Expression of the MutL Homologue hMLH3 in Human Cells and its Role in DNA Mismatch Repair

Elda Cannavo, Giancarlo Marra, Jacob Sabates-Bellver, Mirco Menigatti, Steven M. Lipkin, Franziska Fischer, Petr Cejka, and Josef Jiricny

1Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland; 2Department of Internal Medicine, Faculty of Medicine, University of Modena, Modena, Italy; and Departments of Medicine and Biological Chemistry, University of California, Irvine, Irvine, California

Abstract

The human mismatch repair (MMR) proteins hMLH1 and hPMS2 function in MMR as a heterodimer. Cells lacking either protein have a strong mutator phenotype and display microsatellite instability, yet mutations in the hMLH1 gene account for ~50% of hereditary nonpolyposis colon cancer families, whereas hPMS2 mutations are substantially less frequent and less penetrant. Similarly, in the mouse model, Mlh1<sup>−/−</sup> animals are highly cancer prone and present with gastrointestinal tumors at an early age, whereas Pms2<sup>−/−</sup> mice succumb to cancer much later in life and do not present with gastrointestinal tumors. This evidence suggested that MLH1 might functionally interact with another MutL homologue, which participates in MMR. Correspondingly, hMLH1- or hPMS2-deficient cells have a strong mutator phenotype and high microsatellite instability (reviewed in ref. 1). In <i>in vitro</i> studies, hMutLox could be shown to associate with hMutS<sub>α</sub> on a mismatch-containing substrate (4) and was suggested to act as a "molecular matchmaker" between these protein complexes and the downstream effectors of repair (reviewed in ref. 3). hMutLγ, a heterodimer of hMLH1 and hPMS1, has been biochemically characterized but could not be shown to participate in MMR <i>in vitro</i> (5). This finding was substantiated by <i>in vivo</i> evidence: Mice carrying a disruption in the <i>Pms1</i> gene display neither microsatellite instability nor cancer predisposition (6). hMLH3 was first identified in <i>Saccharomyces cerevisiae</i> and its gene product, scMlh3p, was shown to bind scMlh1p (8, 9) and to be involved in meiotic recombination (reviewed in refs. 10, 11). As <i>mlh3</i> mutants display a mutator phenotype similar to that of <i>msk3</i>-deficient strains (8, 12), it was suggested that the two polypeptides are involved in the repair of a subset of insertion/deletion loops. hMLH3 seems to be involved in meiotic recombination (13, 14) and the same is true for the murine Mlh3 (14). As both Mlh1- and Mlh3-deficient mice are sterile (reviewed in refs. 10, 11), it was suggested that the two polypeptides function together. However, unlike Mlh1<sup>−/−</sup> animals (6), Mlh3<sup>−/−</sup> mice did not succumb to cancer in the first 9 months of life (15). The roles of the various MMR factors and the phenotypes of mice with defects in <i>MMR</i> genes are listed in Table 1.

The involvement of MutL homologue malfunctions in human cancer is not as clear cut as in the case of the MutS homologues. Mutations in <i>hMLH1</i> predominate in hereditary nonpolyposis...
colon cancer, accounting for nearly 50% of all known germ line MMR gene mutations (2). Surprisingly, no germ line mutations have been found in hPMS1 or hPMS2, which was unexpected, given the key role of the latter protein in MMR. Recent immunohisto-chemical analysis of 1,048 unselected colon tumors revealed the lack of hPMS2 in ~1.5%, a proportion similar to that of MSH2-deficient cancers (16). Genetic analysis identified germ line mutations in hPMS2 in a number of these patients and it is likely that the remainder will also be linked to genetic alterations once the problems associated with sequencing of the hPMS2 locus are overcome (there are ~20 hPMS2 pseudogenes on chromosome 7, which interfere with DNA sequencing). However, these patients do not belong to typical hereditary nonpolyposis colon cancer families and the penetrance of these mutations seems to be very low. One possible explanation for this finding is that the defect in hPMS2 is partially compensated for by another MutL homologue, such as hMLH3. Germ line hMLH3 missense and frameshift mutations have been described in familial colorectal cancer cases but the implication of these alterations in carcinogenesis is ambiguous. In some cases, the mutation in hMLH3 was identified in families carrying a second MMR gene mutation, whereas no mutations in the other MMR genes could be identified in other cases (17–19). A similar discrepancy applies also to the microsatellite instability status of the tumors (17, 20). The role of hMLH3 in MMR and of hMLH3 mutations in cancer thus remains open to question. In an attempt to provide answers to these questions, we examined the role of hMLH3 in MMR in vitro.

