Defective Flap Endonuclease 1 Activity in Mammalian Cells Is Associated with Impaired DNA Repair and Prolonged S Phase Delay*  

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Flap endonuclease 1 (FEN-1) is a 5′–3′ flap exo/endonuclease that plays an important role in Okazaki fragment maturation, nonhomologous end joining of double-stranded DNA breaks, and long patch base excision repair. Here, we demonstrate that the wild type FEN-1 binds tightly to chromatin in conjunction with proliferating cell nuclear antigen (PCNA) recruitment after MMS treatment, and the nuclease-defective FEN-1 increased the sensitivity of the cells to methylmethane sulfonate (MMS) and to UV light but not to ionizing radiation. In contrast, the cells expressing the nuclease-defective and PCNA binding-defective double mutant FEN-1 exhibited sensitivities similar to those in the cells expressing the wild type FEN-1. MMS treatment caused a prolonged delay of S phase progression and impairment in colony-forming activity of cells expressing nuclease-defective FEN-1. A comet assay demonstrated that DNA repair after MMS or UV treatment was impeded in the cells expressing nuclease-deficient FEN-1 but not in the cells with double-mutated FEN-1. Taken together, these findings suggest that FEN-1 plays an essential role in the DNA repair processes in mammalian cells and that this activity of FEN-1 is PCNA-dependent.

The FEN-1 nuclease recognizes specific types of DNA structures. The 5′-flap structure is a common DNA structural intermediate occurring during DNA replication, recombination, and repair. In eukaryotic DNA replication, displacement of an upstream primer by incoming polymerase can result in the formation of the 5′-flap structure (1). In vitro, FEN-1 is highly active toward 5′-flap DNA. It acts as an endonuclease to cleave the displaced flap strand at the single strand/double strand junction. It also acts as a 5′–3′ exonuclease. Acting as an exonuclease, FEN-1 participates in hydrolysis of double-stranded DNA substrates containing a nick, gap, or 3′-overhang. During lagging strand DNA synthesis, RNA primers are removed by RNase H1. However, this enzyme cannot excise the final 5′-terminal ribonucleotide at the RNA-DNA junction. The completion of RNA primer removal by FEN-1 is essential for Okazaki fragment processing in reconstituted replication assays (2, 3). The 5′-flap intermediates are also formed during double-stranded break repair, homologous recombination, and excision repair (4).

DNA base damage generated by a variety of physical and chemical agents is a major threat to the genetic integrity of cells. To remove damage and maintain the integrity of the genome, a number of DNA repair pathways are provided. The major repair pathway in mammalian genomic DNA is thought to be the base excision repair (BER), although other pathways such as nucleotide excision repair, recombination repair, and translesion DNA synthesis can also be involved. BER is initiated by the enzymatic removal of the altered base or by chemical hydrolysis of the glycosidic bond connecting the damaged base to the sugar phosphate backbone. Recent evidence has indicated that BER in mammalian cells is mediated through at least two subpathways that are differentially by their repair patch sizes and the enzymes involved and are designated as single-nucleotide BER and long patch BER (5, 6). The choice of subpathways in BER depends on whether the dRP intermediate can be efficiently removed by the polymerase β lyase activity to yield a 5′-phosphorylated DNA strand capable of serving as a substrate for DNA ligase (7). When such processing is inefficient, long patch BER can occur.

The in vitro role of FEN-1 in the DNA repair and of its interaction with proliferating cell nuclear antigen (PCNA) has been mainly assessed in yeast mutants (8–10), but very limited information is available for mammalian cells. In the present study, we demonstrate a critical role of FEN-1 in DNA repair from MMS or UV irradiation-induced damage but not from ionizing radiation in mammalian cells that conditionally express FEN-1. Here, we demonstrate that FEN-1 and PCNA is important for DNA repair processes in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture—T24 human bladder carcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells that express the wild type and mutant FEN-1 proteins under the control of the tetracycline operator were generated by using a mammalian expression system from the complete cytomegalovirus promoter (Invitrogen). T24 cells stably expressing the tetracycline repressor were transiently transfected with a second vector expressing the wild type or mutant fen-1 gene under the control of tetracycline. Protein expression was induced by adding 1 μg of tetracycline to the culture medium for 24 h.

