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Nuclear reorganization of DNA mismatch repair proteins in response to DNA damage

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ABSTRACT

The DNA mismatch repair (MMR) system is highly conserved and vital for preserving genomic integrity. Current mechanistic models for MMR are mainly derived from *in vitro* assays including reconstitution of strand-specific MMR and DNA binding assays using short oligonucleotides. However, fundamental questions regarding the mechanism and regulation in the context of cellular DNA replication remain. Using synchronized populations of HeLa cells we demonstrated that hMSH2, hMLH1 and PCNA localize to the chromatin during S-phase, and accumulate to a greater extent in cells treated with a DNA alkylating agent. In addition, using small interfering RNA to deplete hMSH2, we demonstrated that hMLH1 localization to the chromatin is hMSH2-dependent. hMSH2/hMLH1/PCNA proteins, when associated with the chromatin, form a complex that is greatly enhanced by DNA damage. The DNA damage caused by high doses of alkylating agents leads to a G_2 arrest after only one round of replication. In these G_2-arrested cells, an hMSH2/hMLH1 complex persists on chromatin, however, PCNA is no longer in the complex. Cells treated with a lower dose of alkylating agent require two rounds of replication before cells arrest in G_2. In the first S-phase, the MMR proteins form a complex with PCNA, however, during the second S-phase PCNA is missing from that complex. The distinction between these complexes may suggest separate functions for the MMR proteins in damage repair and signaling. Additionally, using confocal immunofluorescence, we observed a population of hMSH6 that localized to the nucleolus. This population is significantly reduced after DNA damage suggesting that the protein is shuttled out of the nucleolus in response to damage. In contrast, hMLH1 is excluded from the nucleolus at all times. Thus, the nucleolus may act to segregate a population of hMSH2–hMSH6 from hMLH1–hPMS2 such that, in the absence of DNA damage, an inappropriate response is not invoked.

1. Introduction

The DNA mismatch repair (MMR) system plays an essential role in maintaining the fidelity of DNA replication by correcting single nucleotide mismatches and insertion/deletion (ID)-loops [1]. This system is further specified by the presence of three MutsS homologs (hMSH2, hMSH3, and hMSH6) that form two distinct heterodimers with hMSH2 as the common subunit. In addition, four MutL homologs have been identified (hMLH1, hMLH3, hPMS1, and hPMS2) that assemble into three functional heterodimers where the hMLH1–hPMS2 heterodimer is required for DNA MMR [2]. Loss of MMR is associated with a mutator phenotype and the hereditary cancer syndrome hereditary nonpolyposis colorectal cancer (HNPCC) [3–6].

The correction of single nucleotide mismatches has been reconstituted *in vitro* using mammalian cell extracts or purified recombinant proteins and a circular heteroduplex substrate [7–9]. From these studies the minimal factors required to support both 5′- and 3′-nick-directed repair were elucidated and include hMSH2–hMSH6, hMLH1–hPMS2, hEXOI, RPA, RFC, PCNA, DNA polymerase δ, and DNA ligase I. However, the question of how these protein players interact and function to execute the repair process still remains.

One model for MMR that has emerged based on the reconstitution data and experiments illuminating the biochemistry of the hMSH2–hMSH6 and hMLH1–hPMS2 heterodimers is the "molecular switch" model [10–12]. This model proposes that the MMR pathway is initiated by the hMSH2–hMSH6 heterodimer, which recognizes and binds to a single nucleotide mismatch. Exchange of ADP for ATP alters the conformation of the heterodimer such that it can freely diffuse in either direction along the DNA in an ATP hydrolysis-independent manner as a "sliding clamp". Multiple hMSH2–hMSH6 heterodimers load onto the DNA in a similar

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fashion [13], and have been proposed to be important for the recruitment of hMLH1–hPMS2 to the mismatch [14]. Formation of hMLH1–hPMS2/hMSH2–hMSH6 protein complexes is thought to be involved in the recruitment of hEXOI and subsequent strand-specific excision. Resynthesis is carried out by replication factor C (RFC), proliferating cellular nuclear antigen (PCNA), and DNA polymerase δ.

Interactions between the various components of the MMR pathway have been studied extensively using an array of *in vitro* techniques; however, the intricacies remain poorly understood. The current consensus is that formation of the hMLH1–hPMS2/hMSH2–hMSH6 ternary complex is ATP-dependent and enhanced in the presence of a heteroduplex substrate. However, both hMSH2–hMSH6 and hMLH1–hPMS2 heterodimers have some affinity for homoduplex substrates *in vitro* [15–18]. PCNA, the processivity factor for DNA polymerase has also been demonstrated to interact with MSH6, MSH3, and MLH1 via a conserved interaction motif called the PIP box [19–21]. Both MSH6 and MSH3 co-localize with PCNA at replication foci during S-phase [19]. The importance of this interaction is highlighted by the fact that a yeast Msh6 carrying mutations in the PIP box are unable to rescue defects conferred by an *Msh6* deletion strain [21,22]. PCNA–hMSH2, and PCNA–hEXOI interactions have also been demonstrated [15,21,23–25].

