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

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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−/− animals are highly cancer prone and present with gastrointestinal tumors at an early age, whereas Pms2−/− mice succumb to cancer much later in life and do not present with gastrointestinal tumors. This evidence suggested that MLH1 might compensate, at least in part, for a deficiency in PMS2. Sterility intestinal tumors. This evidence suggested that MLH1 might can also assist in the repair of base-base mismatches and single extrahelical nucleotides in vitro. Analysis of hMLH3 expression in colon cancer cell lines indicated that the protein levels vary substantially and independently of hMLH1. If hMLH3 participates in MMR in vivo, its partial redundancy with hPMS2, coupled with the fluctuating expression levels of hMLH3, may help explain the low penetrance of hPMS2 mutations in hereditary nonpolyposis colon cancer families.

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Introduction

Mismatch repair (MMR) proteins are a highly conserved group of polypeptides that play key roles in the correction of mispairs arising during DNA replication. They also prevent recombination between nonidentical sequences and participate in the signaling of certain types of DNA damage. The importance of MMR proteins in the maintenance of genomic integrity is underscored by the finding that germ line mutations in MMR genes predispose to hereditary nonpolyposis colon cancer, a common familial cancer predisposition syndrome (reviewed in refs. 1, 2). The principal MMR players in human cells are homologues of the bacterial MutS and MutL proteins. hMutSo, a heterodimer of the MutS homologues hMSH2 and hMSH6, binds base-base mismatches and small insertion/deletion loops, whereas hMutSβ (a heterodimer of hMSH2 and hMSH3) binds only insertion/deletion loops. This in vitro evidence could be corroborated by analysis of the phenotypes of MMR-deficient cells: Those lacking hMSH2 are fully MMR deficient and display a mutator phenotype and microsatellite instability that is consistent with the loss of repair of both base-base mismatches and insertion/deletion loops. Cells lacking hMSH6 retain a strong mutator phenotype but their microsatellite instability is limited to mononucleotide repeats due to the functional redundancy with hMutSβ in insertion/deletion loop repair. This situation is mirrored in hereditary nonpolyposis colon cancer families, where the penetrance of hMSH2 mutations is substantially higher than that of alterations in the hMSH6 locus (reviewed in ref. 2).

Whereas it is generally accepted that hMutSo and hMutSβ are the mismatch recognition factors that initiate MMR (reviewed in ref. 3), the function of the MutL homologues remains speculative. The human genome contains numerous genes that have significant sequence homology to mutL and to yeast MutL homologue and postmeiotic segregation genes; however, to date, only hMutLo, a heterodimer of hMLH1 and hPMS2, could be shown to be involved in MMR. Correspondingly, hMLH1- or hPMS2-deficient cells have a strong mutator phenotype and high microsatellite instability (reviewed in ref. 1). In in vitro studies, hMutLo could be shown to associate with hMutSo 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). hMutLβ, a heterodimer of hMLH1 and hPMS1, has been biochemically characterized but could not be shown to participate in MMR in vitro (5). This finding was substantiated by in vivo evidence: Mice carrying a disruption in the Pms1 gene display neither microsatellite instability nor cancer predisposition (6). hMLH3 was first identified in Saccharomyces cerevisiae 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 mlh3 mutants display a mutator phenotype similar to that of msh3-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−/− animals (6), Mlh3−/− 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 MMR 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 hMLH1 predominate in hereditary nonpolyposis
Table 1. Overview of mammalian MutS and MutL homologues and their roles in MMR

<table>
<thead>
<tr>
<th>Heterodimer</th>
<th>Components</th>
<th>MMR role</th>
<th>Phenotype of knockout mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMutSo</td>
<td>hMSH2</td>
<td>Repair of base-base mismatches and small loops</td>
<td>Lymphomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td>hMutSo</td>
<td>hMSH6</td>
<td>Repair of loops</td>
<td>Lymphomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td>hMutSβ</td>
<td>hMSH2</td>
<td>Repair of loops</td>
<td>Gastrointestinal tumors</td>
</tr>
<tr>
<td>hMutLα</td>
<td>hMLH1</td>
<td>Repair of all MMR substrates</td>
<td>Lymphomas, gastrointestinal, skin and other tumors; sterility</td>
</tr>
<tr>
<td>hMutLin1</td>
<td>hMLH1</td>
<td>?</td>
<td>Lymphomas, gastrointestinal, skin, and other tumors</td>
</tr>
<tr>
<td>hMutLy</td>
<td>hMLH1</td>
<td>?</td>
<td>No phenotype</td>
</tr>
<tr>
<td></td>
<td>hMLH3</td>
<td></td>
<td>Lymphomas, gastrointestinal, skin, and other tumors</td>
</tr>
</tbody>
</table>

