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 Sµ-Sγ3 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 Sµ 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 *scid* 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

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The Journal of Experimental Medicine

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 (Sμ–Sγ3 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 Sμ or Sγ3 regions (16). In addition, the junctions are restricted to the portion of the Sμ 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 *mlh1*^{−/−} and *msh2*^{−/−} mice used as littermate controls. For the analysis of switching in cultured B cells, all the cells were from littermates. However, the sequence analysis of *msh2/mlh1*^{−/−} 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 littermates.

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

PCR Amplification of Sμ-Sγ3 Junctions and Germline Sμ and Sγ3 Segments. Genomic DNA was isolated as described (16). Sμ-Sγ3 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′-AACAAGCTTGGCTTA-ACCGAGATGAGCC-3′) and g3–2 (5′-TACCCTGACCCA-GGAGCTGCATAAC-3′) (16) for sequences M-M-4–138. Different primers were used for sequences M-M 201–262, Smu1 (5′-TAGTAAGCGAGGCTCTAAAAAGCAT-3′, nts 5031–5055 of MUSIGCD07; reference 13) and Sg3–4 (5′-TGTC-CTATCTACTTGGTTCCTCT-3′, nts 2645–2667 of MUSI-GANA). These primers are located 168 nts 5′ and 40 nts 3′ to the first primer set. The germline Sγ3 and the germline Sμ sequences were determined previously for comparison to Sμ-Sγ3 junctions from cells induced to switch to IgG3 (16). The mutation frequency in the recombinant products was determined by sequencing the entire cloned PCR product. In most cases both Sμ and Sγ3 sequences from one clone could be compared with the germline segments, but occasionally the presence of deletions prevented comparison with one side or the other. The PCR error frequency in our experiments was determined to be 8.3×10^{-4} (9 substitutions in 10799 nts) by sequencing independently amplified Sμ-γ3 junctions from the plasmacytoma TIB114, using procedures identical to ones used for splenic B cell junctions. TIB114, like other plasmacytomas does not have AID and does not undergo SHM (22).

Cloning, Identification, and Sequence Analysis of PCR Products. PCR products were cloned into the vector pGEM®-T Easy (Promega) or into pCR4-TOPO (Invitrogen) using blue/white screening for inserts. DNA was isolated from white colonies using QIAprep spin miniprep kit (QIAGEN). Clones containing Sμ-Sγ3 junction inserts of varying sizes were chosen to optimize the identification of unique junctions. Nucleotide sequencing was performed by the University of Massachusetts Medical School Nucleic Acids Facility, using an ABI 377 DNA sequencer and Big Dyes. Sequences were aligned using the Clustal program of MacVector 6.5.3. Alignments were generally obvious, although in a few cases more than one alignment was possible due to the repetitive nature of the sequences and occasional internal deletions. In these cases, alignments were optimized to reduce nucleotide differences between the germline and recombinant sequences. For statistical analysis, data were grouped according to the number of nts of identity at each junction: 0, 1 and 2, 3 and 4, and 5 or more and the Fisher-Freeman-Halton test was applied. Analysis of the whole table found significant differences between strains in the distribution of nts of junctional identity (P = 0.005).

Results

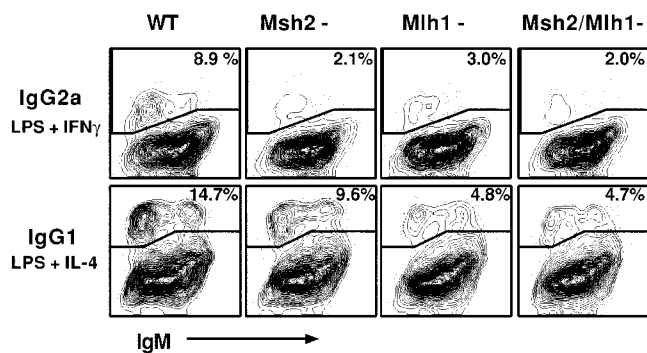To examine the ability of *msh2/mlh1*^{−/−} B cells to undergo CSR, splenic B cells were cultured with inducers of