Cloning and Site-directed Mutagenesis of Human fen-1—cDNA for human FEN-1 was amplified from the human cDNA using the oligonu-
The absence (H11002) of nuclease-defective (T24N), or nuclease- and PCNA binding-defective FEN-1 proteins were exclusively localized in the nuclei of S phase T24 binding-defective FEN-1 proteins. FEN-1 proteins were visualized by collected and suspended in cold hypotonic buffer (10 mM Hepes, 5 mM MgCl2 and 0.5 mM CaCl2) and extracted with CSK buffer (10 mM PIPES-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) containing 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM mg/ml leupeptin for 5 min at 20 °C. Cells were washed with permeabilization buffer and then extracted with high salt nuclear extraction buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 30 mM NaVO4, 10 mM β-glycerophosphate for 10 min at 4 °C). Nuclei were recovered by centrifugation at 1,000 × g for 5 min. Nuclei were washed with permeabilization buffer and then extracted with high salt nuclear extraction buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 30 mM NaVO4, 10 mM NaF, and 1 mM EDTA) supplemented with 10 μg/ml aprotonin, 5 μg/ml leupeptin, 20 μM microcinystein-LR, 1 μM Na3VO4, and 10 mM β-glycerophosphate for 10 min at 4 °C. Nuclei were recovered by centrifugation at 22,000 × g for 10 min. Lysates were either immunoprecipitated as indicated or mixed with 2% SDS-PAGE sample buffer and boiled for 10 min. Aliquots derived from the equivalent cell numbers were separated by 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore Corp.) and immunooblotted using either monoclonal anti-FEN-1 (FEN-1–4E7, 2 μg/ml; GeneTex), polyclonal anti-Myc Tag (residues 409–420; Upstate Biotechnology, Inc., Lake Placid, NY), or monoclonal anti-PCNA (1 μg/ml; Upstate Biotechnology) antibodies. Antibodies used for the cell cycle checkpoint proteins were polyclonal anti-Chk1 (1 μg/ml; Cell Signaling Technology), polyclonal anti-phospho-Chk1 (Ser42, 1 μg/ml; Cell Signaling Technology), polyclonal anti-Chk2 (1 μg/ml; Upstate Biotechnology), polyclonal anti-phospho-Rb (Ser389, 1 μg/ml; Cell Signaling Technology), or polyclonal anti-phospho-Rb (Ser780, 1 μg/ml; Cell Signaling Technology). Immunooblots were visualized by chemiluminescence (ECL; Amersham Biosciences).

Immunofluorescence Microscopy—Cell preparations for immunofluorescence microscopy were performed by a method from Martini et al. (12). Cells grown on coverslips were washed with PBS containing 0.5 mM MgCl2 and 0.5 mM CaCl2 and extracted with CSK buffer (10 mM PIPES-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2) supplemented with 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mg/ml leupeptin for 5 min at 20 °C. Cells were washed three times with PBS and then fixed with methanol at −20 °C for 10 min. Cells were washed with PBS and further fixed with 4% paraformaldehyde. After washing with PBS, cells were blocked in 5% bovine serum albumin, 0.1% Tween in PBS (blocking buffer) for 10 min, and specific antibodies were added at the appropriate dilution (5 μg/ml; antibodies to anti-PCNA antibodies). To visualize the primary antibodies, fluorescein isothiocyanate-conjugated anti-rabbit IgG or Cy3-conjugated mouse IgG was used. Confocal analysis was performed using a Zeiss LMS 5 Pascal confocal laser scanning microscope equipped with an Axioplan 2 and a ×63 objective lens. The images were produced using the Adobe Photoshop 5 software program (Adobe Systems Inc., San Jose, CA).

Colony Formation Assay—Cells (0.25 or 1 × 104) were seeded onto a dish and continued to cultivate for 8 h. After appropriate treatments, cells were left to grow for an additional 8–10 days. The numbers of colonies for each dish were enumerated after Giemsa staining.

