposure of the cells to simple alkylating agents would induce a large number of O^6-methylguanine lesions in the chromosomal DNA, which would trigger apoptosis. The O^6-methylguanine thus formed would persist in the DNA through the progression of the cell cycle and, hence, the conditions and timing required for the formation of an apoptosis-related protein complex could be followed in the cells. Moreover, by comparing the results obtained with O^6-methylguanine-DNA methyltransferase-proficient and -deficient cells, it is possible to ascertain if the molecular events observed are related to O^6-methylguanine (23,24).

Taking advantage of this system, we have investigated an early step of apoptosis triggered by O^6-methylguanine. Since the protein complex formed on O^6-methylguanine-containing DNA is unstable, to stabilize the multi-protein complex formed on the chromatin, we treated cells undergoing the early process of apoptosis with a protein crosslinking agent. After the isolation of the complex, the crosslinks were cleaved and its components were analyzed. In this way, we were able to determine the nature of the complex and resolve the conditions and timing required for its formation.

MATERIALS AND METHODS

Cell lines and cell culture

Human cell lines, HeLa S3 (wild-type), and its O^6-methylguanine-DNA methyltransferase-deficient derivative, HeLa MR (25), were obtained from H. Hayakawa (Kyushu University, Fukuoka, Japan). SW48, a human tumor-derived cell line deficient in both *Mgmt* and *Mlh1* expression, was purchased from American Type Culture Collection. LoVo, a human colorectal adenocarcinoma cell line deficient in *Msh2* (26), was obtained from S. Oda (National Kyushu Cancer Center, Fukuoka, Japan). The HeLa MR line stably expressing FLAG epitope-tagged PMS2 was constructed as follows. A DNA fragment encoding the hPMS2 protein was amplified by PCR using pcDNA3.1/V5-His-hPMS2 plasmid DNA (27), obtained from S. Fukushige (Tohoku University School of Medicine, Miyagi, Japan), as a template and was inserted into the HindIII–BamHI site of the p3xFLAG-CMV-10 expression vector (Sigma). The resulting plasmid, named p3xFLAG-hPMS2, was digested with ScaI, and the linearized DNA was introduced into HeLa MR cells by electroporation using a Genepulser (Bio-Rad). Stable transfectants were selected as G418 (700 μg/ml) resistant colonies, and a line expressing the FLAG-tagged hPMS2 protein was isolated and designated as HeLa MR (ePMS2). These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% CO_2 atmosphere.

Chemicals

N-Methyl-*N*-nitrosourea (MNU) and 3,3′-dithiobis-sulfosuccinimidylpropionate (DTSSP) were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and Pierce, respectively. O^6-benzylguanine and aphidicolin were purchased from Sigma.

Preparation of whole cell extracts

The cells were washed twice with phosphate-buffered saline (PBS) and scraped from the dish. The collected cells by centrifugation at 3000 *g* for 5 min at 4°C were suspended with 2× SDS–PAGE sample buffer [120 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT and 0.002% bromophenol blue], sonicated and boiled. The material was centrifuged at 20 000 *g* for 10 min, and the supernatant fraction was taken as the whole cell extract.

Preparation of chromatin extracts

Approximately 3×10^6 cells were seeded in a 10 cm dish and incubated for 1 day. The cells were washed twice with PBS and then treated with 1 mM MNU in serum-free DMEM for 1 h. After the medium was replaced with DMEM containing 10% FBS, the cells were incubated for the appropriate duration. The cells were permeabilized and washed extensively on the dish with ice-cold buffer A [20 mM HEPES–KOH (pH 7.9), 5 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose and 0.1 mM EGTA] containing 50 μg/ml of digitonin (Wako) and protease inhibitors (Roche), and then were treated with 1 mM DTSSP (Pierce) for 2 h at 4°C. The crosslinking reaction was stopped by the addition of 50 mM Tris–HCl (pH 7.5). The cells were scraped from the dish and collected by centrifugation at 3000 *g* for 10 min at 4°C. The cell pellet was suspended in ice-cold buffer B [20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, 1.5 mM $MgCl_2$, 10% glycerol and 0.1% Triton X-100] containing protease inhibitors and then was sonicated. The material was centrifuged at 20 000 *g* for 15 min at 4°C, and the supernatant fraction was taken as the chromatin extract.

Immunoprecipitation and western blotting

For the immunoprecipitation of the FLAG-tagged PMS2-bound proteins, 10 μl of anti-FLAG M2-agarose (Sigma) were added to 1 ml of chromatin extract, prepared as described

above, and incubated for 4 h at 4°C. After extensive washings of the beads with buffer B, the proteins bound to the beads were eluted in 40 µl of buffer B containing 200 µg/ml of 3× FLAG peptide (Sigma). To immunoprecipitate the proteins bound to MSH2 or PMS2, 10 µl of anti-MSH2 (Zymed) or anti-PMS2 antibody conjugated with Protein G–Sepharose (Amersham Biosciences) were mixed with the chromatin extract in a similar manner as described, and proteins bound to the beads were eluted with SDS–PAGE sample buffer [60 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM DTT and 0.001% bromophenol blue]. The anti-PMS2 antibody was raised in a rabbit immunized with purified protein covering the entire length of human PMS2 expressed in *E.coli* and then was affinity-purified using the antigen. The specificity of the antibody was confirmed by immunoblotting (data not shown). Conjugation of the antibody to Protein G beads was carried out as described previously (28). Appropriate amounts of the eluted samples were subjected to SDS–PAGE and electroblotted onto a PVDF membrane (Bio-Rad). Detection was performed with an ECL Advance western blotting detection kit (Amersham Biosciences). The primary antibodies used were as follows: anti-FLAG M2 (Sigma), anti-MLH1 (BD Biosciences), anti-MSH2 (Zymed), anti-MSH6 (BD Biosciences), anti-PCNA (Santa Cruz Biotechnology) and anti-Histone H3 (abcam). Anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences) were used as the secondary antibody.

siRNA transfection

siRNA for the *PCNA* sequence (siPCNA), 5′-AAGCAC-CAAACCAGGAGAAAG-3′, was purchased from Qiagen. After culturing 1×10^6 cells in a 10 cm dish for 1 day, the cells were transfected with 40 nM of siRNA, using the Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's protocol. For the control transfection, the negative control siRNA (Qiagen) was used.

