Identification of a Second MutL DNA Mismatch Repair Complex (hPMS1 and hMLH1) in Human Epithelial Cells*

Wai K. Leung†‡, Jae J. Kim†, Ling Wu‡, Jorge L. Sepulveda†, and Antonia R. Sepulveda‡†

From the Departments of †Medicine and ‡Pathology, Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas 77030

Deficiencies of MutL DNA mismatch repair-complex proteins (hMLH1, hPMS2, and hPMS1) typically result in microsatellite instability in human cancers. We examined the association patterns of MutL proteins in human epithelial cancer cell lines with (HCT-116, N87, SNU-1, and SNU-638) and without microsatellite instability (HeLa, AGS, KATO-III, and SNU-16). The analysis of hMLH1, hPMS2, and hPMS1 was performed using Northern blot, Western blot, and co-immunoprecipitation studies. Our data provide evidence that MutL proteins form two different complexes, MutL-α (hPMS2 and hMLH1) and MutL-β (hPMS1 and hMLH1). Gastric and colorectal cancer cells lines with microsatellite instability lacked detectable hMLH1. Decreased levels of hMLH1 protein were associated with markedly reduced levels of hPMS2 and hPMS1 proteins, but the RNA levels of hPMS1 and hPMS2 were normal. In this study, we describe the association of hPMS1 with hMLH1 as a heterodimer, in human cells. Furthermore, normal levels of hMLH1 protein appear to be important in maintaining normal levels of hPMS1 and hPMS2 proteins.

The DNA mismatch repair system (MMR) maintains the sequence integrity of the genome by recognizing and repairing mispaired nucleotides that result from misincorporation during DNA replication. The genes encoding for DNA MMR proteins are highly conserved throughout evolution. In humans, there are two sets of MMR proteins, corresponding to homologues of the bacterial MutHLS system. The human MutS proteins consist of hMSH2, hMSH3, and hMSH6 and the human MutL proteins include hMLH1, hPMS1, hPMS2, and hMLH3 (1–3).

DNA mismatch repair requires available MutS-α (MSH2 and MSH6) or MutS-β (MSH2 and MSH3) and MutL protein complexes (4–8). The MutS-α complex recognizes base-base mispairs and small insertion or deletion loops, whereas the MutS-β complex is primarily involved in the correction of small insertion or deletion loops (9, 10). Efficient mismatch repair requires that MutL protein complexes bind MutS-α or MutS-β (1).

The role of hPMS1 in human tissues has not been clarified. In yeast, the two MutL-α homologues (MLH1 and PMS1) are known to form heterodimers and are essential for MMR. In humans, the two MutL-α homologues (hMLH1 and hPMS2) also function as a heterodimer (1). Furthermore, hMSH2, hMLH1, and hPMS2 can be co-immunoprecipitated in the presence of DNA and ATP (11). Another MutL protein, the Saccharomyces cerevisiae MLH3 was shown to interact with MLH1 in a two-hybrid system (2). Similarly, the association of hMLH1 and hMLH3 was described in human cells (12). Recently, using a yeast two-hybrid assay and coexpression of baculoviruses carrying cDNAs encoding hMLH1, hPMS1, and hPMS2 in S9 cells, hPMS1 was shown to form a heterodimer with hMLH1 (3). Although a function of hPMS1 in DNA mismatch repair has not been demonstrated, a role for this protein in DNA repair is supported by studies in hPMS1 knockout mice, which showed that, although not developing tumors, these mice display poly(A) tract mutation frequencies above normal levels (13).

Defects in human mismatch repair mechanisms have been implicated in the susceptibility to some inherited and sporadic colorectal, gastric, and endometrial cancers. Characteristically, these cancers display instability of microsatellite repeats, which is an important trait in the identification of human tumors with MMR gene defects (14–17). Germline mutations of DNA MMR genes occur in more than 90% of hereditary nonpolyposis colorectal cancer (HNPPC) cases, and approximately 70% of the cases are due to germline mutations in hMLH1 and hMSH2 (14). Mutations in other MMR genes (hPMS1, hPMS2, hMSH3, and hMSH6) are infrequent (18). Recently, various missense mutations of hMLH1 in HNPPC were found to have a decreased ability to physically interact with hPMS2 (19). Other functional alterations of the human MutL homologues may be critical in tumorigenesis.