Single Cell Gel Electrophoresis (Comet) Assay—The comet assay was performed using a commercially available kit (Treveniv) under alkaline conditions. After appropriate treatment, cells were trypsinized for 4 min and suspended in low point melting agarose at 1 × 109/ml. Immediately after mixing, aliquots (75 μl) of cell suspension were placed onto a slide. After solidification of agarose at 4 °C in the dark for 10 min, the slides were placed in ice-cold lysis solution (2.5 mM NaOH, 100 mM EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10, 10% Me2SO, and 1% Triton X-100) for 1 h. The slides were then incubated in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA for 40 min at room temperature). Electrophoresis was carried out at 300 mA for 20 min. The slides were neutralized, and DNA was stained. The tail moment of fragmented DNA was quantitated using a public domain image analysis program for the comet assay.

Cell Growth and Cytotoxicity Assays—Cell growth and cytotoxicity of cells after MMS treatment were assessed using commercially available...
kits, the Cell Counting Kit-8 (Dojindo) and the LDH-Cytotoxic Test (Wako).

RESULTS

Conditional Expression of the Wild Type or Mutant Human FEN-1 Proteins—Three functional motifs of FEN-1 are proposed to be responsible for its nuclease activities, interaction with PCNA, and nuclear localization (13). To examine the role of FEN-1 in DNA repair in mammalian cells, we first established human cell lines that expressed the wild type or nuclease-defective human FEN-1 protein under the control of tetracycline (Fig. 1A). FEN-1 can interact with PCNA to increase its nuclease activity during the course of DNA replication, DNA repair, or both, implying a critical role of the interaction between FEN-1 and PCNA (14). Therefore, we also created an additional human cell line that expressed the nuclease-defective and PCNA binding-defective double mutant protein. Mock-transfected T24 cells (T24TR) contained endogenous FEN-1 protein, which did not significantly change in expression levels after tetracycline treatment (Fig. 1B). Cells transfected with a plasmid containing the wild type (WT) or either of the mutant FEN-1 genes (nuclease-defective (N) and nuclease and PCNA binding-defective (NP)) expressed the corresponding FEN-1 protein in the presence of tetracycline, whereas a trace amount of the proteins was detected without tetracycline. Western blot analysis confirmed that the introduced FEN-1 proteins were at similar levels among the three cell lines. The densitometric analysis showed that the ratios of exogenous to endogenous FEN-1 proteins were 1.3, 1.7, and 1.5 for the WT, nuclease-defective, and nuclease and PCNA binding-defective FEN-1 proteins, respectively (Fig. 1B). These FEN-1 proteins were localized predominantly in the nucleus (Fig. 1C).

We next examined the function of exogenously expressed FEN-1 proteins. To this end, we immunoprecipitated the FEN-1 proteins from cells that were mock-transfected or were expressing either the wild type, nuclease-defective, or nuclease-defective and PCNA binding-defective FEN-1 proteins and assessed the PCNA binding activity and the flap endonuclease activity of the FEN-1 complexes. The immunoprecipitates from cells expressing the wild type FEN-1 contained PCNA and exhibited flap endonuclease activity (Fig. 2A). Confocal microscopy demonstrated that FEN-1 and PCNA were colocalized in the nucleus of these cells in the S phase of the cell cycle (Fig. 2B). In contrast, the immunoprecipitates from cells expressing nuclease-defective FEN-1 did not display endonuclease activity, although a substantial amount of PCNA protein was detected (Fig. 2A). As expected, the immunoprecipitates from cells expressing the double mutated FEN-1 did not contain PCNA protein and did not exhibit flap endonuclease activity. Importantly, any cell line expressing the wild type or mutated FEN-1 grew at similar rates compared with the mock-transfected cell line, independent of the absence or presence of tetracycline.

MMS Treatment Induces FEN-1 and PCNA—We reasoned that FEN-1 and PCNA should bind tightly to chromatin in the nucleus for participating in DNA repair, since PCNA was re-
ported to bind tightly to chromatin in cells with DNA damage by alkylating agents (15). Therefore, we assessed in confluent cells (non-S phase cells) whether the affinity of endogenous FEN-1 and PCNA proteins to the chromatin is selectively altered after MMS treatment. To this end, we fractionated cell lysates into low salt buffer (cytosolic fraction), high salt buffer (nuclear soluble fraction), and further into chromatin-bound extracts. The chromatin-bound fraction was recovered as a nuclear remnant fraction after Triton treatment of and then centrifugation of the high salt extracts. Then we immunoblotted the endogenous FEN-1 and PCNA in each of the cell lysates. MMS treatment did not significantly affect the level of FEN-1 either in cytosolic or nuclear soluble extracts (Fig. 3A).