Materials and Methods
cDNA Vectors
pFastBac1-His6-hMLH3. The cDNA of hMLH3 (Swiss-Prot entry Q9UHC1) was used as template for a PCR reaction where (His)6 tag was added at the NH2 terminus of some cases, the mutation in hMLH3 have been described in familial colorectal cancer cases but the penetrance of these mutations seems to be very low. One possible explanation for this finding is that the defect in hPMS2 is overcome (there are ~20 hPMS2 pseudogenes on chromosome 7, which interfere with DNA sequencing). However, these patients do not belong to typical hereditary nonpolyposis colon cancer families and the penetrance of these mutations seems to be very low. One possible explanation for this finding is that the defect in hPMS2 is partially compensated for by another MutL homologue, such as hMLH3. Germ line hMLH3 missense and frameshift mutations have been described in familial colorectal cancer cases but the implication of these alterations in carcinogenesis is ambiguous. In some cases, the mutation in hMLH3 was identified in families carrying a second MMR gene mutation, whereas no mutations in the other MMR genes could be identified in other cases (17–19). A similar discrepancy applies also to the microsatellite instability status of the tumors (17, 20). The role of hMLH3 in MMR and of hMLH3 mutations in cancer thus remains open to question. In an attempt to provide answers to these questions, we examined the role of hMLH3 in MMR in vitro.

<table>
<thead>
<tr>
<th>Heterodimer</th>
<th>Components</th>
<th>MMR role</th>
<th>Phenotype of knockout mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMutSc</td>
<td>hMSH2</td>
<td>Repair of base-base mismatches and small loops</td>
<td>Lympomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td>hMutScβ</td>
<td>hMSH6</td>
<td>Repair of loops</td>
<td>Lympomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td>hMutLα</td>
<td>hMLH1</td>
<td>Repair of all MMR substrates</td>
<td>Gastrointestinal tumors</td>
</tr>
<tr>
<td>hMutLβ</td>
<td>hPMS2</td>
<td>?</td>
<td>Lympomas, gastrointestinal, skin and other tumors; sterility</td>
</tr>
<tr>
<td>hMutLγ</td>
<td>hMLH1</td>
<td>?</td>
<td>Lympomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td></td>
<td>hMLH3</td>
<td>Sterility</td>
<td></td>
</tr>
</tbody>
</table>

Expression of hMutLγ
The Bac-to-Bac baculovirus expression system (Life Technologies, Gaithersburg, MD) was used according to the instructions of the manufacturer. Spodoptera frugiperda Sf9 cells (2 × 10⁶; Life Technologies) were infected with either a single recombinant baculovirus or with a combination of two viruses at a multiplicity of infection of 10. Cells were harvested 72 hours after infection and total extracts were prepared as described (21). Partial purification of hMutLγ from Sf9 extracts was done using Ni-NTA agarose (Qagen, Hilden, Germany), and the QIAexpressionist system was used according to the instructions of the manufacturer using 5 ml of 50% Ni-NTA slurry per 100 mg of protein extract.

hMLH3 Antibody Production and Purification
The COOH-terminal polypeptide of hMLH3 (amino acids 961-1,453) was expressed using the Impact-CN-System (New England Biolabs) in BL21 E. coli transformed with pTXB1-hMLH3 (amino acids 961-1,453). The peptide was purified using fast protein liquid chromatography on a MiniQ 4.6/50 PE column (Amersham Pharmacia, Upsala, Sweden) and used to immunize rabbits at Eurogentec (Seraing, Belgium). The rabbit polyclonal antibody was then affinity-purified using the COOH-terminal polypeptide immobilized on a nitrocellulose membrane. In brief, 100 μg of the purified polypeptide were blotted onto a nitrocellulose membrane by standard electrophoretic transfer, visualized by Ponceau S staining, and the corresponding band was cut out. The membrane was blocked with 5% nonfat dry milk in TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% (W/V) Tween 20] for 60 minutes, incubated with 700 μL of the polyclonal antibody for 4 hours at 4°C, and washed three times with TBST for 15 minutes. The membrane was then cut into small pieces (1 × 0.5 cm) and the antibody was eluted from the membrane by incubation for 20 minutes at room temperature in 0.1 mol/L glycine (pH 2.5). The supernatant was collected and the pH was neutralized by an equal volume of 1 mol/L Tris-HCl (pH 8.0). The purified antibody was stored at ~−20°C in 50% glycerol.