Alterations of the MutL complex are also important in subsets of sporadic colorectal and gastric cancers displaying microsatellite instability (MSI) associated with reduced expression of hMLH1 in the absence of germline mutations and derived colorectal and gastric cancers were shown to have MSI associated with decreased expression of hMLH1 (15, 20, 21). Epigenetic silencing of the hMLH1 gene caused by hypermethylation of a CpG island in the promoter region was recently found to be an important cause of MMR deficiency in sporadic gastric cancer (21–23). In this study, we used established epithelial cancer cell lines to identify the pattern of association of the human MutL proteins (hMLH1, hPMS2, and hPMS1) in cells with and without MSI. We show that epithelial cells from colorectal, cervical, and gastric cancers express the hPMS1 gene and that this protein associates with hMLH1 but not with hPMS2. Furthermore, we examined the RNA expression of MutL proteins to assess if loss of MutL proteins in cells with MSI was associated with absence of mutL transcripts.
EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—The gastric cancer cell lines included 1739-CRL (AGS), 5822-CRL (NCI-N87), HTB-103 (KATO-III), 5971-CRL (SNU-1), and 5974-CRL (SNU-638) from ATCC and SNU-638, kindly provided by Dr. Lance Ferrin and previously reported (24). The cell line SNU-1 has been reported to have a nonsense mutation at codon 226 of hMLH1 (CGA→TGA) resulting in a premature stop codon (25). The colorectal cancer cell lines (HCT-116) and the cervical cancer cell line CCL-2 (HeLa) (ATCC) were also used. The hMLH1 gene in HCT-116 cells contains a base substitution that results in a termination signal at codon 252 (TCA→TAA) (26). All gastric cancer cell lines were maintained in RPMI 1640 medium with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), except KATO-III cells, which were grown in 20% fetal bovine serum. HCT-116 cells were maintained in McCoy’s medium with 10% fetal bovine serum, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.).

MSI Assay Using Polymerase Chain Reaction—To characterize the MSI status of gastric cancer cell lines, DNA was extracted from eight or nine different cell clones per marker examined as described previously (27). The microsatellite markers included the BAT26 mononucleotide repeat markers and three dinucleotide markers, as described previously (17). Briefly, one of the primers in each set was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega Biotec, Madison, WI). Polymerase chain reactions (PCRs) were performed for 35 cycles, consisting of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, with the exception of BAT-40, which required annealing temperatures of 40 °C. The PCR products were separated through 7% polyacrylamide gels containing 32% formamide and 3% formaldehyde (28).

Western Blot—Whole cell protein extracts from cultured cancer cell lines were prepared in Laemmli sample buffer (28). The same amounts of protein (15 μg) were resolved through SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated overnight at 4 °C with antibodies against hMLH1 (clone G168-15), anti-hPMS2 polyclonal antibodies (C-20 and K-20), or antibodies against hMLH1 monoclonal antibody (clone FE11; Oncogene Research, Cambridge, MA), hMSH2 (clone FE11; Oncogene Research, Cambridge, MA), hMSH1 (clone C-20 and K-20; Santa Cruz Biotechnology), and hPMS1 (C-20 and K-20; Santa Cruz Biotechnology). Antibody concentrations were 1:1000 for all antibodies used. After washing, the filters were incubated with peroxidase-labeled anti-mouse antibodies (hMLH1, hMSH2, and hPMS1), or peroxidase-labeled anti-rabbit antibodies (hPMS1, hPMS2) (Amersham Pharmacia Biotech) for 1 h at room temperature. The proteins were then detected using an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech) and exposed to x-ray film. The membranes were then stripped and reprobed with anti-human actin antibody (Roche Molecular Biochemicals, Indianapolis, IN).

Co-immunoprecipitation—Cultured cell lines were lysed with buffer containing 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% SDS, 0.1% bromphenol blue, and 10% glycerol. The proteins were resolved through SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane. The membranes were incubated overnight at 4 °C with antibodies against hMLH1, hMSH2, or peroxidase-labeled anti-rabbit antibodies (hPMS1, hPMS2) (Amersham Pharmacia Biotech) for 1 h at room temperature. The proteins were then detected using an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech) and exposed to x-ray film. The membranes were then stripped and reprobed with anti-human actin antibody (Roche Molecular Biochemicals, Indianapolis, IN).