Assay of caspase-3 activity

One day after the siRNA transfection, the cells ($1–5 \times 10^5$) were seeded in a 6 cm dish and incubated at 37°C for 1 day. The cells were treated with 1 mM MNU for 1 h and incubated for another 3 days. The cell lysate preparation and the caspase activity assay were performed, according to the instructions in the EnzChek caspase-3 assay kit #2 (Molecular Probes). To assay the caspase-3 activity, the rate of hydroxylation of the synthetic substrate Z-DEVD-R110 was measured in the presence or absence of the inhibitor, Ac-DEVD-CHO. The hydrolysis products were quantified using a spectrofluorometer, Fusion α (PerkinElmer), with excitation at 496 nm and emission at 520 nm. The values obtained with the inhibitor were subtracted from those obtained without the inhibitor to express the specific activity of caspase-3.

Flow cytometric analysis

Cells (1×10^6) on a 10 cm dish were harvested by a 0.25% trypsin–0.02% EDTA treatment, washed with PBS and suspended in 400 µl of PBS containing 0.1% Triton X-100, 25 µg/ml of propidium iodide and 0.1 mg/ml of RNase A. Samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson), with 10 000 events per determination.

RESULTS

Formation of a protein complex on O^6-methylguanine-containing DNA

Accumulating evidence suggests that an initial step in the apoptosis induced by O^6-methylguanine would be the binding of mismatch recognition proteins to the DNA with the lesion. However, it was difficult to isolate such a protein–DNA complex from the cells, since it is unstable and decomposed during ordinary extraction procedures. To overcome this difficulty, we treated the cells with a crosslinking agent, before the materials were isolated. The cells were permeabilized with digitonin and then treated with DTSSP, to stabilize the complex. After sonication of the cells, the chromatin was extracted and then the crosslinks were cleaved by a treatment with a reducing agent. In this way, we were able to resolve the components of the complex and follow the conditions needed for its formation.

O^6-methylguanine, which is produced in DNA by treating cells with simple alkylating agents, can be repaired by a specific DNA repair enzyme, O^6-methylguanine-DNA methyltransferase. HeLa MR cells are defective in this enzyme and readily undergo apoptosis after exposure to relatively low doses of MNU. We used this cell line and its parental line, HeLa S3, which is proficient in the methyltransferase activity, to determine whether a protein–DNA complex is formed in an O^6-methylguanine-dependent manner. The two types of cells were treated with 1 mM MNU for 1 h and incubated in MNU-free growth medium for another 11 h, and then the cells were collected for examination of the protein complexes. From the same amounts of chromatin fractions prepared from each sample, DTSSP-linked protein complexes were immunoprecipitated with the use of an anti-MSH2 monoclonal antibody, and then the protein components were dissociated by the treatment with the reducing agent. The materials thus obtained were subjected to SDS–PAGE, followed by the detection of each component by specific antibodies.

Figure 1 shows analyses of the MSH2-bound protein components on the chromatin DNA of the two types of cells, with or without MNU treatment. In HeLa MR cells without MNU treatment, certain amounts of MSH6 and PCNA were co-precipitated with MSH2, indicating that low, but significant amounts of MutSα as well as PCNA are associated with the chromatin, even under normal conditions. When the cells were exposed to MNU, the association of these components with the chromatin increased, and moreover, new bands, corresponding to MLH1 and PMS2, appeared. Since only very low signals for these proteins were detected in untreated cells, it is likely that the association of MutLα with the chromatin is strictly dependent on the formation of O^6-methylguanine in the DNA. This notion is supported by the results obtained with HeLa S3 cells, in which the O^6-methylguanine is repaired efficiently by its intrinsic repair enzyme activity. With and without MNU treatment, there were no significant changes in the patterns of these protein bands.

Association of the protein components with chromatin after MNU treatment

To address this hypothesis for the complex formation on the damaged chromosome in detail, we constructed HeLa MR

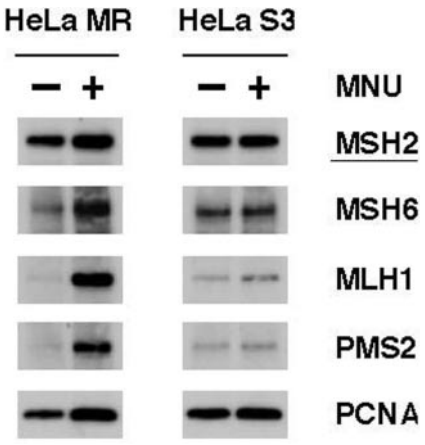

Figure 1. Formation of a PCNA–MutSα–MutLα complex in methyltransferase-deficient cells upon exposure to MNU. HeLa MR (methyltransferase-deficient) and HeLa S3 (methyltransferase-proficient) cells were incubated in serum-free medium with or without 1 mM MNU for 1 h, and then were placed in 10% FBS-containing medium and incubated further for 11 h. The cells were treated with hypotonic buffer containing 50 μg/ml of digitonin on the dish and were then collected. After sonication of the cells, the chromatin extracts were prepared by centrifugation and the same amount of extracts were used for each immunoprecipitation assay with an anti-MSH2 antibody. The immunoprecipitated materials were separated by SDS–PAGE and immunoblotted with specific antibodies against the MSH2, MSH6, MLH1, PMS2 and PCNA proteins.