It was used to perform all the experiments described in this study except for the immunoprecipitation of hMLH3 from human cell extracts.

Human Cell Lines and Preparation of Cell Extracts
All the colon cancer cell lines, HEK293, and HeLa cell lines used in this study were obtained from the cell line repository of Cancer Network Zurich. The hPMS2-deficient cell lines HeLa clone 12 (22) and HeLa clone 25 (23) were kindly provided by Dr. Margherita Bignami (ISS, Rome, Italy). The cell line HEK293T was derived from HEK293 by immortalization with adenovirus 5 DNA and transfection with SV40 large T antigen (24). The hMLH1 gene in this cell line is epigenetically silenced by promoter hypermethylation (25). The 293T Lo cell line was derived in our laboratory (26). In these cells, the hMLH1 cDNA was stably integrated under the control of the tetracycline response promoter.
using the Tet-Off system (Clontech, Palo Alto, CA). In the absence of doxycycline, these cells express hMLH1 and are MMR proficient. All the cell lines were cultured at 37°C in a 5% CO₂-humidified atmosphere and maintained in the appropriate media. Whole cell extracts from these cell lines were prepared as described (26) without modifications. The origin and MMR status of the cell lines used in this study is listed in Table 2.

**Western Blot Analysis**

Western blots were done as previously described (26) using the following primary antibodies: rabbit polyclonal anti-hMLH3 (1:400), anti-hMLH1 and anti-hPMS2 from BD PharMingen (San Diego, CA) (1:4,000 and 1:1,000, respectively), and anti-β-tubulin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA), which amplified a 229 bp product. The methylation-specific primers were 5'-CGGCTAGTTTCCGAGTC-3' (sense) and 5' CTAAAACCTAACGACAG CG 3' (antisense), which amplified a 205 bp product. The PCR conditions are available on request.

To reactivate the expression of hMLH1 and hMLH3, 2.5 × 10⁵ HEK293T cells were seeded on a 78 cm² dish on day 0 and treated with 3 μg/mL of 5-aza-2'-deoxycytidine (Fluka, Buchs, Switzerland) on days 2 and 5. The medium was changed 24 hours after each addition of the drug and the cells were harvested on day 8.

**Microarray Experiments**

Microarray experiments were done as described previously (27). Gray columns in the graphs represent mRNA levels based on raw signals detected in the corresponding cell lines with the Affymetrix HG-U133A microarray.

**Mismatch Repair Assays**

The assays were done as described previously (28, 29).

**Results**

**Expression of hMutLγ in SF9 cells and production of anti-hMLH3 antibody.** To produce the recombinant hMLH3 and hMutLγ factors, *S. frugiperda* SF9 cells were infected with baculoviruses carrying cDNAs encoding hMLH1 and/or hMLH3. Infection of SF9 cells with the *hMLH3* virus alone yielded the protein in an amount that was hardly detectable by Western blotting. The amount of expressed protein was significantly increased when the cells were coinfected with both hMLH1 and hMLH3 vectors (Fig. 1A), suggesting that the presence of hMLH1 is necessary for the stabilization of hMLH3 in SF9 cells. This is reminiscent of hMSH6 and hPMS2, both of which require their heterodimeric partners (hMSH2 and hMLH1, respectively) for stability. However, the amount of the recombinant heterodimer obtained was too low to be measured by Western blotting.