Microsatellite Instability in Established Gastrointestinal Cancer-derived Cell Lines—Established cancer cell lines have been productive models to study DNA mismatch repair deficiencies underling microsatellite instability in gastrointestinal cancers. We therefore used a number of gastric cancer cell lines and a colorectal cancer cell line to investigate alterations of the MutL complex. The MSI-positive cell lines included the colorectal cancer line HCT-116 (30) and the gastric cancer cell line SNU-1 (31). Control mismatch repair-competent, MSI-negative HeLa (30) and KATO-III cells were also tested (Table II). In addition, we tested additional gastric cancer cell lines (AGS, N-87, and SNU-16) and determined their patterns of microsatellite instability with four microsatellite markers that included three dinucleotide markers and one mononucleotide marker (Table II). Eight or nine different cell clones were examined with each microsatellite marker. SNU-1 cells displayed marked instability in both the dinucleotide and in the BAT marker (Fig. 1), whereas N-87 cells only showed one clone with microsatellite instability at the D13S170 dinucleotide repeat (Table II).

Expression of DNA Mismatch Repair Proteins in Gastrointestinal Cancer Cell Lines—The expression of the MutL protein homologues hMLH1, hPMS2, and hPMS1 and of the MutS homologue hMSH2 were examined by Western blot analyses (Fig. 2). Western blot analyses of MSI-positive SNU-1 gastric cancer cells showed that this cell line lacked detectable hMLH1 and hPMS2 and showed markedly reduced levels of hPMS1. HCT-116 cells (Fig. 2) and other gastric cancer lines (SNU-638, data not shown) also showed decreased levels of the three MutL proteins. Expression of hMSH2 was similar in all lines tested (Fig. 2). Next, we examined whether the RNA levels of the three MutL proteins were similarly decreased. Northern blot analyses of the MutL genes revealed normal levels of hPMS1 and hPMS2 mRNA in both SNU-1 (Fig. 3) and HCT-116 cells (data not shown), whereas hMLH1 mRNA was almost undetectable in these two cell lines (Fig. 3).
creased in SNU-1 and HCT-116 cells. hMLH1, and hPMS2 are undetectable and hPMS1 is markedly decreased. Note that all cell lines show hMSH2 expression, whereas the same blots with anti-actin antibody, used as a control for protein top

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input. Protein and RNA expression of MutL and hMSH2 MMR genes in cultured cancer cell lines.

TABLE II

MSI in gastric cancer cell lines by multiple cell clone analysis

| Cell line | D1S170 | D2S123 | TP53 | BAT26 |
|-----------|---------|---------|-------|--------|
| AGS       | 0/9     | 0/9     | 0/9   | 0/9    |
| N-87      | 1/9     | 0/9     | 0/9   | 0/9    |
| SNU-16    | 0/9     | 0/9     | 0/9   | 0/9    |
| KATO-III  | 0/9     | 0/9     | 0/9   | 0/9    |
| SNU-1     | 4/8     | 4/8     | 4/8   | 3/7    |
| HCT-116   | 4/9     | 4/9     | 7/9   | 3/8    |

TABLE III

Protein and RNA expression of MutL and hMSH2 MMR genes in cultured cancer cell lines

| Cell line | MSI | hMSH2 | hMLH1 | hPMS1 | hPMS2 |
|-----------|-----|-------|-------|-------|-------|
| Hela      | No  | Yes   | Yes   | Yes   | Yes   |
| HCT-116   | Yes | Yes   | No    | No    | No    |
| N-87      | Yes | Yes   | Yes   | Yes   | Yes   |
| AGS       | No  | Yes   | Yes   | Yes   | Yes   |
| SNU-1     | Yes | Yes   | Yes   | Yes   | Yes   |
| KATO-III  | No  | Yes   | Yes   | Yes   | Yes   |

