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Brief Definitive Report

Mlh1 Can Function in Antibody Class Switch Recombination Independently of Msh2

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Abstract
Mismatch repair proteins participate in antibody class switch recombination, although their roles are unknown. Previous nucleotide sequence analyses of switch recombination junctions indicated that the roles of Msh2 and the MutL homologues, Mlh1 and Pms2, differ. We now asked if Msh2 and Mlh1 function in the same pathway during switch recombination. Splenic B cells from mice deficient in both these proteins were induced to undergo switching in culture. The frequency of switching is reduced, similarly to that of B cells singly deficient in Msh2 or Mlh1. However, the nucleotide sequences of the Spu-Sy3 junctions resemble junctions from Mlh1– but not from Msh2-deficient cells, suggesting Mlh1 functions either independently of or before Msh2. The substitution mutations within S regions that are known to accompany switch recombination are increased in Msh2- and Mlh1 single-deficient cells and further increased in the double-deficient cells, again suggesting these proteins function independently in class switch recombination. The finding that MMR functions to reduce mutations in switch regions is unexpected since MMR proteins have been shown to contribute to somatic hypermutation of antibody variable region genes.

Key words: B cells • immunoglobulin isotypes • mismatch repair • switch region mutations • switch junctions

Introduction
Antibody class switching occurs in B cells after activation by antigen and results in a switch from IgM and IgD isotype expression to IgG, IgE, or IgA isotype expression, thereby changing the antibody effector functions while maintaining the identical antigen specificity. Isotype switching occurs by an intrachromosomal deletional recombination within tandemly repeated switch (S) region sequences located upstream of each Ig heavy chain constant region gene. Recombination occurs anywhere within each S region segment, which differ in sequence from each other and also vary in length from 2 to 10 kb (for a review, see reference 1). Activation-induced cytidine deaminase (AID) is required for class switch recombination (CSR) and for somatic hypermutation (SHM) of antibody variable region genes (2, 3). Recent data from Neuberger et al. (4, 5) indicate that AID directly deaminates genomic DNA. A current model for the role of AID in CSR is to deaminate dC residues in S regions, which generates U:G mismatches and initiates base-excision repair, resulting in single-strand DNA breaks (6). Consistent with this model, mice found deficient in uracil DNA glycosylase (UNG), which is the first enzyme in the base excision repair pathway, have greatly impaired (~10-fold reduced) CSR (5). One possible explanation for the low levels of CSR remaining is that mismatch repair (MMR) proteins, specifically Msh2 and Msh6, might recognize the U:G mismatch created by AID and initiate MMR by introducing DNA breaks. Fibroblasts transfected with an AID expression plasmid can recombine plasmid switch recombination substrates, indicating that AID is the only B cell–specific component essential for CSR (7).

After creation of DNA breaks, recombination of Spu with downstream S regions appears to occur by nonhomologous end-joining (NHEJ), because S-S junctions often occur at very short microhomologies (0 to 2 nts) between the upstream and downstream S regions (8). Furthermore, the KU protein complex, thought to be essential for NHEJ, is required for CSR (9, 10). Interestingly, the kinase activity of DNA-PKcs is not required for CSR, as shown by examination of sad mice (11); however, when the DNA-PKcs gene is deleted by gene targeting, CSR to all isotypes except IgG1 is eliminated (12).

Examination of mice deficient in the mismatch repair (MMR) proteins Msh2, Mlh1, and Pms2 has shown that
these proteins have roles in CSR (13–16). Splenic B cells from mice deficient in Msh2, Mlh1, and Pms2 show two- to fivefold reductions in CSR, compared with wild-type B cells when stimulated in culture, and also show altered switch recombination junctions. MMR proteins in eukaryotes fall into two classes: (a) the MutS homologues (Msh1–6), which recognize DNA mismatches, loops, and other distortions, and (b) the MutL homologues (Pms2, Mlh1, and Mlh3 in mammals), which bind to MutS homologues bound to DNA and are thought to recruit endonuclease, exonuclease, and helicase activities (17). In addition to roles in post-replicative MMR, i.e., the correction of mutations introduced during DNA replication, MMR proteins have roles in homologous recombination during meiosis and in double-strand break repair (18, 19).