**Table 2. Characteristics of the human cell lines used in this study**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin</th>
<th>MMR status</th>
<th>MMR protein defect</th>
<th>Genetic complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>Embryonic kidney epithelium</td>
<td>+</td>
<td>hMLH1, hPMS2, hMLH3</td>
<td></td>
</tr>
<tr>
<td>293T</td>
<td>Embryonic kidney epithelium</td>
<td>–</td>
<td>hMLH3</td>
<td>hMLH1 cDNA</td>
</tr>
<tr>
<td>293T Lα+</td>
<td>Embryonic kidney epithelium</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCo2</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO115</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH1, hPMS2</td>
<td></td>
</tr>
<tr>
<td>Colo741</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX-1</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP5D</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMSH2, hMSH6, hMSH3, hMLH3</td>
<td></td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH1, hPMS2, hMSH3</td>
<td></td>
</tr>
<tr>
<td>HCT116+Ch.3</td>
<td>Colon carcinoma</td>
<td>+</td>
<td>hMSH3</td>
<td>Chromosome 3</td>
</tr>
<tr>
<td>HT29</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hec1A</td>
<td>Endometrial adenocarcinoma</td>
<td>–</td>
<td>hPMS2, hMSH6</td>
<td>Chromosome 7</td>
</tr>
<tr>
<td>Hec1A-Ch.7</td>
<td>Endometrial adenocarcinoma</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa clone 12</td>
<td>Cervical carcinoma</td>
<td>–</td>
<td>hPMS2</td>
<td></td>
</tr>
<tr>
<td>LS411</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH1, hPMS2</td>
<td></td>
</tr>
<tr>
<td>SW48</td>
<td>Colon carcinoma</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW480</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW837</td>
<td>Colon carcinoma</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*+, MMR proficient; –, MMR deficient (these cell lines are unable to repair both base-base mismatches and insertion/deletion loops, with the exception of Hec1A-Ch.7, which is able to repair insertion/deletion loops).

The primary alteration of MMR protein expression is reported in bold. Lack of hMLH1 or hMSH2 lead to proteolytic degradation of hPMS2, or hMSH6 and hMSH3, respectively. The hMSH3 gene in HCT116 cells is mutated as a consequence of the MMR defect. The hMLH3 alterations are those described in this study; other alterations have been reported elsewhere (see text).
cells (Fig. 1C). The purified antibody was then tested using extracts of various human colon cancer cell lines. The antibody highlighted a double band migrating at the expected size of hMLH3 (Fig. 1D, bottom). As the faster migrating band was also observed in Western blots done with the preimmune serum (data not shown), and as the abundance of the slower-migrating band correlated with hMLH3 mRNA expression levels in the same cell lines (Fig. 1D, top), we concluded that the latter is the specific band. As shown in Fig. 1D, the levels of hMLH3 fluctuate significantly in the tested cell lines and seem to be independent of the amount of hMLH1 and hPMS2 expressed in the same cells.

Relative abundance of hMLH3 in human cells and its interaction with hMLH1. Given that hMLH3, hPMS2, and hPMS1 interact with the same region of hMLH1 (30), we wanted to ask whether the relative abundance of the three different heterodimers can be correlated with the phenotype of the cells. Therefore, we did semiquantitative Western blots where we compared the intensity of bands due to endogenous hMLH3 and hPMS2 proteins in HeLa cells with that of bands due to known amounts of the corresponding recombinant proteins (Fig. 2A). These experiments revealed that hMLH3 is ~60 times less abundant than hPMS2. Considering that hPMS1 is ~10 times less abundant than hPMS2 in human cells (5), hMLH3 exists in the cells at levels significantly to permit extensive purification. The reasons underlying the low levels of expression are unknown at this time, but it is possible that high amounts of the full-length protein may be toxic (7).

Commercially available antibodies could detect the recombinant hMLH3 protein on Western blots but failed to detect the endogenous protein in all the human cell lines used in this study (data not shown). Therefore, we raised our own polyclonal rabbit antiserum, directed against the COOH terminus of hMLH3, which contains the hMLH1-interacting domain. The affinity-purified antibody (Fig. 1B) detected a band of the expected size (~160 kDa) in SF9 lysates infected with the hMLH1 and hMLH3 vectors, whereas no signal was visible when we probed lysates of uninfected human cells (5), hMLH3 exists in the cells at levels significantly lower than those of hPMS2 and hPMS1, which are present at much higher levels. Considering that hPMS1 is ~10 times less abundant than hPMS2 in human cells (5), hMLH3 exists in the cells at levels significantly lower than those of hPMS2 and hPMS1, which are present at much higher levels.
lower than those of the other two hMLH1-interacting partners hPMS2 and hPMS1. In spite of this difference, hMLH3 was found to physically interact with hMLH1 in Far Western experiments (7) and in mammalian two hybrid assays (30). We could confirm this interaction using immunoprecipitation experiments in which the anti-hMLH1 antibody could immunoprecipitate both hMLH3 and hPMS2 from human cell lysates (Fig. 2B, top) and the anti-hMLH3 antibody precipitated the endogenous hMLH1 (Fig. 2B, bottom). No proteins were detected in control experiments where the precipitating antibody was omitted. The interaction between hMLH3 and hMLH1 was not mediated by bound DNA because treatment with DNase before incubation with the antibodies failed to abolish the interaction between the two proteins (Fig. 2B, top).