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 ^{3}H-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 Sμ-Sγ3 junctions from *msh2$^{-/-}$* B cells are strikingly different from *mlh1$^{-/-}$* Sμ-Sγ3 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. Sμ-Sγ3 junctions were amplified by PCR, cloned and sequenced (Fig. 3). We compared the lengths of overlap, or identity, between the Sμ and Sγ3 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 2. (A) In vitro class switching in splenic B cells from Mlh1- and Msh2-single-deficient and double-deficient mice expressed as percent of switching in B cells from wild-type littermates. Culture conditions for each isotype have been described (reference 16), except for IgG2a which was induced by treatment with LPS + interferon-γ (10 U/ml). The average of three experiments is shown (except n = 2 for Mlh1- IgG1, IgG2a, IgG2b, and IgG3) and error bars represent the SEM. (B) DNA synthesis measured by ^{3}H-thymidine incorporation in 3d cultures with the same inducers used in panel A. Bars show the average of triplicate cultures plus 2 SD, and are representative of two experiments. (C) Cell cycle analysis by propidium iodide staining of cells induced with LPS and anti-δ-dextran for 65 h. The markers estimate the percent of cells in G_0/G_1, S, and G_2/M (left to right) phases of cell cycle.

Figure 1. Class switching is similarly reduced in Mlh1- and Msh2-single-deficient and double-deficient mice. Representative FACS® analysis of splenic B cells induced as indicated for 4d, surface stained with anti-IgM and anti-IgG2a or anti-IgG1 and analyzed by flow cytometry.

The Journal of Experimental Medicine

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129XB6 Sm    654  TGAGCTGAGCTGAGCTGGAAGTGAGCTGAGCTGGGCTGAGC
                  *************** *  *   *    *   *
M-M-4        654  TGAGCTGAGCTGAGCTGGAGTGATGGGGTAGGTTGGAGTAT    4 nt insert
                  *  *    *   *  ** ***************
129xB6 Sg3   248  GGCTGGGCAGCTCTCAGGTGAACTGGGGTAGGTTGGAGTAT

129XB6 Sm    246  GAGCTGGGTGAGCTGAGCTAAGCTGGGTGAGCTGAGCTGA
                  ******************* ***  *   **** *
M-M-5        246  GAGCTGGGTGAGCTGAGCTAACTAGGGTAGGTGGAAGCATA    0 nt identity
                  **    *    *** *  *
129xB6 Sg3   441  GACTGGGCAGCTCTGGGGGACCTAGGGTAGGTGGAAGCATA

129XB6 Sm    589  GAGCTGAGCTGAGCTGAGCTGGGGTGAGCTGAGCTGAGCTG
                  *******************  *   *  * ***   *   *
M-M-6        321  GAGCTGAGCTGAGCTGAGCTGAGCTAGGGTAAGTGAGGGTA    1 nt identity
                  ****  *  ***       *****************
129xB6 Sg3   584  AGGCTGGGCAGCTCTGGAGCGAGCTAGGGTAAGTGAGGGTA

129XB6 Sm    501  GGGTGAGCTGAGCTGGGGTGAGCTGAGCTGAGCTGGGGTG
                  *******************  *   *  *   *   *   *
M-M-9        501  GGGTGAGCTGAGCTGGGGTGAAGCTGGGGAGGTGGAGCTG    0 nt identity
                  *   *   ****
129xB6 Sg3   536  CAGGCTGGGCAGCTCTCAGGCGAGCTGGGGAGGTGGAGCTG

129XB6 Sm    634  TGAGCTGGGGTGAGCTGGGGTTGAGCTGAGCTGAGTGGAAG
                  ** *   ****
M-M-21       634  TGAGCTGGGGTGAGCTGGGGTGTGAGGATGTGGGGACCAGG    1 nt identity
                  ** *   **    *     **
Sg3-Balb/c   641  CTCTCGGGGGTAGCTAGGGTAAGTGAGGATGTGGGGACCAGG