PCNA is, however, more efficiently extractable from the nuclear soluble fraction after MMS treatment. In contrast, FEN-1 and PCNA were much more bound to the chromatin matrix (nuclear remnant) in MMS-treated cells. The MMS-induced chromatin bindings of PCNA and FEN-1 treatment were dose-dependent, whereas both protein levels did not change in the total cell lysates, indicating that chromatin-bound PCNA and FEN-1 were selectively increased after MMS treatment (Fig. 3B). These findings suggest that FEN-1 and PCNA proteins form tight complexes with the chromatin in response to MMS treatment.

To test whether exogenously expressed FEN-1 that is tightly bound to chromatin is also increased in response to MMS treatment, we performed confocal microscopy to visualize the subcellular localization of exogenously expressed FEN-1 and endogenous PCNA in T24 cells expressing the wild type or either of the mutant proteins after MMS treatment. These cells were first permeabilized with 0.2% Triton X-100 to visualize the chromatin-bound form of the proteins. Similarly to endogenous FEN-1, the exogenously expressed wild type and nuclease-defective FEN-1 proteins were induced to bind chromatin by MMS treatment (Fig. 4A). However, the double-mutated FEN-1 was not induced to bind chromatin by the agent, consistent with the notion that PCNA is an adapter protein for FEN-1 to recruit the protein onto the appropriate site for its action on DNA (16). The introduced wild type and nuclease-defective FEN-1 proteins both colocalized with the PCNA protein in MMS-treated cells.

To substantiate these findings, we determined exogenous FEN-1 protein levels that were bound to chromatin by Western blotting (Fig. 4B). Chromatin-bound wild type FEN-1 was detectable in T24WT cells in the absence of MMS. This appears to be inconsistent with the findings of confocal studies. A possible explanation for this discrepancy is that anti-Myc tag antibodies were not accessible to the epitope in vivo, probably due to the conformational masking of the epitope in the complexes containing FEN-1. The wild type and nuclease-defective FEN-1 were found to be more tightly bound to the chromatin matrix in cells treated with MMS, while the double mutated FEN-1, which lacks a binding property to PCNA, was not recovered from the chromatin fraction, implying that the binding of the FEN-1 protein to the chromatin is via the PCNA (Fig. 4B). Collectively, these findings suggest that the introduced FEN-1 proteins were bound to chromatin in these cells in a PCNA- and DNA damage-dependent fashion.

Cells Expressing Nuclease-defective FEN-1 Are Sensitive to MMS—Given that the exogenously expressed wild type human FEN-1 protein is functional and behaves like the endogenous protein in response to MMS, we next asked whether the single mutation in the nuclease domain or the double mutation also in the PCNA-binding domain of the FEN-1 protein may affect the responses of the cells expressing either of these mutant proteins to MMS. We first examined the colony-forming capacities of the cells expressing the wild type, nuclease-defective, or nuclease-defective and PCNA binding-defective protein in the presence of MMS. Consistent with the findings that the exogenously expressed proteins did not affect the cell growth in the absence of MMS (Fig. 2C), the colony-forming capacity was not
significantly different among the cell lines expressing either the wild type or mutant FEN-1 in the absence of MMS (Fig. 5A). Furthermore, in the absence of tetracycline, all cell lines tested showed a dose-dependent, parallel decrease in the number of colonies formed after MMS treatment. MMS inhibited in a parallel fashion the colony formation in the mock-transfected cells and the cells expressing the wild type or double mutated FEN-1. In contrast, the cells expressing the nuclease-defective FEN-1 showed a markedly increased sensitivity to MMS for the colony-forming capacity, with differences in colony numbers being apparent at as low a dose as 0.25 mM.

The lactate dehydrogenase and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays, which detect dead and intact cells, respectively, indicated that the defective colony-forming capacity of T24N cells was not due to accelerated cell death; the nuclease-defective FEN-1 decreased the number of intact cells after MMS treatment, whereas the mutant FEN-1 did not accelerate the cell death (Fig. 5, B and C).