cells stably expressing FLAG epitope-tagged PMS2, which produced about twice as much of the FLAG-PMS2 as the endogenous PMS2 protein (data not shown). We first used this strain, named HeLa MR (ePMS2), to check whether the amounts of protein components of the mismatch recognition complex increase by the treatment of MNU. As shown in Figure 2A and B, immunoblotting experiments using whole cell extracts clearly indicates that the amounts of mismatch repair proteins and PCNA are almost constant during times even after MNU treatment. We then monitored the amounts of these proteins that are bound to chromatin. As shown in Figure 2C and D, dramatic increases in the levels of MLH1 and FLAG-tagged PMS2, the components of MutLα, were observed in the chromatin extracts upon the treatment of MNU. Such increases were also shown in HeLa MR cells, ruling out the possibility that this is due to the effect of the exogenous expression of FLAG-PMS2 protein in HeLa MR (ePMS2) cells (data not shown). PCNA increases moderately and MSH2 and MSH6, the components of MutSα, do slightly. The observations suggest that the association of these proteins with the damaged chromatin is induced, without changing the total amounts of the proteins, after MNU treatment.

Time course of the complex formation after MNU treatment

To follow the process of the protein complex formation on the damaged chromosome, we used the chromatin extracts at each time point (Figure 2C) for immunoprecipitation experiments. First, an anti-MSH2 antibody was employed and the proteins interacting with MSH2 were detected by immunoblotting (Figure 3A). Without MNU treatment, certain amounts of PCNA and the two components of MutSα, MSH2 and MSH6, were detected, and the amounts of these proteins increased slightly with the progression of cell growth. Significant increases in the amounts of these proteins were observed after MNU administration, and MLH1 and PMS2, the latter of which was FLAG-tagged, in the complex increased rapidly and concomitantly up to 12 h after the MNU treatment (Figure 3A and B). It seems that certain amounts of MutSα and PCNA are associated with the normal form of the chromatin, and when the DNA is damaged, MutLα, composed of MLH1 and PMS2, is further bound to the complex.

To monitor the time course of formation of the complex, the chromatin-associated PMS2 was immunoprecipitated with the use of its FLAG-tag, and the components of the mismatch protein complex were examined. As shown in Figure 3C and D, relatively small amounts of PMS2 were present on the chromatin without the MNU treatment, and the associations of the other components with PMS2 were hardly detected. After the MNU treatment, the amounts of the PMS2-associated forms of the protein components increased dramatically and reached a maximum level at the 12 h of treatment, consistent with the observation made in the preceding experiment (Figure 3B). Thus, the sequential binding of MutSα and MutLα was indicated by the reciprocal immunoprecipitation experiments using the chromatin extracts.

Mutations affecting the complex formation

To confirm this notion, we examined the complex formation in various cell lines with defects in some of the mismatch repair proteins. The cell line SW48, derived from a human colorectal adenocarcinoma, is deficient in O^6-methylguanine-DNA methyltransferase activity and lacks the MLH1 protein, owing to transcriptional silencing of the genes (29,30). In immunoprecipitation assays using the same amount of chromatin extracts from cells treated with or without MNU, the MNU-induced interactions of MSH2 with the components of MutLα, MLH1 and PMS2, were detected in the HeLa MR, but not in the SW48 (Figure 4A). Moreover, the interaction between MSH2 and PCNA was observed, regardless of the MNU treatment, in SW48 as well as in HeLa MR. These findings indicate that the MLH1 function is dispensable for the interaction between MutSα and PCNA on chromatin.

Suppression of the complex formation was observed with the cell line LoVo, which is defective in the MSH2 function. Since this cell line has functional O^6-methylguanine-DNA methyltransferase, the experiment was performed with the use of O^6-benzylguanine, a specific inhibitor of the methyltransferase (31,32). In the presence of O^6-benzylguanine, methyltransferase-proficient HeLa S3 cells, which are otherwise defective in MNU-induced complex formation (Figure 1), were able to produce the complex in response to the MNU treatment. Under the same conditions, LoVo cells showed no response to the MNU treatment (Figure 4B). These results indicate that the complex formation is indeed triggered by the O^6-methylguanine lesions produced on the chromosomal DNA and also that the association of MutLα with the chromatin is MutSα-dependent.