129XB6 Sm    440  GAGCTGGGGTGAGCTGAGCTGGGTGAGCTGAGCTGAGCTG
                  *******************  *   *** *   *
M-M-28       440  GAGCTGGGGTGAGCTGAGCTCAAGCTGGGCAGCTCTGGGG    0 nt identity
                  **   *   **    *  *
129xB6 Sg3   321  GATAAGTGAGGATGTGGGGACCAAGCTGGGCAGCTCTGGGG

129XB6 Sm    325  GAGCTGAGCTGAGCTGCGGTAAGCTGGGATGAGCTGGGGTGA
                  ***************** ***********
M-M-30       325  GAGCTGAGCTGAGCTGGGTAAGCTGGGGTAGGAGGGAGTGT    10 nt identity
                  *    *** *******************
129xB6 Sg3   488  TAAGCTGAGCACTACAGGTGAGCTGGGGTAGGAGGGAGTGT

MUSIGCD09    755  GCTGAGCTGGGGTGAGCTGAGCTGAGCTGGGGTGAGCTGAG
                  *************  **    *         ***    ****
M-M-35       594  GCTGAGCTGGGGTAAGGCTGGGATGAGCTGGGGTAAGTGAG    8 nt insert
                  *    ***  ***       *****    *********
129xB6 Sg3   629  GACCAGGCTGGGCAGCTCTGGAGGGAGCTAGGGTAAGTGAG

129XB6 Sm    353  ATGAGCTGGGGTGAGCTGACTGAGCTGGAGTGAGCTGAG
                  ******************* **  *  *  *  **
M-M-39       353  ATGAGCTGGGGTGAGCTGAGCTGGACAGTTCTGGGGGAGC    4 nt identity
                  *   *   **    *** ************
129xB6 Sg3    38  GTTGGAGCATGGGAAACAGCTGGACAGCTCTGGGGGAGC

129XB6 Sm    531  AGCTGAGCTGAGCTGGGGTGAGCTGAGCTGGGGTGAGCTG
                  ******************* *** *    *   *
M-M-41       531  AGCTGAGCTGAGCTGGGGTGCTCTGGGGAAGCTGGGGTAC    1 nt identity
                  *   **   *****  ********************
129xB6 Sg3   143  ATGGGAACCAGGCTGGACAGCTCTGGGGAAGCTGGGGTAC

129XB6 Sm    245  TGAGCTGGGTGAGCTGAGCTAAGCTGGGGTGAGCTGAGCTGA
                  ******************** ***  *  **
M-M-45       245  TGAGCTGGGTGAGCTGAGCTAGGGTTAGATGGAAAATGTGAAT    0 nt identity
                  *   *    ************
129xB6 Sg3   818  TTGGGCAGCTACAGGTGAGCTGGGTTAGATGGAAAATGTGAAT

MUSIGCD09    630  GGTGAGCTGAGCTGAGCTGAGCTGAGCTGGGGTGAGCTGAG
                  ******************* **  *     **
M-M-47      ~900  GGTGAGCTGAGCTGAGCTGGGCAGCTGGGGTTGTTAGGAGT    1 nt identity
                                *****************
Sg3-Balb/c   436  CCAAGCTGGGCAGCTCTGGGGCAGCTGGGGTTGTTAGGAGT

129XB6 Sm    342  GGTAAGCTGGGATGAGCTGGGGTGAGCTGAGCTGAGCTGG
                  *******************    *    *  *   *****
M-M-48       342  GGTAAGCTGGGATGAGCTGGGAGTGTGGGGACCAGGCTGG    3 nt identity
                  *      **   ************************
129xB6 Sg3   504  AGGTGAGCTGGGGTAGGAGGGAGTGTGGGGACCAGGCTGG