Next, we examined the cell cycle profiles of MMS-treated cells expressing nuclease-defective FEN-1 (Fig. 6). Cells were treated for 1 h with 0.5 mM MMS and then washed to remove MMS from the culture medium. Fluorescence-activated cell sorting analysis of T24N cells in the absence or presence of tetracycline demonstrated that cells in both conditions displayed gradual increases in the S phase fractions at 12 h after MMS removal from the culture medium. However, at 24 h after MMS removal, cells in the culture medium without tetracycline were still accumulated at S phase of the cell cycle. A further observation up to 36 h of cell cultures in the presence of tetracycline demonstrated that these cells were arrested at S phase and the G2/M boundary in the presence of tetracycline. These findings suggest that cells expressing nuclease-defective FEN-1 exhibit a significantly prolonged delay in S phase progression after MMS treatment.

**Repair of MMS-induced DNA Damage Is Retarded in Cells Expressing Nuclease-defective FEN-1**—Taken together, these
findings suggest that nuclease-defective FEN-1 may act as a dominant negative mutant and may cause a prolonged delay in the repair process from MMS-induced DNA damage. We tested this possibility by conducting the single cell gel electrophoresis (comet) assay, where the amount of damaged DNA was quantitated by calculating the tail moment (Fig. 7). Cells were precultured for 24 h in the absence or presence of tetracycline, treated for 1 h with 0.25 mM MMS, and washed, and then cultivation continued in the medium without MMS for an additional 2 h to allow recovery from MMS-induced DNA damage. Tetracycline was present in the recovery medium.

MMS treatment of the mock-transfected T24TR cells resulted in a marked shift of the tail moments to the right, indicating that DNA was fragmented to varying degrees (Fig. 7A). By 2 h after MMS removal from the culture medium, the tail moments had approximated the control profile, indicating that the damaged DNA had been efficiently repaired. Such kinetics did not change in the presence of tetracycline. T24WT cells showed changes in the tail moment profile similar to those seen in the mock-transfected cells in the absence or presence of tetracycline (Fig. 7A). T24NP cells in the absence of tetracycline displayed kinetics indistinguishable from that of T24WT cells (Fig. 7A and B). However, T24N cells expressing nuclease-defective FEN-1 exhibited a further shift to the right of the tail moments throughout the course of the MMS-induced DNA damage and its repair (Fig. 7A and B). These findings suggest that, compared with the cells expressing the wild type FEN-1, MMS yielded significant DNA damage, and the DNA repair was significantly impaired in cells expressing nuclease-defective FEN-1.

In contrast, a further mutation in the PCNA-binding domain of the FEN-1 protein created cells that respond to MMS in a similar fashion to the mock-transfected cells (T24TR) and to the T24WT cells. Given that the nuclease-defective FEN-1 protein exerted its dominant-negative function through the binding with PCNA, these findings suggest that FEN-1 is critical for DNA repair from MMS-induced damage and that its function is dependent upon the binding to PCNA. Furthermore, the finding that the double-mutated FEN-1 protein, which lacks both nuclease and PCNA-binding capacities, did not bind chromatin in MMS-treated cells suggests that nuclear localization and recruitment onto chromatin along with the PCNA is a critical step for DNA repair after MMS treatment.

**FEN-1 Is Also Involved in the Repair from UV-induced but Not in Ionizing Radiation-induced DNA Damage**—Given the significant contribution of FEN-1 in the repair process from MMS-induced DNA damage, we next tested whether the repair from other DNA-damaging agents, such as UV and ionizing radiation (IR), might require FEN-1. UV-C caused dose-dependent decreases in the number of colony formations in mock-transfected T24N cells (Fig. 8A). When nuclease-defective FEN-1 was introduced by tetracycline in these cells, the survival rate was further decreased by UV-C irradiation. Comet assay showed that DNA repair was moderately but significantly impaired in T24N cells in the presence of tetracycline (Fig. 8B). The T24TR and T24WT cells in the presence or absence of tetracycline exhibited similar sensitivity to UV-C as the N cells expressing dominant-negative FEN-1 (data not shown). Therefore, these findings suggest that FEN-1 is also involved in the repair process from DNA damage by UV irradiation. The role of FEN-1 was also tested for IR. We used x-rays as an IR source. In contrast to the findings with MMS and UV, T24N cells expressing nuclease-defective FEN-1 showed similar levels of sensitivity to IR (Fig. 8C). No significant difference was observed for the comet assay (data not shown). Together, these findings suggest that FEN-1 is significantly involved in the repair processes from DNA damage by alkylating agents such as MMS and by UV irradiation but not from DNA damage by IR.