**hMLH1 is not required for the stability of hMLH3 in human cells.** hPMS2 and hPMS1 are stabilized by the presence of hMLH1 (1, 31). Considering this characteristic of these two MutL homologues, together with the finding that hMLH1 was required for the stabilization of hMLH3 in baculovirus-infected Sf9 cells (Fig. 1A), we expected to observe substantially decreased levels of endogenous hMLH3 in human cell lines lacking hMLH1. Surprisingly, we could detect hMLH3 in hMLH1-deficient HCT116 cells, and the restoration of hMLH1 expression by chromosome 3 transfer resulted in no appreciable increase in hMLH3 level (Fig. 3A, bottom left). This shows that the presence of hMLH1 is not required for hMLH3 stability in human cells. The relative amounts of intracellular hMLH3 were also unaffected by hPMS2 levels, as shown by comparison of hMLH3 band intensity in Western blots of extracts of the hPMS2-deficient Hec-1A cells with those of a Hec-1A clone in which the expression of hPMS2 was restored by chromosome 7 transfer (Fig. 3A, bottom right). hMLH3 protein levels failed to correlate with hMLH1 and hPMS2 expression also in other colon cancer cell lines, such as SW480 or Caco2, that express both hMLH1 and hPMS2, or LS411, CO115, or SW48 that lack hMutLα (Fig. 3B).

Having established that the level of hMLH3 in cells is not dependent on hMLH1 but that it correlates well with hMLH3 mRNA levels (Fig. 3B), we wondered whether the fluctuation of hMLH3 expression in the tested cell lines could be linked with cytosine methylation, which is known to silence several key genes in colon cancer (32). The human embryonic kidney cell line 293T is deficient in both hMLH1 and hMLH3 (Fig. 3C) and it could be shown that the CpG islands that constitute the promoters of hMLH1 (25) and other genes (33) are silenced by hypermethylation in these cells. As the hMLH3 promoter also contains a CpG island, we reasoned that the lack of hMLH3 expression in this cell line might also be linked to the transcriptional inactivation of its promoter. This prediction was substantiated in two independent experiments. First, treatment of 293T cells with the demethylating agent 5-aza-2’-deoxycytidine partially restored the expression of hMLH3 (Fig. 3C). In the second experiment, we treated genomic DNA of 293T and the parental 293 cells (which express both hMLH1 and hMLH3; Fig. 3C) with sodium bisulfite, which deaminates cytosines to uracils, but leaves 5-methycytosines unchanged. Methylation-specific PCR showed that the promoter of the hMLH3 gene in 293T cells was indeed methylated (data not shown). As expected, expression of high amounts of hMLH1 in the 293T-derived 293T Lox+ cells resulted in the stabilization of hPMS2 (26) but did not affect hMLH3 levels (Fig. 3C). The promoter of the hMLH3 gene can thus be silenced by cytosine methylation, but this is most likely not the only mechanism that results in the lack of expression of the protein, as 5-aza-2’-deoxycytidine treatment failed to induce the expression of hMLH3 in GP5D cells (Fig. 3D).
Role of hMutLγ in in vitro mismatch repair. The observation that extracts from 293T-Lax+ cells are MMR proficient (26) despite their lack of hMLH3 suggested that hMutLγ does not play a major role in MMR in vitro. However, the possibility that it acts as a backup to hMutLα in the absence of hPMS2 could not be excluded. Therefore, we tested extracts of the human cell line HeLa clone 12, which expresses hMLH1, hPMS1, and hMLH3 but lacks hPMS2. As shown in Fig. 4A, these extracts were deficient in the repair of heteroduplex substrates containing either a G/T mismatch or an insertion/deletion loop of one or two nucleotides, but their repair proficiency on all tested substrates could be restored by the addition of recombinant hMutLα. Before concluding that hMutLγ does not participate in MMR, we considered the possibility that the expression level of endogenous hMLH3 in the tested human cell lines might be too low to be detectably active in our in vitro assay. Therefore, we decided to test whether in vitro MMR activity may be detected in the presence of higher amounts of the heterodimer. These experiments were done with the hMutLα, β, and γ deficient extracts of 293T cells supplemented with whole cell extracts from S9 cells expressing comparable amounts of hMutLα or hMutLγ (Fig. 4B, inset). As shown previously, extracts of S9 cells overexpressing hMutLα could complement the MMR defect in the 293T extracts very efficiently, whereas extracts of uninfected S9 cells failed to do so (Fig. 4B; ref. 26). Interestingly, when extracts of S9 cells expressing hMutLγ were used, we observed an increase in repair activity of ~20%. Similar results were obtained when the hMutLγ was enriched by Ni-agarose chromatography, showing that the observed MMR activity was specific to hMutLγ. We detected similar repair activities on substrates containing a G/T mismatch or a 1-base loop with a nick located either 5′ or 3′ from the mismatch, but no activity was observed on a substrate containing insertion/deletion loops of two or four nucleotides (Fig. 4B; data not shown). These experiments show that although physiologic levels of hMutLγ are insufficient to mediate mismatch correction in our in vitro MMR assays, the factor can participate, albeit with low efficiency, in the correction of base-base mispairs and one-nucleotide insertion/deletion loops.