129XB6 Sm    306  AGCTGGGCTGAGCTGGGGTGAGCTGAGCTGAGCTGGGGTA
                  ******************* *  * ** *
M-M-49       306  AGCTGGGCTGAGCTGGGGTGAGGTAGGTGGAAGCATAGGA    4 nt identity
                  *     **   **************
129xB6 Sg3   723  GAGACCTGGCTGGGGAGCTGAGGTAGGTGGAAGCATAGGA

129XB6 Sm    392  GAGCTGGGCTGAGCTGGGGTGAGCTGGGCTGAGCTGGGCT
                  ******************* *   ** ***   *  *
M-M-51       392  GAGCTGGGCTGAGCTGGGGTGCTGGGGTAGGAGGGAGTAT    1 nt identity
                  *     **  **   ********************
129xB6 Sg3   768  AGCTGAGCAGCTACAGGTGAGCTGGGGTAGGAGGGAGTAT

129XB6 SmN    83  TGATTACTCTGAGGTAAGCAAAGCTGGGCTTGAGCCAAAG
                  *******************  *  **     *    *   *
M-M-255       83  TGATTACTCTGAGGTAAGCGGGGTAGGAGGGAGTATGAGG    0 nt identity
                  **  *    *********** *** ******
129xB6 Sg3N  772  GAGCAGCTATAGGTGAGCTGGGGTAGGAGGGAGTATGAGG
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129XB6 Sm    246  GAGCTGGGTGAGCTGAGCTAAGCTGGGTGAGCTGAGCTG
                  ******************* **  *  ***
M-M-53       246  GAGCTGGGTGAACTGAGCTAAGGAGGGAGTATGAGGACTA    2 nt identity
                  *     ***************
129xB6 Sg3   776  AGCTACAGGTGAGCTGGGGTAGGAGGGAGTATGAGGACTA

129XB6 Sm    540  GAGCTGGGGTGAGCTGAGCTGGGGTGAGCTGAGCTGAGCT
                  *******************
M-M-63       540  GAGCTGGGGTGAGCTGAGCTCTAGGTTGGGCAGCTACAGG    0 nt identity
                  *   **********************
129xB6 Sg3   793  GGTAGGAGGGAGTATGAGGACTAGGTTGGGCAGCTACAGG

129XB6 Sm    251  CTTGGCTGAGCTAGGGTGAGCTGGGCTGAGCTGGGGTGAG
                  ********** **********  *  ** **  *
M-M-64       251  CTTGGCTGAGTTAGGGTGAGCTGGGGTAGGAGGGAGTATG    11 nt identity
                  **  *     **   ********************
129xB6 Sg3   744  GCTGAGCAGCTACAGGTGAGCTGGGGTAGGAGGGAGCATG

129XB6 Sm    297  GGGTGAGCTGAGCTGGGCTGAGCTAGACTGAGCTGAGCTA
                  ***************** ***      *   **    *
M-M-75       297  GGGTGAGCTGAGCTGGGCTGAGCTTAGTGGAAGTGTAGGG    6 nt identity
                  ***      *****  *****************
129xB6 Sg3   397  GGGCAGCTCTGGGCAGCTGAGCTTAGTGGAAGTGTAGGG

129XB6 Sm    234  CTAGGGTGAGCTGAGCTGGGTGAGCTGAGCTAAGCTGGGGT
                  ********* **** ***
M-M-100      235  CTAGGGTGAGCTGAGGTGGGTCCAGGTTATGCAGCTCCTG    4 nt identity
                  **  **    *  **************
129xB6 Sg3   886  CTGGGTCAGGGTACACAGGGTCCCAGGTTATGCAGCTCCTG

129XB6 Sm     52  TAGGGTTGAATAGAGCTAAACTCTACTGCCTACACTCGACTG
                  ******** **** *** **  *    *  *
M-M-104       52  TAGGGTTGAGTAGAGATAAATAGGATATTAAGCTGAGCAGCT    1 nt identity
                  **  *  *   *  *   *************
129xB6 Sg3   739  GCTGAGGTAGGTCGGGAACATAGGGTTAAAGCTGAGCAGCT