Switch recombination junctions obtained from both IgG- and IgA-expressing MMR-deficient B cells differ from junctions from wild-type B cells, suggesting that MMR proteins are directly involved in CSR (13, 14, 16). Interestingly, the characteristics of S junctions from Msh2-deficient B cells differ from those of Mlh1- and Pms2-deficient cells. Junctions formed during switching from IgM to IgG3 (Sp-Sy3 junctions) in Msh2-deficient B cells show decreased lengths of the microhomology normally observed at S junctions from wild-type cells and increased occurrence of small insertions at the junctions which do not correspond to the sequences of either the Sp or Sy3 regions (16). In addition, the junctions are restricted to the portion of the Sp region containing tandem consensus repeats, although CSR normally also occurs both 5' and 3' to this segment (13). By contrast, about one-fourth of the junctions from Mlh1- and Pms2-deficient B cells showed increased lengths of microhomology in comparison to wild-type junctions and no obvious restriction in location of recombination sites (14, 16). These data indicate that the role of Msh2 differs from the role of Mlh1 and Pms2 in CSR. However, these data do not indicate whether Msh2 is acting in a different pathway from Mlh1 or Pms2.

In this study we investigated whether Msh2 and Mlh1 function in the same or in different pathways in CSR. We reasoned that if they were to function in different pathways, splenic B cells deficient in both Msh2 and Mlh1 might show a greater deficiency in CSR than either mutant alone. In addition, the nucleotide sequences of the S junctions should indicate if the proteins function in the same or different pathways. If the proteins function in the same pathway, it seemed likely that the junction sequences would resemble those from cells deficient in the protein which acts earlier in the pathway, which presumably would be Msh2.

Materials and Methods

Mice. Mlh1-deficient mice were made by gene targeting and were obtained from R.M. Liskay, Oregon Health Sciences University, Portland, OR (20). Msh2-deficient mice were obtained from T. Mak, University of Toronto, Toronto, CA (21). Mice heterozygous for Mlh1 were bred to Msh2-heterozygotes to obtain mice heterozygous for both genes. These double heterozygotes were bred to generate mice deficient in both Mlh1 and Msh2, as well as wild-type and single-deficient msh2+/− and msh2−/− mice used as littermate controls. For the analysis of switching in cultured B cells, all the cells were from littersmates. However, the sequence analysis of msh2/msh1+/− mice was initiated after the analysis of the wild-type and single-deficient junctions was completed, and thus these mice are from the same colony but are not true littersmates.

B Cell Isolation and Cultures. Splenic B cells were isolated and cultured as described (15, 16).

PCR Amplification of Sp-Sy3 Juncions and Germline Sp and Sy3 Segments. Genomic DNA was isolated as described (16). Sp-Sy3 junctions were amplified from genomic DNA by PCR using the Expand Long Template Taq and Pfu polymerase mix (Roche) and the primers μ3-H3 (5'-AACAGGTTGGCTTAAACCGAGATGAGCCC-3') and g3–2 (5'-TACCTGTGACGAGGAGCTGCTAACAAGCTTGGCTTA-3').

Segments.

Results

To examine the ability of msh2/msh1+/− B cells to undergo CSR, splenic B cells were cultured with inducers of
isotype-specific CSR for 4 d and then analyzed by FACS® for surface Ig expression. The wild-type mice and mice deficient in Msh2, Mlh1, or both Msh2 and Mlh1 were tested in the same experiment and were always littermates. Fig. 1 presents two examples of FACS data showing surface isotype expression of wild-type and MMR-deficient B cells stimulated to switch to IgG2a and IgG1. Fig. 2 A presents the average amount of switching to each isotype as a percentage of switching in WT cells. CSR to all of the isotypes assayed was reduced in msh2/mlh1/B cells to 22 to 45% of WT, but the reduction was no more than was observed in cells from msh2/− or mlh1/− single-deficient mice.

To ensure that the reduced switching was not due to problems in cell proliferation or the cell cycle, we examined proliferation in B cells from the wild-type and 3 strains of MMR-deficient mice cultured to induce isotype-specific CSR. Proliferation, assayed by 3H-thymidine incorporation on day 3 of culture, was identical in wild-type and all MMR-deficient cells under all induction conditions (Fig. 2 B). The cell cycle, assayed by propidium iodide incorporation, was also not altered in MMR-deficient B cells induced with LPS and anti-δ-dextran for 65 h (Fig. 2 C).

As S-S3 junctions from msh2/− B cells are strikingly different from mlh1/− S-S3 junctions, we reasoned that the nucleotide sequence of the junctions in msh2/mlh1/− B cells would indicate whether these proteins are functioning together in CSR. Genomic DNA was prepared from cells induced to switch to IgG3 with LPS and anti-δ-dextran for 4 d. S-S3 junctions were amplified by PCR, cloned and sequenced (Fig. 3). We compared the lengths of overlap, or identity, between the S and Sy3 sequences (boxed nucleotides) and found that junctions from msh2/mlh1/− mice have significantly longer overlaps than those from msh2/− mice (P = 0.034) and are similar but not identical to those from mlh1/− mice (P = 0.173; references 14 and 16). Like Mlh1-deficient cells, there are junctions with extended microhomology (5–11 nts); however, the frequency of junctions with extended microhomology seems to be lower (Table I). It is clear that cells from msh2/mlh1/− B cells have junctions that differ from msh2/− B cells, and therefore that Mlh1 must have a role in CSR that does not require Msh2.