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, which was added at the NH2 terminus of some cases, the mutation in hMLH3 implied of these alterations in carcinogenesis is ambiguous. In have been described in familial colorectal cancer cases but the mutations in hMLH3. Germ line partially compensated for by another MutL homologue, such as possible explanation for this finding is that the defect in hPMS2 is not belong to typical hereditary nonpolyposis colon cancer families 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*.

Expression of hMutLy

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 × 10^6; 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 hMutLy from Sf9 extracts was done using Ni-NTA agarose (Qagen, Hilden, Germany), and the Qiaplexion system was used according to the instructions of the manufacturer using 5 mL of 50% Ni-NTA slurry per 100 mg of protein extract.

hMutLy was expressed also in bacteria using a bicistronic vector pET11b-His6-hMLH3/MHLH (cloning information on request) in the BL21 strain of *Escherichia coli*. After induction of expression at 37°C for 4 hours with 0.4 mmol/L isopropyl-β-D-thiogalactopyranoside, the heterodimer was expressed but was insoluble. Nevertheless, the protein could be used to quantify the relative abundance of hMLH3 in HeLa cells.

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, Uppsala, 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% Tween 20] for 60 minutes, incubated with 700 μL of the polyclonal antibody for 4 hours at 4°C, and washed thrice 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 Hec-1A (23) were kindly provided by Dr. Margherita Bigianni (IS, 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 Lc cell line was developed 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 (1:100), anti-hMLH1 and anti-hPMS2 from BD PharMingen (San Diego, CA) (1:1,000 and 1:1,000, respectively), and anti-β-tubulin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA).

**Commmunoprecipitation Analysis of hMLH1 and hMLH3**

HeLa whole cell extract (1 mg) was incubated in a total volume of 500 μL in NP40 Lysis Buffer [50 mmol/L Tris-HCl (pH 8.0), 125 mmol/L NaCl, 1% NP40, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland)] for 3 hours at 4°C with the anti-hMLH1 (6 μg; BD PharMingen) or anti-hMLH3 (10 μg; Santa Cruz Biotechnology) antibodies. The immunoprecipitates were captured by incubation for 30 minutes at 4°C with 50 μL of 50% slurry of Protein A/G PLUS agarose (Santa Cruz Biotechnology). The agarose beads were then washed thrice with cold NP40 Lysis Buffer and the proteins were eluted with SDS sample buffer and subjected to Western blot analysis. Control experiments were done either in the absence of antibody or in the presence of 25 units of Benzonase (Merck, Whitehouse Station, NJ).

**Analysis of the hMLH3 Promoter and Treatment of Cells with 5-Aza-2’-deoxycytidine**

The hMLH3 5’ flanking region was analyzed for CpG content with the CpG plot software of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) and its methylation status was evaluated with 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.

**Mismatch Repair Assays**

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 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.