Discussion

Like its S. cerevisiae homologue (8), the mammalian MLH3 gene (7) could be shown to be involved in meiotic recombination (13–15). However, whereas the S. cerevisiae (8) and Schizosaccharomyces pombe (12) proteins play a small but distinct role in the repair of a subset of insertion/deletion loops, no similar evidence existed for mammalian MLH3. In this present study, we set out to search for this evidence.

We first wanted to study the expression of hMLH3 and confirm the existence of hMutLγ in vivo. Using a newly generated antibody, we showed that hMLH3 is much less abundant than the other two known hMLH1 interactors, hPMS2 and hPMS1. Despite this, we could confirm the physical interaction between hMLH3 and hMLH1 in HeLa cells by immunoprecipitation experiments. Surprisingly, although hMLH1 was required for hMLH3 stability in S9 cells (Fig. 4A), no such requirement was apparent in human cells where no degradation of hMLH3 occurred in the absence of hMLH1 (Fig. 3A and B). We also failed to observe any significant competition between hMLH3 and hPMS2 for hMLH1, showing that in human cells hMLH3 might be stabilized by interaction with another, as yet unidentified, protein. This finding is supported by evidence from meiosis in mice, where Mlh3 was seen to bind to pachytene chromosomes before Mlh1 and, after Mlh1 recruitment to these sites, foci containing Mlh3 alone persisted (11, 15). It was, therefore, suggested that Mlh3 could either exist alone or interact with a different partner (11). Immunoprecipitation experiments revealed a direct interaction of scMlh3p with Sgs1 helicase in meiotic S. cerevisiae cells (34) and hMLH3 was shown to bind hMSH4 in meiotic human cells (14); however, the identification of the putative hMLH3 partners that might help stabilize it in mitotic cells must await the results of future experiments.

The ultimate objective of this work was to elucidate the role of hMLH3 in human MMR. We first tested extracts of human HeLa clone 12 cells, which lack hPMS2 (22) and thus contain only hMutLβ and hMutLγ. As the former heterodimer is devoid of MMR activity in our in vitro MMR assay (31), any observed repair activity could be ascribed to hMutLγ. The extracts were MMR deficient on all tested substrates (Fig. 4A), which suggested that the hMutLγ heterodimer does not participate in MMR. However, as hMLH3 is generally much less abundant in human cells than hPMS2, we
Role of hMutLγ in MMR