Nucleotide Mutations in S Regions Are Increased in MMR-deficient B Cells. Frequent nucleotide substitutions are observed in the S regions surrounding switch recombination
Figure 3. Nucleotide sequences surrounding the switch recombination junctions cloned from Mlh1/Msh2-double-deficient cells induced with LPS and anti-δ-dextran. The sequences are of PCR products amplified from 13 independent cultures from two mice. Each cloned sequence (designated M-M) is shown aligned with the sequence of germline Sα (AF466347, upper sequence) and germline Sγ3, (lower sequence) from 129/SvJ-C57BL/6 mice (16), or occasionally from BALB/c mice (MUSIGCD09) as necessary. The boxed nucleotides represent the overlap in sequence between Sα and Sγ3 with the number of these nucleotides shown to the right of each sequence. A vertical line represents no overlap (no identity). Underlined, boldface nucleotides are designated as insertions as these nucleotides do not appear to be templated by either S region.
Table I.  

<table>
<thead>
<tr>
<th>Mouse</th>
<th>≥2 bp</th>
<th>≥5 bp</th>
<th>≥8 bp</th>
<th>≥10 bp</th>
<th>No. of sequences</th>
<th>Percentage w/inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>44</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>Mlh1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>49</td>
<td>30</td>
<td>17</td>
<td>11</td>
<td>47</td>
<td>2</td>
</tr>
<tr>
<td>Pms2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>40</td>
<td>24</td>
<td>16</td>
<td>16</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msh2&lt;sup&gt;-/-&lt;/sup&gt;/Mlh1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>38</td>
<td>13</td>
<td>9</td>
<td>9</td>
<td>32</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sequences of WT, msh1<sup>-/-</sup>, pms2<sup>-/-</sup>, msh2<sup>-/-</sup> junctions were reported previously (reference 20), except for 24 additional sequences from another msh2<sup>-/-</sup>/mouse from the same colony.  
<sup>b</sup>Distribution of lengths of microhomology is significantly different from msh2<sup>-/-</sup>, P = 0.034, but not from msh1<sup>-/-</sup>, P = 0.173.

juncions (8, 23). These mutations are interpreted as indicating that error-prone DNA synthesis is involved in creating S-S junctions (23). The mutations are reminiscent of those observed during Ig V gene SHM, which have been shown to occur less frequently in mice deficient in Msh2, Mlh1, or Pms2 (24, 25). We asked if MMR deficiency also decreases mutation frequency in the cloned recombined msh2/mlh1<sup>-/-</sup> S region segments in comparison to previously determined sequences from wild-type mice (16). We found that MMR deficiency does indeed affect the mutation frequency, but unlike V gene mutations introduced by SHM, S region mutations are increased in MMR-deficient B cells (Table II). These data suggest that MMR is involved in removing mutations introduced into S regions, presumably by AID and/or by the error-prone DNA synthesis proposed to accompany CSR (4, 23, 26). Furthermore, these data provide further evidence for the hypothesis that Mlh1 can function independently of Msh2, as the frequency of mutations is significantly higher in sequences from the double-deficient cells than in WT cells, whereas sequences from the single-deficient cells are not as highly mutated. These data indicate that Mlh1, in the absence of Msh2, can function in mutation repair within S regions. The role of Mlh1 in the absence of Msh2 appears to affect the sequences of the junctions and surrounding sequences without further reducing the efficiency of class switching.

Discussion

We had hypothesized that a combined deficiency of Msh2 and Mlh1 would have the phenotype of Msh2-deficiency in CSR, as binding of a Msh2 heterodimer with either Msh3 or Msh6 to DNA is the first step in the postreplicative MMR pathway, and as Msh2 functions in double-strand break repair in yeast without the MutL homologues (27). Instead, we found that a portion of the switch recombination junctions from Msh2/Mlh1 double-deficient cells showed a phenotype similar to Mlh1- or Pms2 deficiency, i.e., increased junctional microhomology relative to WT junctions, which is significantly different from Msh2 deficiency. From these data, we conclude that the presence of Mlh1/Pms2 heterodimers prevents the formation of switch junctions with extended microhomologies, whether or not Msh2 is present.