Role of PCNA in O^6-methylguanine-induced apoptosis

The results of these experiments indicated that PCNA is concomitantly associated with MutSα on normal chromatin

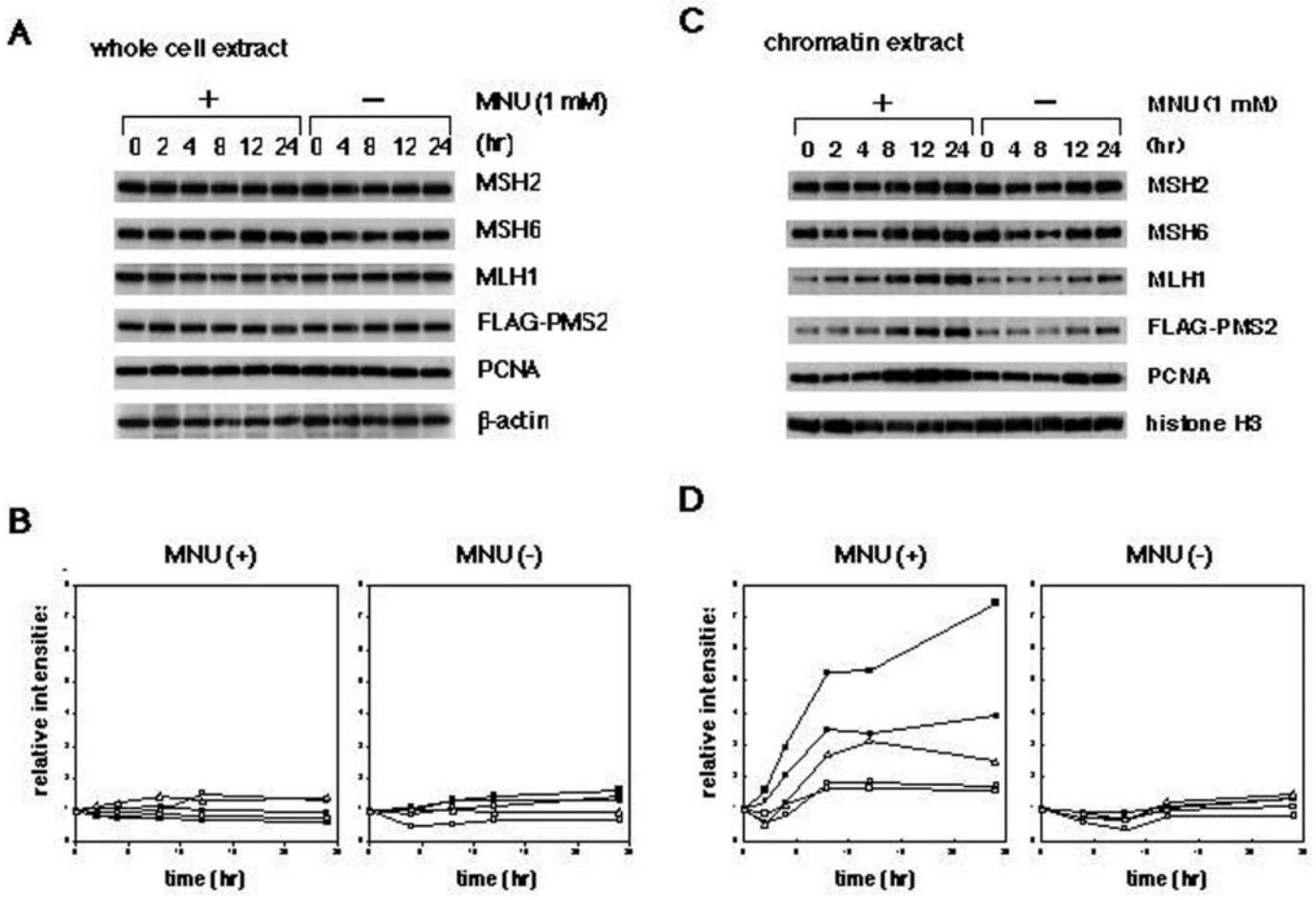

Figure 2. Association of the protein components with chromatin after MNU treatment. (**A**) HeLa MR (ePMS2) cells were treated with or without 1 mM MNU for 1 h and collected at the indicated times and the whole cell extracts were prepared as described in Materials and Methods. Immunoblotting was performed by the use of antibodies that specifically recognize MSH2, MSH6, MLH1, FLAG-PMS2, PCNA and β-actin. β-Actin is a loading control. (**B**) The intensities of the bands for each protein, shown in (A), were quantified, and the relative intensities as compared with that at 0 h are plotted. Open squares, MSH2; open circles, MSH6; closed squares, MLH1; closed circles, FLAG-PMS2; and open triangles, PCNA. (**C**) HeLa MR (ePMS2) cells treated in the same way in (A) were collected and the chromatin extracts were prepared as described in Figure 1. The amounts of proteins bound to chromatin were analyzed by immunoblotting using specific antibodies against mismatch repair proteins, PCNA and histone H3. Histone H3 is a loading control. (**D**) The relative intensities of the bands for each protein, shown in (C), are calculated in the same way as in (B), and are plotted. Open squares, MSH2; open circles, MSH6; closed squares, MLH1; closed circles, FLAG-PMS2; and open triangles, PCNA.

and further binds to MutLα to form a PCNA–MutSα–MutLα complex upon MNU treatment of the cells. To determine whether PCNA is prerequisite for the complex formation and if such a complex is involved in the progression of apoptosis, we employed the RNA interference method (33). For this, siPCNA, a siRNA specific for a part of the *PCNA* mRNA sequence, and siCont., a negative control RNA, were synthesized and introduced into HeLa MR (ePMS2) cells. At 48 h after the application of siPCNA, the cellular level of PCNA decreased to 39% of the level attained with cells that received the siCont., whereas the expression levels of other mismatch-related proteins were not affected (Figure 5A). Then, the siPCNA-treated cells and control cells were exposed to 1 mM MNU, and the chromatin extracts were prepared after 12 h for immunoprecipitation. Under *PCNA* knockdown conditions, the loading of the mismatch repair proteins to chromatin was severely inhibited (Figure 5B). A significantly lower amount of FLAG-PMS2 was immunoprecipitated with an anti-FLAG antibody, as compared with that of the control cells (Figure 5C). Simultaneously, the amounts of the PMS2-associated forms of MSH2 and MLH1 decreased to 60% (for MSH2) and 47% (for MLH1) of levels for the control, respectively. Thus, PCNA is actually required for the recruitment of the mismatch repair proteins to chromatin and for the formation of the mismatch recognition complex on the damaged chromosome. Similar observation was reported in a cell-free reconstituted system, by using purified PCNA, MutSα (MSH2/MSH6) complex and mismatch-containing heteroduplex DNA (34).