It is likely that hPMS1 acts like the yeast homologue MLH3 and binds to hMLH1. To test this hypothesis we performed immunoprecipitation of protein extracts from the mismatch repair competent cell lines (HeLa, AGS, KATO-III, and SNU-16) and from the MSI-positive gastric cancer cell line (SNU-1) using an antibody that specifically recognizes hMLH1. After blotting to polyvinylidene difluoride membranes, the transferred proteins were identified with antibodies against hMLH1, hPMS1, and hPMS2 (Fig. 4A). To further examine the physical association pattern of MutL proteins, we investigated whether hPMS1 binds hPMS2 by performing protein co-immunoprecipitation. Whole cell protein extracts from MSI-negative AGS cells, which express the three MutL proteins, were immunoprecipitated with hPMS2 antibody, blotted to polyvinylidene difluoride membranes, and reacted with either hMLH1, hPMS1, or hPMS2 antibodies (Fig. 4B). The hMLH1 and hPMS2 antibodies recognized their target proteins after immunoprecipitation by the hPMS2 antibody, but hPMS1 antibodies did not (Fig. 4B). In addition, proteins immunoprecipitated with anti-hPMS1 reacted with anti-hMLH1 and anti-hPMS1 antibodies but not with anti-hPMS2 antibody, further supporting the notion that hPMS1 binds hMLH1 but not hPMS2. This finding demonstrates that hPMS2 and hPMS1 are present in the cell bound to hMLH1, but that hPMS1 and hPMS2 do not associate with each other. Interestingly, the amount of hPMS1 immunoprecipitated by anti-hPMS1 was similar to the amount of this protein immunoprecipitated by hMLH1 (Fig. 4B). Similarly, the amount of hPMS2 immunoprecipitated by anti-hPMS2 was similar to the amount of this protein immunoprecipitated by hMLH1 (Fig. 4B). These findings suggest that hPMS1 and hPMS2 proteins exist in the cell predominantly bound to hMLH1, with a minimal free-protein pool (Fig. 4B). Several theoretical possibilities for the association of hMLH1, hPMS1, and hPMS2 are depicted in Fig. 4C. Our data suggest that the most likely model of MutL complex formation in human cells is depicted as F and G in Fig. 4C, i.e. mutually exclusive heterodimers of hPMS2-hMLH1 (MutL-a) and hPMS1-hMLH1 (MutL-b).
levels were also low in SNU-1 and HCT-116 cells. This finding might be explained by data from recent studies indicating that hMLH1 promoter methylation was associated with low levels of hMLH1 RNA and protein in gastric cancers with a high level of MSI (21–23). Similar findings have been described in MSI-H colorectal cancers (15, 20, 34).

The requirement of hMLH1 for normal cellular levels of hPMS1 and hPMS2 also indicates that these proteins probably interact with each other. A complex containing hMLH1 and hPMS1 was only recently described by co-expression of baculoviruses carrying cDNAs encoding hMLH1, hPMS1, and hPMS2 in Sf9 cells, and in a yeast two-hybrid assay (3). To determine the patterns of interaction of the three MutL proteins in vivo, we performed co-immunoprecipitation combined with Western blot analysis using cell lines with competent DNA mismatch repair function. Our results showed that a specific antibody against hMLH1 not only precipitated hMLH1, but also hPMS1 and hPMS2 (Fig. 4). Therefore, co-immunoprecipitation confirmed that the MutL proteins form a complex consisting of hMLH1, hPMS1, and/or hPMS2. Several models for the association of the three MutL proteins are possible, as represented in Fig. 4C. The results of co-immunoprecipitation studies indicate that hPMS2 and hPMS1 are present in the cell bound to hMLH1, but that hPMS1 and hPMS2 do not associate in the cell, resulting in two types of MutL heterodimers. The model of MutL protein association can thus be depicted as F (hMLH1-hPMS2 or MutL-α, because it was the first MutL complex described) and G (hMLH1-hPMS1 or MutL-β) (Fig. 4C). The identification of two different forms of the MutL complex in human cells potentially increases the complexity of DNA MMR deficiencies associated with human disease, namely, in HNPCC and in sporadic cancers. In view of the apparent redundancy of the protein partners of hMHS2 (hMSH3 and hMSH6) and of hMLH1 (hPMS2, hMLH3, and hPMS1), it is logical that the most critical genes in DNA MMR are hMHS2 and hMLH1, matching the most frequent MMR gene deficiencies in the hereditary colorectal cancer syndromes and MSI-positive sporadic cancers (14, 21). In other words, the redundancy of hPMS1 and hPMS2 may explain why these genes are rarely found mutated in cancers with the mutator phenotype.

In conclusion, our data provide evidence for the association of hPMS1 with hMLH1 as a heterodimer in human epithelial cancer cells. In addition, normal levels of hMLH1 protein appear to be important in maintaining normal levels of hPMS1 and hPMS2 proteins, suggesting that PMS proteins are unstable in the absence of hMLH1. These findings raise the possibility that the assembly and stability of MutL complex proteins might be regulated by post-translational mechanisms.

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