Atomic force microscopy has shown that yeast Mlh1/Pms1 can directly bind two different DNA molecules (28), suggesting it might participate in synopsis of S region junctions in the absence of Msh2 or other MutS homologues. In addition, the NH<sub>2</sub>-terminal domain of human Pms2 can bind DNA as a monomer (29). Perhaps the Mlh1/Pms2 heterodimer is involved in forming the synaptic structure between the S<sub>µ</sub> and S<sub>γ3</sub> sequences. In its absence, the increased microhomology we observe at the junction may provide stability to the structure. The Msh2 heterodimer might function subsequently to process DNA ends before ligation. Perhaps Msh2 excises single-strand ends that if not removed result in mutations that resemble inserts at the junction. It is notable that the lengths of microhomology and presence of inserts in junctions from the msh2/msh1<sup>-/-</sup> cells seem somewhat intermediate between msh2<sup>-/-</sup> and msh1<sup>-/-</sup> junctions (Table I), suggesting that Msh2 also has a function in the absence of Mlh1. Although comparison of junctions from wild-type and msh2<sup>-/-</sup> cells supports a role for Msh2 in end-processing, the msh2/msh1<sup>-/-</sup> junctions clearly show that Msh2 is not needed to obtain extended microhomologies.

Table II.  

<table>
<thead>
<tr>
<th>S&lt;sub&gt;µ&lt;/sub&gt; mutation freq (×10&lt;sup&gt;4&lt;/sup&gt;)</th>
<th>WT</th>
<th>msh2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>msh1&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>msh2/msh1&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&lt;sub&gt;µ&lt;/sub&gt; mutations</td>
<td>25.0</td>
<td>44.6</td>
<td>36.3</td>
<td>63.7</td>
</tr>
<tr>
<td>Nucleotides sequenced</td>
<td>9,598</td>
<td>6,278</td>
<td>15,976</td>
<td>9,730</td>
</tr>
<tr>
<td>Signif. of diff. from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.046</td>
<td>NS</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>msh2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>msh1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Sy3 mutation freq (×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>19.2</td>
<td>34.3</td>
<td>26.9</td>
<td>44.9</td>
</tr>
<tr>
<td>Sy3 mutations</td>
<td>18</td>
<td>23</td>
<td>43</td>
<td>45</td>
</tr>
<tr>
<td>Nucleotides sequenced</td>
<td>9,384</td>
<td>6,694</td>
<td>15,985</td>
<td>10,023</td>
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<td>Signif. of diff. from:</td>
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</tr>
<tr>
<td>WT</td>
<td>NS</td>
<td>NS</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>msh2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
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<tr>
<td>msh1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant.

<sup>a</sup>By Fisher’s exact test.
Alternatively, it is possible that Mlh1 may be functioning with another MutS homologue, instead of directly binding to DNA itself. Mlh1 is thought to function in meiosis with Msh4 and 5, and Msh4 and Msh5 have been shown to bind Holliday junctions and to be required for crossing over during meiosis (18, 20, 30). However, the process of meiosis involves homologous recombination, which is not involved in CSR, as different S regions do not share the required lengths of homology. In addition, the sites of recombination used indicate that the repeat units do not align in register (8). Whether Mlh1/Pms2 functions alone or with other MutS homologues, our data suggest that it directs switching to an end-joining type of pathway and prevents an alternative short-homology pathway. This activity clearly does not depend on Msh2.

The use of an alternate pathway may explain why class switching in vitro is not further reduced in the Mlh1/Msh2-deficient mice, relative to those deficient in Mlh1 or Msh2 alone, as the frequency of switching would be governed by the rate of this alternate pathway. This alternate pathway may also be used when the kinase Ataxia-Telangectasia Mutated (ATM) is absent, since these switch junctions have been shown to have increased lengths of microhomology as well (31). Our data suggest that the Mlh1/Pms2 heterodimer is involved during normal CSR, perhaps to hold the DNA ends together in a particular structure thus focusing recombination to the DNA ends, but when this heterodimer is not available, an alternative pathway can be used. Although DNA-PK has also been proposed to focus recombination to DNA ends, the role of Mlh1 differs, as mice deficient in DNA-PKcs do not manifest increased lengths of microhomology at Sµ-Sγ1 junctions (12).

Altogether the data support the model that during CSR, the Mlh1/Pms2 heterodimer stabilizes and provides a structure to the synaptic complex, perhaps inhibiting the generation of relatively long stretches of junctional homology, and focusing the recombination to the DNA ends. The Mlh1/Pms2 heterodimer may function alone or with a MutS heterodimer that does not include Msh2. The data also suggest that Msh2 acts subsequently or independently to recruit end-processing machinery that trims DNA ends, resulting in Sµ-Sγ3 junctions that normally manifest one to two nucleotides of microhomology and infrequent appearance of sequence insertions.

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