**Results**

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 complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>Embryonic kidney epithelium</td>
<td>+</td>
<td>hMLH1, hPMS2, hMLH3</td>
<td>hMLH1 clDNA</td>
</tr>
<tr>
<td>293T</td>
<td>Embryonic kidney epithelium</td>
<td>–</td>
<td>hMLH3</td>
<td></td>
</tr>
<tr>
<td>293T La +</td>
<td>Embryonic kidney epithelium</td>
<td>+</td>
<td>hMLH1, hPMS2</td>
<td></td>
</tr>
<tr>
<td>CaCo2</td>
<td>Colon carcinoma</td>
<td>+</td>
<td>hMLH1</td>
<td></td>
</tr>
<tr>
<td>C0115</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMSH2, hMSH6, hMSH3, hMLH3</td>
<td></td>
</tr>
<tr>
<td>Colo741</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH1, hPMS2, hMSH3</td>
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</tr>
<tr>
<td>CX-1</td>
<td>Colon carcinoma</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>GP5D</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH1, hPMS2</td>
<td></td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon carcinoma</td>
<td>–</td>
<td>hMLH3</td>
<td></td>
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<tr>
<td>HCT116+Ch.3</td>
<td>Colon carcinoma</td>
<td>+</td>
<td>hMSH3</td>
<td>Chromosome 7</td>
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<td>Colon carcinoma</td>
<td>+</td>
<td>hPMS2, hMSH6</td>
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<td>Hec1A</td>
<td>Endometrial adenocarcinoma</td>
<td>–</td>
<td>hMSH6</td>
<td></td>
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<tr>
<td>Hec1A+Ch.7</td>
<td>Endometrial adenocarcinoma</td>
<td>–</td>
<td>hMSH6</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
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<td>+</td>
<td>hPMS2</td>
<td></td>
</tr>
<tr>
<td>HeLa clone 12</td>
<td>Cervical carcinoma</td>
<td>–</td>
<td>hMLH1, 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>hMLH1, hPMS2</td>
<td></td>
</tr>
<tr>
<td>SW480</td>
<td>Colon carcinoma</td>
<td>+</td>
<td>hMSH3</td>
<td></td>
</tr>
<tr>
<td>SW837</td>
<td>Colon carcinoma</td>
<td>+</td>
<td>hMLH3</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 endogeneous 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

Figure 1. Expression of hMutLγ in SF9 cells, anti-hMLH3 antibody specificity, and endogeneous levels of hMLH3 in colon cancer cell lines. A, expression of hMLH3 in SF9 cells infected either with a baculovirus vector expressing hMLH3 or with a mixture of hMLH1- and hMLH3-expressing viruses. This Western blot shows that hMLH3 is stabilized in this system by hMLH1. B, Coomassie blue staining of rabbit polyclonal anti-hMLH3 serum before (Serum) and after (Anti-hMLH3) affinity purification. C, Western blot analysis of whole cell extracts of SF9 cells uninfected (TE Ctrl, 2 μg) or coinfected with the hMLH1/hMLH3 baculoviruses (TE MutLγ, 2 μg). The recombinant polypeptide (amino acids 961-1,453) used for the generation of the antibody was loaded in the third lane (1 ng). Anti-hMLH3, affinity-purified serum used at 1:400 dilution; Pre-serum, preimmune serum used at the same dilution. D, microarray analysis of mRNA expression levels (top) and Western blot analysis of protein levels (bottom) of hMLH3 in a series of colon cancer cell lines (50 μg of whole cell extract per lane); n.s., nonspecific band detected by the anti-hMLH3 antibody in human cell extracts.

Figure 2. Relative abundance of hMLH3 and its interaction with hMLH1 in vivo. A, the recombinant hMLH3 and hPMS2 proteins were loaded onto a denaturing polyacrylamide gel in the indicated amounts and visualized by Western blotting with the respective antibodies. The relative abundance of the two polypeptides was calculated by comparing the intensity of the hMLH3 and hPMS2 bands with those of the endogenous proteins present in 50 μg of HeLa whole cell extract. The blot is representative of two independent experiments, and the intensities of the bands were calculated by densitometry. B, communoprecipitation of hMLH3 and hMLH1 in HeLa cells. One milligram of whole cell extract was incubated with or without 6 μg of anti-hMLH1 antibody (top) or 10 μg of anti-hMLH3 antibody (bottom). DNase, reaction done in the presence of 25 units of Benzonase. Ponceau staining for IgG is also shown to show equal loading. Ctrl, 50 μg of HeLa whole cell extract.
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 S9 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 both hMLH1 and 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-methylcytosines 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-La+ cells are MMR proficient (26) despite their lack of hMLH3 suggested that hMutLγ does not play a major role in MMR in vitro. However, 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 Sf9 cells expressing comparable amounts of hMutLα or hMutLγ (Fig. 4B, inset). As shown previously, extracts of Sf9 cells overexpressing hMutLα could complement the MMR defect in the 293T extracts very efficiently, whereas extracts of uninfected Sf9 cells failed to do so (Fig. 4B; ref. 26). Interestingly, when extracts of Sf9 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 Sf9 cells (Fig. 1A), 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 Scs1 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
that hMLH3 was able to bind hMLH1 (data not shown). The experiments done with Sf9 extracts expressing hMutLα 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 hMutSβ. 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 hMutLex 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).

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**References**

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

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