Caspase-3, a member of the cysteine protease family, is induced during the course of apoptosis, induced by TNF, gamma rays and many other means (35). This is also the hallmark of the progression of apoptosis triggered by O^6-methylguanine; caspase-3 activation occurred in *Mgmt*-deficient cells, but not in cells defective in both *Mgmt* and *Mlh1*, when these cells were treated with MNU (18). Therefore, we next examined whether the caspase-3 induction in HeLa MR (ePMS2) cells exposed to MNU is affected by the *PCNA* knockdown. The result shown in Figure 5D indicates that the treatment of the cells with siPCNA significantly

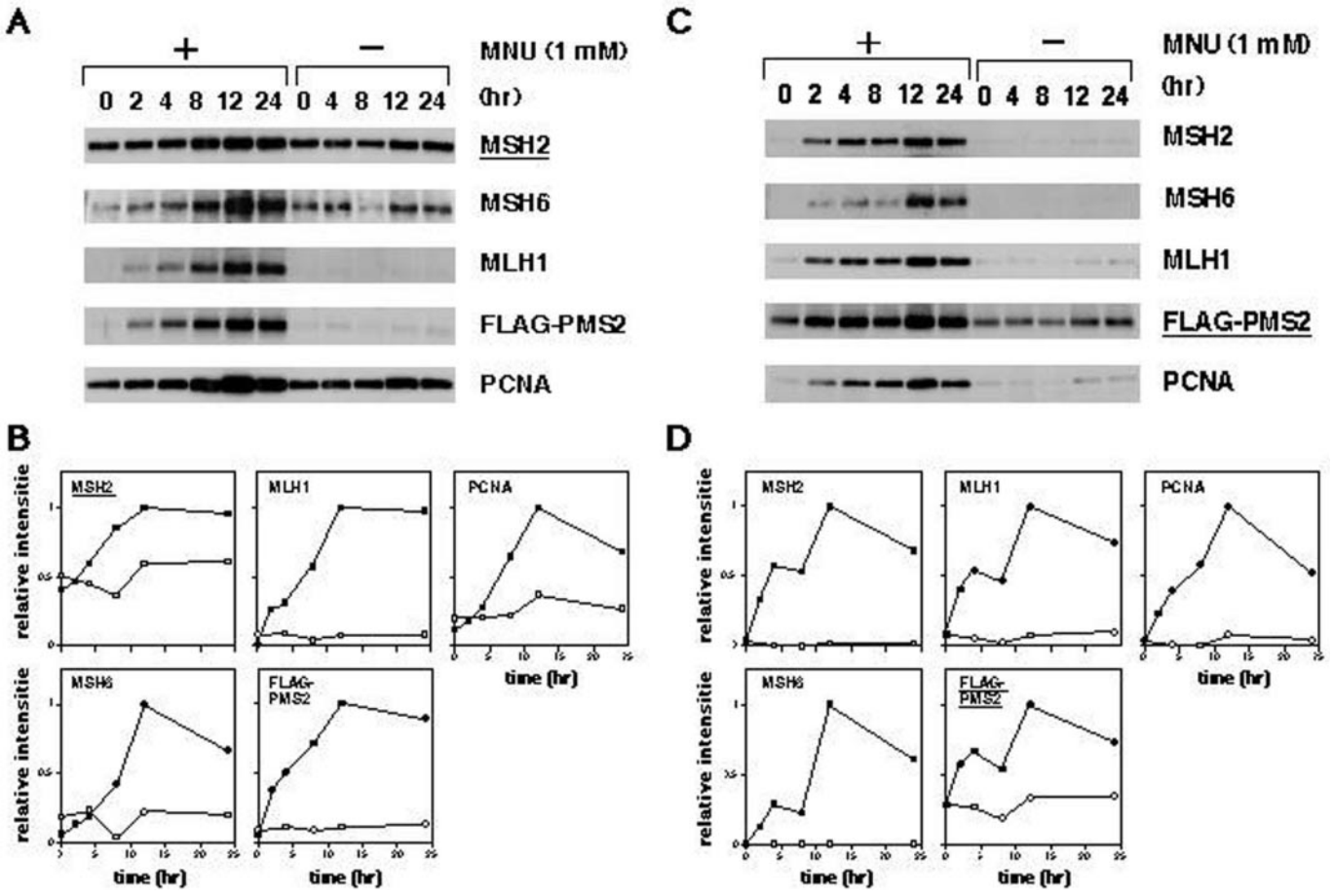

Figure 3. Time course of the formation of the mismatch recognition complex after MNU treatment. HeLa MR (ePMS2) cells were treated with or without 1 mM MNU for 1 h and collected at the indicated times for examination, as described in the legend to Figure 1. (**A**) Immunoprecipitation with the anti-MSH2 antibody was performed using the chromatin extracts shown in Figure 2C. Antibodies that specifically recognize MSH2, MSH6, MLH1, FLAG-PMS2 and PCNA were used for immunoblotting. (**B**) The intensities of the bands for each protein, shown in (A), were quantified, and the relative intensities as compared with that at 12 h with MNU treatment (peak signal) are plotted for each protein. Closed and open circles represent signals obtained with and without MNU treatment, respectively. (**C**) The same amounts of chromatin extracts from HeLa MR (ePMS2) cells were used for immunoprecipitation with the anti-FLAG antibody, followed by immunoblotting. (**D**) The relative intensities of the bands for each protein, shown in (C), are calculated in the same way as in (B), and are plotted. Closed and open circles represent signals obtained with and without MNU treatment, respectively.

reduced the induction of caspase-3. This implies that PCNA is a necessary component of the mismatch recognition complex, which is required for the execution of MNU-induced apoptosis.