wished to exclude the possibility that the lack of repair activity is linked to insufficient amounts of hMutLγ. Therefore, we tested the MMR activity of extracts of 293T cells, which are deficient in all three MutL homologues, supplemented either with recombinant hMutLo or hMutLγ (Fig. 4B). The former factor complemented the hMutLγ-deficient activity of 293T extracts on all tested substrates. While comparable amounts of hMutLγ were used, we observed a small but significant (~20%) repair with both G/T and +1 insertion/deletion loop substrates. This repair activity was not due to an intrinsic repair activity of the SF9 extracts per se, as extracts from uninfected SF9 cells were repair deficient in the complementation experiments. As there are no available functional assays to test the activity of hMutLγ, a possibility exists that this heterodimer was isolated in a partially inactive form. However, we consider this possibility unlikely because all the procedures used were identical to those used for the preparation of the SF9 extract expressing hMutLLo, which was fully active. Moreover, immunoprecipitation experiments done with SF9 extracts expressing hMutLγ showed that hMLH3 was able to bind hMLH1 (data not shown). The sensitivity of the in vitro MMR assay remains, however, rather low so that the contribution of hMutLγ to the repair process in vivo might be higher. Interestingly, the repair activity of hMutLγ was limited to G/T mismatch and 1-base loops, as we failed to observe any repair activity using +2- and +4-base-loop substrates. The latter result indicates that hMutLγ seems to be involved in the repair of substrates recognized by hMutSo rather than insertion/deletion loops of more than one extrahelical nucleotide recognized by hMutSγ. This is in contrast to the data obtained in S. cerevisiae where the role of scMlh3p seems to be in the repair of a subset of insertion/deletion loops together with scMutSβ. The role of hMLH3 in mammals thus might differ from that in lower eukaryotes.

Our findings, suggesting that hMutLγ may play a backup role in human MMR, are supported by evidence from the mouse model. As noted above, Mlh3 null mice were not cancer prone in the first 9 months of life and showed no gross defects in MMR (15). However, a long-term study of these animals, coupled with a highly sensitive analysis of their genomic DNA, provides evidence for the involvement of Mlh3 defects in both MMR and tumorigenesis. Mlh3−/− mice have a shorter life span than the wild-type controls and more than half of the animals develop cancers, including gastrointestinal tumors after the 9-month time span. Importantly, Mlh3 deficiency increased the levels of mutations in long mononucleotide repeats, although to a lesser extent than in Pms2−/− mice (35). Taken together, our results and the mouse model data suggest that the hMutLγ heterodimer functions in the repair of base-base mismatches and small insertion/deletion loops.

Considering the possible involvement of hMLH3 in human MMR, the identification of hMLH3 silencing through promoter hypermethylation is of particular interest. We showed that the hMLH3 promoter is methylated in 293T cells and that the protein is consequently not expressed. In this particular cell line, the methylation could be caused by the presence of the SV40 large T antigen. However, using methylation-specific PCR, we could detect partially methylated hMLH3 promoters in the colon cancer cell line LS411 and in the ovarian cancer cell line A2780/CP70, and fully methylated in the leukemia cell line Jurkat (data not shown), which shows that hMLH3 silencing via promoter hypermethylation can also be unrelated to the presence of SV40 large T antigen.

Although recombinant hMutLγ possessed detectable repair activity in our in vitro MMR assays, hPMS2-deficient cells expressing hMLH3 display a strong mutator phenotype (refs. 16, 23; this study). This suggests that hMLH3, most likely in the form of hMutLγ, does not play a major role in MMR in vivo. However, the detection of sequence variants of hMLH3 in the germ line of families predisposed to colorectal cancer (17, 20), coupled with our detection of epigenetic silencing of hMLH3 in human cell lines, suggests that this gene may play a role in human cancer, possibly in combination with other risk factors. If hMutLγ does indeed play a backup role for hMutLo in vivo, the fluctuating abundance of hMLH3, such as that observed in the tested cell lines (Figs. 1 and 3), might help explain the variable penetrance of hPMS2 mutations in hereditary nonpolyposis colon cancer families (16).

Acknowledgments

Received 7/21/2005; revised 8/25/2005; accepted 9/19/2005.

Grant support: Swiss Bridge (P. Cejka and J. Jiricny), Swiss National Science Foundation grant 3100/068182.02 (E. Cannavo and J. Jiricny), Union Bank of Switzerland AG (F. Fischer and J. Jiricny), and Swiss Cancer League (J. Sabatés-Bellver and G. Marra).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Christine Hemmerle for technical assistance and Dr. Pavel Janscak for help with protein purification.

References


www.aacjournals.org

10765

Cancer Res 2005; 65: (23), December 1, 2005

Downloaded from cancerres.aacjournals.org on July 23, 2017. © 2005 American Association for Cancer Research.
Expression of the MutL Homologue hMLH3 in Human Cells and its Role in DNA Mismatch Repair

Elda Cannavo, Giancarlo Marra, Jacob Sabates-Bellver, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/65/23/10759

Cited articles  This article cites 34 articles, 14 of which you can access for free at: http://cancerres.aacrjournals.org/content/65/23/10759.full#ref-list-1

Citing articles  This article has been cited by 19 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/65/23/10759.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.