How these *in vitro* interaction data translate to the function of the MMR pathway in the cell during DNA replication remains an important question. Studies of the MMR pathway within the cell have focused primarily on its role in signaling a DNA damage response. MMR-deficient cells have been shown to be much more resistant to the cytotoxic effects of several DNA damaging agents [26]. Most intensely studied are the DNA methylating compounds such as the S_N1 type alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). MNNG generates a variety of DNA lesions including the cytotoxic O^6-methyl-guanine (O^6-meG) adduct. This lesion can be repaired by methylguanine methyltransferase (MGMT) in an irreversible one-step reaction whereby the methyl group is transferred from the donor guanine to MGMT [27]. However, if DNA replication occurs prior to repair, there is a propensity for O^6-meG-thymine (O^6-meG/T) mismatches to form which are recognized by the hMSH2–hMSH6 heterodimer [28]. MMR-proficient cells induce a G_2 arrest that, interestingly, requires two rounds of DNA replication following MNNG treatment [29,30]. The arrest is followed by induction of apoptosis as cells with intact MMR are approximately 100-fold more sensitive to alkylating agents than a MMR-deficient cell line [26,31]. Alternatively, as a recently published report suggests, these cells may not be arresting in G_2, but rather are proceeding through mitosis into a third cycle in the absence of cell division, ultimately resulting in cell death [32].

Though a MMR-dependent response to alkylation damage has been characterized, the mechanistic role of the MMR proteins in activating this response is not clear. Using simple predictions based on MMR mechanistic models derived from *in vitro* studies, we attempted to characterize the interactions between the major MMR proteins and with DNA in cells treated with an alkylating agent. We analyzed the localization of MMR proteins in the nucleus as well as the formation of MMR protein complexes on and off the chromatin during an unperturbed cell cycle or in cells treated with MNNG.

2. Materials and methods

2.1. Cell lines and small interfering RNA (siRNA)

HeLa S3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen). Hec59 cells were grown in DMEM:Nutrient Mixture F-12 (DMEM/F-12). Hec59 + chromosome 2 cells were grown in DMEM/F-12 supplemented with 500 μg/mL G418 sulfate (Gibco). All medium contained 10% fetal bovine serum (FBS; Gibco). Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. Transfections of siRNA were performed with Lipofectamine 2000 (Invitrogen). The hMSH2 target sequence was TCCAGGCATGCTTGTGTTGAA. The MLH1 target sequence was GTGGCTCATGTTACTATTACA. All siRNA oligonucleotides were purchased from QIAGEN.

2.2. Chemicals, antibodies, and reagents

N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) (obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository; CAS: 70-25-7) was dissolved in DMSO to a concentration of 10 mM and stored at −20 °C until use. O^6-Benzylguanine (O^6-BG; CAS: 19916-73-5) was purchased from Sigma, dissolved in DMSO to a concentration of 25 mM and stored at −80 °C until use. Thymidine was purchased from Sigma (T1895), dissolved in 1 N NaOH to a final concentration of 1 M and stored at room temperature until use. Antibodies against Nucleolin (SC-55486 mouse), PCNA (SC-56), and rabbit IgG (SC-2027) were purchased from Santa Cruz Biotechnology. Antibodies against Phospho-Chk1 S317 (#2344), Phospho-Chk2 Thr68 (#2661), Chk1-total (2G1D5), and Chk2-total (#2662) were purchased from Cell Signaling. Antibodies against hMSH6 (A300-023A) and hMLH1 (A300-015A for nucleolar immunofluorescence) were purchased from Bethyl Laboratories. Antibodies against hMLH1 (550838 for Western blot analysis and immunoprecipitations), hMLH1 (554073 for immunofluorescence studies), hMSH2 (556349 for Western blot analysis) and Orc2 (551178) were purchased from BD Biosciences. Antibody against hMSH2 (NA27 for immunoprecipitation) was purchased from Oncogene. Antibody against Actin (A5060) was purchased from Sigma. Alexa Fluor-488, -594 anti-rabbit IgG or Alexa Fluor-488, -594 anti-mouse IgG were purchased from Molecular Probes.

2.3. Cell synchronization

Synchronization at the G_1/S boundary was performed by a double thymidine block (DTB) [33]. HeLa cells were grown for 18 h in complete medium containing 2 mM thymidine, an additional 10 h without thymidine, and then incubated again for 16 h with 2 mM thymidine. At 14 h into the second block, 25 μM O^6-BG, a competitive inhibitor of MGMT, was added until the cells were released into serum free medium containing 0.2, 2, or 10 μM MNNG and 25 μM O^6-BG. After 1 h, cells were incubated in complete medium until harvested. For cell synchronization at G_2/M, HeLa cells were grown in 300 ng/mL nocodazole for 16 h at which time cells were detached by mechanical shake-off and replated in fresh complete medium without nocodazole. After 3 h, 25 μM O^6-BG was added and cells were incubated for an additional 2 h. Serum free medium was then added containing 10 μM MNNG and 25 μM O^6-BG. After 1 h, cells were incubated in complete medium until harvested.

2.4. Cell cycle analyses

Cell cycle analyses were performed using propidium iodide (PI) staining for DNA content and subsequent detection by flow cytometry. Briefly, cells were collected at various time points post-release from either double thymidine or nocodazole block and fixed in 70% ethanol at −20 °C. Cells were then treated with 20 μg/mL PI and 200 μg/mL RNase A and incubated at 37 °C for 1 h, filtered, and analyzed with a FACS Calibur flow cytometer (BD Biosciences). The resulting data was analyzed by Modfit analysis software (Verity Software House). Time points were performed in duplicate.