129XB6 Sm    235  TAGGGTGAGCTGAGCTGGGTGAGCTGAGCTAAGCTGGGGTG
                  *************** ***
M-M-109      235  TAGGGTGAGCTGAGGTGGGTCCCAGGTTATGCAGCTCCTGG    4 nt identity
                  **  **    ***************
129xB6 Sg3   889  GGGCTATCAGATCACAGGGTCCCAGGTTATGCAGCTCCTGG

129XB6 Sm    196  GGGGTGAGCTGAGCTGGGCTGAGCTAGACTGAGCTGAGCTA
                  ***************** ***      **   *   *  *
M-M-113      196  GGGGTGGGCTGAGCTGGGCTACAGGTGAGCTGGGTTGGATG    3 nt identity
                  *  *       *   ****************
129xB6 Sg3   808  GAGGACTAGGTTGGGCAGCTACAGGTGAGCTGGGTTTAGATG

MUSIGCD09    722  TGAGCTGAGCTGAGCTGAGCTGGGGTGAGCTGAGCTGGGGT
                  **************** ****  **  *   *  *  *  *
M-M-114      330  TGAGCTGAGCTGAGCTGAGCTAGGGTAAGTGAGGGTATGGG    11 nt identity
                  **  **    *************************
129xB6 Sg3   588  TGGGCAGCTCTGGAGGGAGCTAGGGTAAGTGAGGGTATGGG

129XB6 Sm    325  GAGCTGAGCTGAGCTGGGCTAAGCTGGGATGAGCTGGGGTG
                  ******************* *  *   *    *
M-M-130      324  GAGCTGAGCTGAGCTGGGCGAGGGAGCTAGGGTAAGTGAGG    1 nt identity
                  *     *   **************
129xB6 Sg3   630  ACCAGGCTGGGCAGCTCTGGAGGGAGCTAGGGTAAGTGAGG

129XB6 Sm    132  ACGCTGTGTTGGGGTGAGCTGATCTGAAATGAGATACTCTG
                  ******************* *   ***
M-M-138      132  ACGCTGTGTTGGGGTGAGCAGGGTAAGTGGGAGTATGGGAGA    1 nt identity
                  **    *   **  **********************
129xB6 Sg3   686  GGGCAGCTACAGGTGAGCAGGGTAAGTGGGAGTATGGAGA

129XB6 Sm    361  GGGTGAGCTGAGCTGAGCTGGAGTGAGCTGAGCTGGGCTGA
                  ****************** *  *  *     ****
M M-201      361  GGGTGAGCTGAGCTGAGCTTGTGGGGACCAGCTGGGAAAC    1 nt identity
                  *    *   *  **   ****************
129xB6 Sg3   171  AAGCTGGGGTACATGGGGTTGTGGGGACCAGGCTGGGAAAC

129XB6 Sm    247  AGCTGGGTGAGCTGAGCTAAGCTGGGTGAGCTGAGCTGAG
                  ******************* *   **** *
M-M-206      247  AGGTGGGTGAGCTGAGCTAGGGCTAACCTGGGCAGCTCTG    0 nt identity
                  **    *  *   ************
129xB6 Sg3   367  TGGGGTAGGTTCGAGTATGGGCAACCTGGGCAGCTCTG

129XB6 Sm    513  GTGAGCTGAGCTGGGGTGATCTGAGCTGAGCTGGGGTGAGC
                  ******************* *  *  *   *** * *  *
M-M-218      513  GTGAGCCGAGCTGGGGTGATCTCAGGGAACTGGGGAGGTGG    0 nt identity
                  *  *   ******   ***************
129xB6 Sg3   530  GGGGACCAGGCTGGGCAGCTCTCAGGGAGCTGGGGAGGTGG