Requirement of DNA replication for the recognition of O^6-methylguanine base pairs

Previous studies with *Mgmt*-knockout mice (5) revealed that bone marrow, spleen, thymus and intestinal mucosa, in which cells are actively growing, are most severely damaged by MNU administration, implying that DNA replication is required for O^6-methylguanine-triggered apoptosis. To determine whether DNA replication is a prerequisite for the formation of the mismatched base-associated protein complex, we examined the effects of aphidicolin, an inhibitor of DNA polymerase, on the formation of the complex. HeLa MR (ePMS2) cells were incubated for 16 h in a medium containing aphidicolin, to inhibit DNA replication, and then were treated with MNU for 1 h. The cell culture was divided into two portions; one incubated in the absence of aphidicolin and the other in the presence of the drug. The cells were collected at the indicated times after MNU treatment and were subjected to flow cytometry and immunoprecipitation analyses. As shown in Figure 6A, most of the cells treated with aphidicolin are arrested at the G_1/S boundary and remained at this stage as long as the drug was present in the medium. When the aphidicolin was removed, the treated cells progressed into the S phase of the cell cycles. Immunoblotting analyses of these samples revealed that total amounts of each mismatch repair protein and PCNA were almost constant (data not shown); however, the level of PCNA bound to chromatin remarkably increased when the cells progressed into S phase (Figure 6B). In accordance with this increase, immunoprecipitation analyses showed that in the absence of aphidicolin, the association of MSH2, MLH1 and PCNA with FLAG-tagged PMS2 occurs gradually when the cells progress into the S phase, whereas the formation of the complex is severely inhibited when the drug is present in the medium (Figure 6C). Thus, it seems that the formation of the mismatch recognition complex induced by MNU treatment is dependent on the progression of DNA replication.

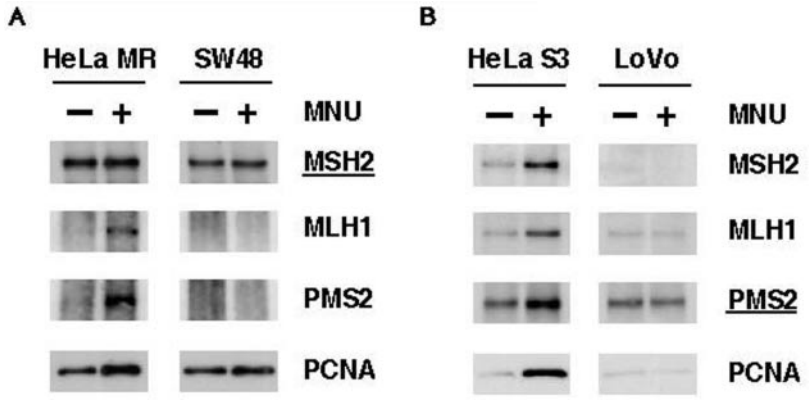

Figure 4. Inability of MLH1- and MSH2-defective cells to form the protein complex. (**A**) Effect of MLH1 deficiency. HeLa MR (methyltransferase-deficient) and SW48 (deficient in both methyltransferase and MLH1) cells were treated with or without 1 mM MNU for 1 h and then were incubated for another 11 h. The chromatin extracts were prepared as described in Figure 1 and the same amount of extracts were subjected to immunoprecipitation with an anti-MSH2 antibody, followed by immunoblotting. (**B**) MSH2-dependent interaction of MutLα with PCNA. HeLa S3 and LoVo (MSH2-deficient) cells were pre-incubated in a medium containing 25 μM O^6-benzylguanine for 2 h and then exposed to MNU for 1 h. After incubation for another 11 h in the presence of O^6-benzylguanine, the chromatin extracts were prepared, immunoprecipitated with the anti-PMS2 antibody, and immunoblotted.