**S Phase Delay Caused by Dominant Negative FEN-1 Is Associated with Alterations in Checkpoint Protein Levels**—Given that the loss of FEN-1 activity in T24 cells expressing dominant-negative FEN-1 leads to a prolonged S phase delay of the cells in response to DNA damage by MMS or UV, we examined the effects of this dominant-negative FEN-1 on the checkpoint pathway in these cells. To this end, we monitored the protein levels of Chk1 and Chk2, which are considered to play important roles in S phase response to DNA damage (17–19). Phosphorylated Chk1 is the active form of the protein. Thus, we assessed the level of phosphorylated Chk1 using antibodies specific for the phosphorylated form of Chk1 (Fig. 9A, upper panel). In the absence of tetracycline, MMS treatment resulted in a slight increase in the level of phosphorylated Chk1, but the level had returned to the control by 24 h after the removal of MMS in the culture medium. In contrast, the MMS treatment-induced phosphorylated Chk1 level was sustained at 24 h of recovery. The total Chk1 protein level was not significantly different between cells at 0 h and those at 24 h after MMS removal and between cells with and without tetracycline (Fig. 9A, lower panel). MMS treatment did not induce Chk2 protein in these cells (data not shown).

The Rb is a negative regulator of the cell cycle, and dephosphorylation of Rb in S phase leads to the inhibition of DNA synthesis and thus S phase progression (20). Therefore, we also assessed the Rb protein levels in cells expressing dominant-negative FEN-1. MMS treatment resulted in rapid increases in the total and hypophosphorylated Rb protein levels in T24N cells (Fig. 9, B and C). No significant difference in the phosphorylation levels was observed between T24N cells expressing nuclease-defective FEN-1 and those without FEN-1 expression in control cells and cells at 0 h. However, at 24 h after MMS removal from the culture medium, the level of hypophosphorylated Rb protein was greater in amount in T24N cells express-
ing nuclease-defective FEN-1. These findings were consistent with the notion that hypophosphorylated Rb represents a biologically active form of Rb in vivo that assembles with E2F and E1A (22).

DISCUSSION

Nuclear Translocation and Subsequent Chromatin Binding of FEN-1 and PCNA—In the present study, we showed FEN-1 recruitment to chromatin during repair processes from DNA-damaging agents as well as during S phase of the cell cycle, supporting a physiological role of PCNA in linking FEN-1 association with chromatin to DNA repair. FEN-1 translocates into the nucleus in a cell cycle-dependent manner (22), with the protein accumulating in the nucleus of cells in S phase of the cell cycle. The assembly and maintenance of the
nucleoprotein complex is important to ensure regulated DNA metabolism. In proliferating cells, the histone synthesis and chromatin assembly occur during S phase of the cell cycle (23). Reassembly of chromatin also occurs during DNA metabolism and maintenance other than S phase. For instance, the detergent (Triton)-resistant, tight association of human chromatin assembly factor with chromatin increased after UV irradiation, and this occurred in parallel with the recruitment of PCNA to chromatin (12).

Role of FEN-1 in the DNA Repair from MMS-induced Damage—The general concept is that cells exposed to genotoxic stress cease proliferating to provide time for DNA repair, to protect genome integrity and ensure survival. Consistent with this notion, we found that the prolonged S phase delay in cells expressing nuclease-defective FEN-1 in response to MMS implies that DNA repair from the damage caused by this alkylating agent is significantly retarded in these cells. In Saccharomyces cerevisiae, MMS slows down S phase progression by blocking initiation from late origins, via a checkpoint gene rad53-dependent mechanism (24). T24N cells expressing nuclease-defective FEN-1 displayed a prolonged arrest during S phase, implying that the checkpoint mechanism remained activated for a longer period than the control cells. Supportive of this notion may be the findings that the active Chk1 protein (phosphorylated Chk1) levels remained elevated even at 24 h after MMS removal from the culture medium, while the Chk1 activation was transient in T24N cells that do not express the nuclease-defective FEN-1. T24N cells expressing nuclease-defective FEN-1 may be escaping from apoptotic death by slowing the S phase progression.