129XB6 Sm    521  AGCTGGGGTGAGCTGAGCTGAGCTGGGGTGAGCTGAGCTGG
                  ******************* *  *  *   *  *
M-M-225      521  AGCTGGGGTGAGCTGAGCTGTAGGGTAGGTGGAAGCATAG    1 nt identity
                  *   **     *   ********************
129xB6 Sg3   442  ACTGGGCAGCTCTGGGGGACTAGGGTAGGTGGAAGCATAG

129XB6 SmN   140  AACTCTACTGCCTACACTCGATGTTCTGAGCTGAGATGAGC
                  ******************* *  *
M-M-262      140  AACTCTACTGCCTACACTGGAGGAGTATGAGGACTAGGTTG    3 nt identity
                  *    **   ************************
129xB6 Sg3   779  TATAGGTGAGCTGGGGTAGGAGGGAGTATGAGGACTAGGTTG
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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 Sμ (AF446347, upper sequence) and germline Sγ3, (lower sequence) from 129 × C57BL/6 mice (16), or occasionally from BALB/c mice (MUSIGCD09) as necessary. The boxed nucleotides represent the overlap in sequence between Sμ 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.

1380   Mlh1 Functions Independently of Msh2 in Isotype Switching

Table I. *Sμ-Sγ3 Junctions in msh2/mlh1$^{-/-}$ B Cells Differ from Junctions in msh2$^{-/-}$ Cells*

	Percentage of junctions with indicated length of microhomology					
Mouse	≥2 bp	≥5 bp	≥8 bp	≥10 bp	No. of sequences	Percentage w/inserts
WT[a]	44	3	0	0	32	3
Mlh1$^{-/-}$	49	30	17	11	47	2
Pms2$^{-/-}$	40	24	16	16	25	4
Msh2$^{-/-}$	19	0	0	0	32	19
Msh2[b]/ Mlh1$^{-/-}$	38	13	9	9	32	6

[a]Sequences of WT, *mlh1$^{-/-}$*, *pms2$^{-/-}$*, *msh2$^{-/-}$* junctions were reported previously (reference 20), except for 24 additional sequences from another *mlh1$^{-/-}$* mouse from the same colony.
[b]Distribution of lengths of microhomology is significantly different from *msh2$^{-/-}$*, P = 0.034, but not from *mlh1$^{-/-}$*, P = 0.173.

Table II. *Mutations Are Increased in Recombined Sμ and Sγ3 Segments from Mismatch Repair-deficient Cells*

	WT	*msh2$^{-/-}$*	*mlh1$^{-/-}$*	*msh2/mlh1$^{-/-}$*
Sμ mutation freq ($\times 10^4$)	25.0	44.6	36.3	63.7
Sμ mutations	24	28	58	62
Nucleotides sequenced	9,598	6,278	15,976	9,730
Signif. of diff. from:[a]				
WT		0.046	NS	0.0001
msh2$^{-/-}$			NS	NS
mlh1$^{-/-}$				0.002
Sγ3 mutation freq ($\times 10^4$)	19.2	34.3	26.9	44.9
Sγ3 mutations	18	23	43	45
Nucleotides sequenced	9,384	6,694	15,985	10,023
Signif. of diff. from:				
WT		NS	NS	0.002
msh2$^{-/-}$			NS	NS
mlh1$^{-/-}$				0.021

NS, not significant.
[a]By Fisher's exact test.

junctions (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$^{-/-}$* 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 synapsis of S region junctions in the absence of Msh2 or other MutS homologues. In addition, the NH$_2$-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 Sμ and Sγ3 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/mlh1$^{-/-}$* cells seem somewhat intermediate between *msh2$^{-/-}$* and *mlh1$^{-/-}$* junctions (Table I), suggesting that Msh2 also has a function in the absence of Mlh1. Although comparison of junctions from wild-type and *msh2$^{-/-}$* cells supports a role for Msh2 in end-processing, the *msh2/mlh1$^{-/-}$* junctions clearly show that Msh2 is not needed to obtain extended microhomologies.

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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