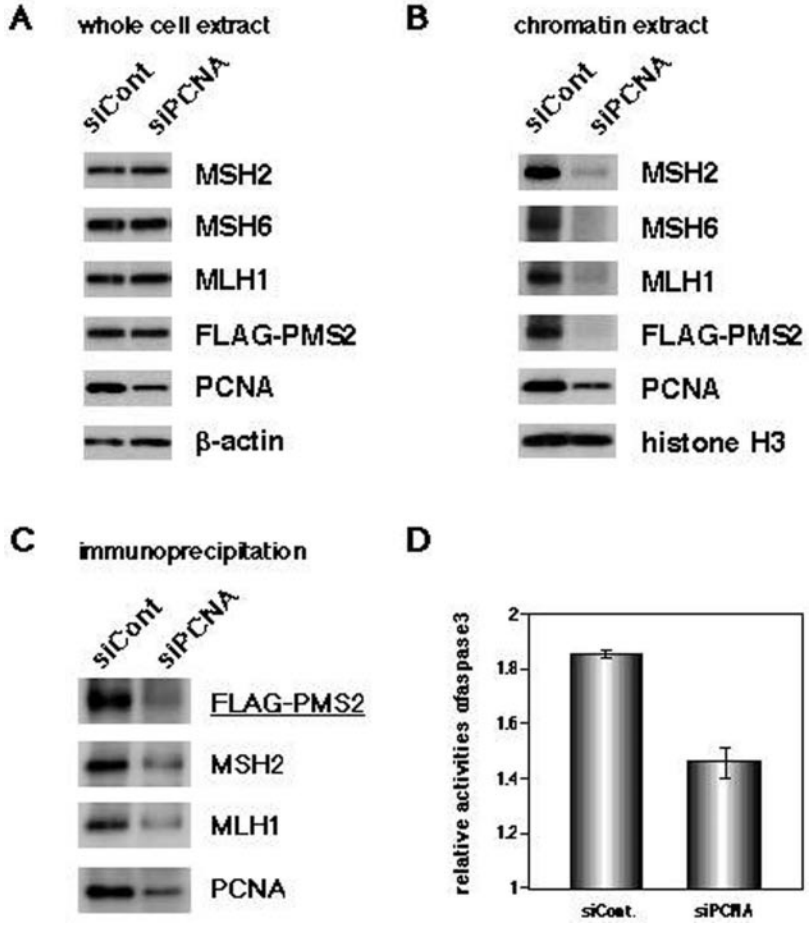

Figure 5. Effects of a *PCNA* knockdown on the formation of the mismatch recognition complex and the apoptotic induction. (**A**) *PCNA* knockdown by siRNA. HeLa MR (ePMS2) cells were transfected with siRNAs for *PCNA* and negative control sequences, and then were cultivated for 48 h. The whole cell extracts prepared were used for immunoblotting to analyze the total amounts of mismatch-related proteins. β-Actin is a loading control. (**B**) Chromatin-bound proteins under *PCNA* knockdown condition. siRNA-transfected cells in (A) were treated with 1 mM MNU. After 12 h incubation, chromatin extracts were prepared and the amounts of mismatch-related proteins and histone H3 were analyzed by immunoblotting. Histone H3 is a loading control. (**C**) Complex formation determined by immunoprecipitation analysis. The chromatin extracts were used for immunoprecipitation with the anti-FLAG antibody and then subjected to immunoblotting with the anti-FLAG, MSH2, MLH1 and PCNA antibodies. (**D**) Induction of apoptosis as measured by caspase-3 activity. Caspase-3 activity was determined at 72 h after treatment with or without 1 mM MNU. Values obtained with the MNU-treated cells were divided by those of the untreated cells, and the relative caspase-3 activities are shown. Experiments were performed three times and the standard deviations are shown in bars.

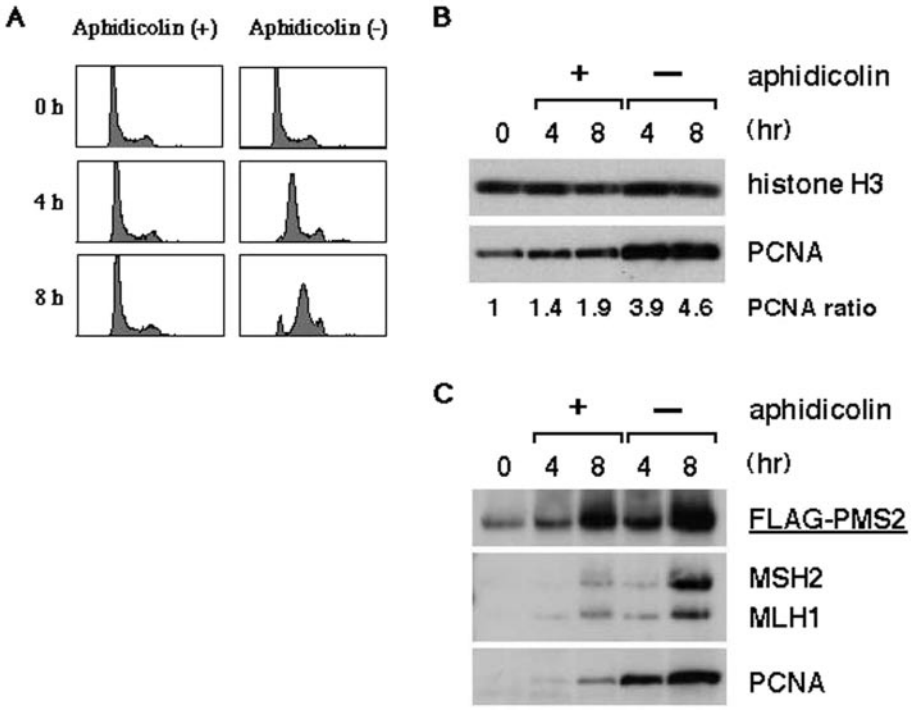

Figure 6. Effects of aphidicolin on the formation of the mismatch recognition complex. (**A**) Arrest of cell cycle progression by aphidicolin. HeLa MR (ePMS2) cells were cultivated in a medium containing 5 μM aphidicolin for 16 h and then treated with 1 mM MNU for 1 h. The culture was divided into two portions, one incubated in the presence of aphidicolin and the other in the absence of the drug. Cells were collected at the indicated time points and subjected to flow cytometric analyses to monitor cell cycle progression. (**B**) Chromatin-bound PCNA during DNA replication. Chromatin extracts were prepared from cells at 0, 4 and 8 h after MNU treatment, in the presence or absence of aphidicolin, and the amounts of histone H3 and PCNA were analyzed by immunoblotting. The relative ratios of PCNA are also shown. (**C**) Formation of protein complex during DNA replication. Chromatin extracts in (B) were used for immunoprecipitation with an anti-FLAG antibody. Immunoblotting was performed with the anti-FLAG, MSH2, MLH1 and PCNA antibodies.