Importance of the Interaction between FEN-1 and PCNA in DNA Repair—An in vitro system using mouse or human cell-free extracts demonstrated that PCNA can stimulate the FEN-1-dependent long patch BER in the presence of DNA polymerase β (25, 26). A more recent study showed that stimulation of human FEN-1 nuclease activity by PCNA was independent of its in vitro interaction with PCNA (27). Here, we showed that the PCNA binding ability of FEN-1 was indispensable for the dominant negative action of nuclease-defective FEN-1. This, in turn, may suggest that FEN-1 exerts its nuclease function through PCNA binding. In support of this possibility, confocal microscopy and Western blot analysis demonstrated that 1) PCNA always translocates into the nucleus and binds tightly to chromatin in response to MMS, irrespective of the FEN-1 state; 2) PCNA binding-defective FEN-1 translocates into the nucleus but does not bind tightly to chromatin; and 3) most of the PCNA colocalized with FEN-1 harboring intact PCNA binding sites. These findings are consistent with the FEN-1-PCNA interaction model, where PCNA stabilizes the binding of FEN-1 after it arrives at the cleavage site (14).

Role of FEN-1 in DNA Repair from UV or Ionizing Radiation—As in the case with S. cerevisiae carrying a deletion of the RAD27 gene (9), T24 human bladder carcinoma cells expressing nuclease-defective FEN-1 exhibited significantly increased sensitivity to MMS and UV light but not to ionizing radiation. It is not evident why deletion of the FEN-1 function does not

FIG. 8. FEN-1 activity is important in DNA repair from UV-induced, but not from IR-induced, damage. A, impaired colony growth after UV-C irradiation in cells expressing nuclease-defective FEN-1 (T24N). B, DNA repair was retarded in cells expressing nuclease-defective FEN-1 after UV-C irradiation (7.5 J/m²). C, colony-forming activity of T24N cells after IR treatment was not significantly different between cells with and without nuclease-defective FEN-1 expression.
confer such hypersensitivity to ionizing radiation. Different from other genotoxic agents such as MMS and UV light, ionizing radiation can directly produce single and double strand breaks by depositing energy onto the deoxyribose phosphate backbone (28). FEN-1 may have little if any role in the repair process for ionizing radiation-specific DNA damage. Nonhomologous end joining and homologous recombination are the two major pathways for repairing double strand DNA breaks in mammalian cells. The nonhomologous end joining pathway is preferentially used in repairing double strand breaks during G1 to early S phase, while homologous recombination is responsible during S to G2 phase (29). FEN-1 was implicated to be involved in nonhomologous end joining but not in homologous recombination (30, 31). Therefore, we speculate that FEN-1 does not play a major role if any in repairing ionizing radiation-induced double strand breaks in cells lacking functional p53.

Nuclear excision repair is a major mechanism for repairing UV-induced lesions. Recently, however, a UV damage endonuclease was identified to stimulate the repair process in mammalian cells by introducing a single strand break immediately 5’ to UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidine (6–4) phosphoproduts, leaving 3’-hydroxyl and 5’-phosphoryl groups at the site of cleavage (32). A single strand break induced by UV damage endonuclease was shown to be a substrate for cleavage by FEN-1 in vitro (33). Therefore, these results suggest that there was moderately increased sensitivity to UV observed in T24 cells expressing nucleotide-defective FEN-1 because the DNA repair process responsible for the UV damage endonuclease-FEN1 pathway was impaired but the repair process for the nucleotide excision repair pathway was still intact.

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FIG. 9. Loss of FEN-1 function affects DNA damage checkpoint mechanism. A, expression of nucleotide-defective FEN-1 caused a sustained activation of Chk1. The total protein level was not significantly different during the recovery from MMS treatment (1 h) between T24N cells with or without nuclease-defective FEN-1 expression (lower panel). In contrast, the phosphorylated (Ser\(^{\text{345}}\)) Chk1 (ppChk1) level was markedly increased during the recovery period (upper panel). B and C, Rb protein remained inactivated during the recovery from MMS treatment. The levels of total Rb protein (pRb, lower panels) and of phosphorylated Rb protein (ppRb, upper panels) were higher in T24N cells expressing nucleotide-defective FEN-1 compared with those of the controls.