DISCUSSION

There are two types of DNA lesions that cause apoptosis, one that blocks DNA replication and another that allows progression of the DNA replication forks. Unless otherwise repaired, both types of lesions can induce cell death, but in different ways. Blockage of DNA replication by bulky DNA lesions is sensed by the ATM/ATR system, which activates the mitochondria-dependent apoptotic pathway for cell death (36). However, modified bases, which allow DNA replication, are recognized by a protein complex, composed of the bacterial mismatch repair protein homologs MutSα and MutLα, to induce apoptosis (18,24). The latter process is of utmost biological and medical significance, since the survival of cells carrying such mismatches would cause base substitution mutations, leading to the induction of tumors and the disposition of hereditary diseases.

Studies on the mechanism of apoptosis caused by replication-permitting base mispairs have been hampered by the lack of an appropriate *in vivo* system, since such mispairs are rarely formed during the normal DNA replication. *In vitro* reconstitution systems with synthetic DNA strands carrying such mismatches have been devised, and protein complexes interacting with the DNA fragments were isolated (37,38). However, it was sometimes difficult to analyze the relevant roles of these complexes, found *in vitro*, in the induction of apoptosis, since base mismatches would cause entirely different outcomes, including DNA repair, apoptosis and some others, according to the conditions *in vivo*. In this regard, O^6-methylguanine-induced mispairs are unique, since most of the cells with these lesions undergo apoptosis (18). O^6-methylguanine can be produced in the chromosomal DNA *in situ* by exposing cells or organisms to simple alkylating agents and, moreover, the amount of the mismatched bases in the DNA can be controlled by regulating the doses of the agents. In the present study, we have taken advantage of this system to identify a protein complex that interacts with mismatched DNA under physiological conditions, in which the protein interactions on the intact chromatin are fixed by a chemical crosslinker introduced into permeabilized cells, and have related it to the induction of apoptosis.

When human cells deficient in O^6-methylguanine-DNA methyltransferase activity were exposed to appropriate doses of MNU, a protein complex composed of PCNA, MutSα and MutLα was formed on the chromosomal DNA. Without exposure to the alkylating agent, no such complex was formed. In normal cells, PCNA and MutSα exist as forms associated with the chromatin, and this association was observed even in cells that lack MLH1, a component of MutLα. In the absence of MutSα, owing to the lack of its component MSH2, no association of MutLα with the chromatin was achieved. Time course experiments revealed that the formation of the PCNA–MutSα–MutLα complex occurs gradually after MNU treatment and reaches a maximum of ~12 h after the treatment, implying that at least one cycle of DNA replication is required for initiating the complex

formation. This notion was supported by our finding that the prevention of DNA replication by a treatment with aphidicolin, a DNA polymerase inhibitor, caused significant decrease in the levels of complex formation. After one round of DNA replication, the O^6-methylguanine–cytosine pair, produced by the action of alkylating agents, can be converted to an O^6-methylguanine–thymine pair, which may be the target for the MutSα–PCNA complex. Notably, the pancytopenia observed in MNU-treated *MGMT*$^{-/-}$ mice is associated with the death of actively growing cells in bone marrow and intestinal mucosa, and this impairment is completely suppressed by the introduction of the *Mlh1* mutation into the mice (5,17).

PCNA is a component of the DNA replication machinery, and its role in mismatch repair has also been implicated. In *in vitro* reconstitution experiments, physical interactions of MutSα with PCNA were detected, and co-localization of MutSα and PCNA in the nucleus was also shown (37,39–41). In the present study, we found that PCNA persists on the chromatin in a form associated with MutSα and, on the exposure of cells to an alkylating agent, it further makes a complex with MutLα. When siRNA for *PCNA* was introduced, the amount of PCNA as well as those of the components of MutSα and MutLα, forming the complex on the chromatin, decreased considerably. Consistent with this reduction, the induction of caspase-3, an event associated with apoptosis, was significantly suppressed. These results support the view that PCNA is required for the progression of O^6-methylguanine-induced apoptosis, in addition to its fundamental roles in DNA replication and repair.

How is the O^6-methylguanine-triggered apoptosis executed? The PCNA–MutSα complex may act as a genome surveillant and bind to the O^6-methylguanine–thymine pair, which is produced after one round of DNA replication. This would then facilitate further binding of MutLα to the complex, and the PCNA–MutSα–MutLα complex thus formed might exert its action on certain molecules, which would transmit the apoptotic signal to downstream members. Since the activation of caspase-3 occurs during the course of O^6-methylguanine-induced apoptosis (18), as observed in the processes triggered by TNF and blockage of DNA replication forks (42,43), it seems that the signals delivered from different sources converge at the step of caspase-3 induction, which then activates the subsequent common steps necessary for the execution of apoptosis, including DNA fragmentation and the formation of apoptotic bodies. To understand the entire scheme of the O^6-methylguanine-induced apoptotic pathway, the components functioning downstream of mismatch repair proteins must be identified. Studies aimed at elucidating this process by both biochemical and genetical means are in progress in the laboratory.

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