Requirement of Non-canonical Activity of Uracil DNA Glycosylase for Class Switch Recombination*

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Nasim A. Begum1, Nakako Izumi1, Momoko Nishikori1, Hitoshi Nagaoka, Reiko Shinkura, and Tasuku Honjo2

From the Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan

Activation-induced cytidine deaminase (AID) and uracil DNA glycosylase (UNG) are required for class switch recombination (CSR). AID is involved in the DNA cleavage step of CSR, but the precise role of UNG is not yet understood. Mutations and deletions are footprints of abortive DNA cleavage in the immunoglobulin switch region in splenic B cells stimulated to undergo CSR. However, a UNG deficiency did not reduce the number of such footprints, indicating UNG is dispensable for the DNA cleavage step. Mutagenesis experiments revealed that the role of UNG in CSR depends on its WXXMF motif. This motif is also essential for the interaction of UNG with the HIV viral peptide Vpr, which recruits UNG to the HIV particle. Furthermore, exogenous Vpr had a dominant-negative effect on CSR. These results suggest that UNG is recruited to the CSR machinery through its WXXMF motif by a Vpr-like host factor and plays a novel non-canonical role in a CSR step that follows DNA cleavage.

Activation-induced cytidine deaminase (AID)3 is necessary and sufficient for two major immunoglobulin (Ig) gene alterations, somatic hypermutation (SHM), and class switch recombination (CSR), which take place in the germinal center by the antigen stimulation of B cells (1–3). SHM introduces point mutations in the variable (V) region of Ig genes, resulting in an increase of the antibody affinity to antigens when coupled with selection of B cells by a limited amount of antigen. In the SHM reaction, DNA is first cleaved by single strand nicks, and mutations are subsequently introduced by erroneous DNA repair (4).

CSR replaces the expressed heavy-chain constant (C_H) region exons from C_H with other downstream C_H exons to generate antibodies with unaltered antigen specificity but with altered effector functions. In CSR, single strand nicks are introduced into both strands of the switch (S) regions to generate staggered cleavage ends with 3’ or 5’ overhangs, which are repaired to form blunt ends by gap-filling DNA synthesis and/or exonucleolytic digestion (4, 5). Finally, the blunt ends of the S and other S regions are joined by the non-homologous end joining (NHEJ) repair machinery (6–9), giving rise to a large deletion in the chromosome and a looped-out circle of deleted DNA (10–12). During this repair process, mutations and deletions are often introduced into the rearranged S regions. Some cleavages in the S regions are abortive and repaired without CSR, but mutations and deletions nonetheless arise during their repair (13–15).

A deficiency of uracil DNA glycosylase (UNG), which removes U from DNA and triggers the base excision repair pathway, causes a severe reduction of CSR activity, to 10% of wild type (16). Interestingly, UNG deficiency does not significantly affect the SHM frequency, unlike deficiencies of the mismatch repair enzymes, which reduce the SHM frequency to 20–50% (17–19). However, UNG deficiency does bias the target base specificity of SHM toward GC, as does a deficiency of MSH2 (16, 20). The phenotypes of the UNG deficiency in CSR and SHM have been interpreted to support the DNA deamination model: DNA cleavage in CSR and SHM is initiated by the AID-dependent deamination of cytosine (C) to form uracil (U) in the S and V DNA regions, respectively (21). Subsequently, in this model, U bases are removed by UNG, and a single strand DNA cleavage is made at the abasic site by a base excision repair pathway enzyme, apurinic/apyrimidinic endonuclease (22).

Recently, however, catalytically inactive UNG mutants were shown to restore full CSR activity in UNG-deficient B cells (23). Moreover, a specific UNG inhibitor, Ugi, blocks CSR activity without inhibiting double-strand DNA breaks (DSBs), as assessed by histone γ-H2AX focus formation at the immunoglobulin heavy chain (IgH) locus (23). Similarly, Ugi did not inhibit AID-dependent DNA cleavage in the IgH gene during SHM (24). These results suggest that UNG is not essential for the cleavage step of CSR or SHM and has an unknown function besides its U-removal activity, which is required in the repair step of CSR. However, other groups, who used a ligation-mediated PCR assay to determine DSB, subsequently reported that UNG is required for DSB in CSR (25, 26). Thus, the role of UNG in SHM and CSR reactions has to be re-evaluated.

We re-examined the involvement of UNG in DNA cleavage in CSR by analyzing mutations and deletions in the germ line Sμ region. The frequencies of deletions in the Sμ region in ung-/- and ung-/- msh2-/- IgM+ B cells were indistinguish-
able from the frequency in wild-type IgM+ B cells. A UNG deficiency increased the frequencies of mutations in the germ line sequence as well as in the rearranged S regions in B cells stimulated to undergo CSR. In addition, mutations in a conserved UNG motif, WXXF, abolished CSR without loss of the U-removal activity UNG. This WXXF motif is a UNG-binding site required for binding to the HIV virion (27, 28). Furthermore, expression of exogenous Vpr inhibited CSR, apparently by competing with some endogenous factor for the WXXF site. These results indicate that UNG plays a hitherto unknown function in CSR, in a step that is downstream of DNA cleavage.

EXPERIMENTAL PROCEDURES

Mice and Spleen-Cell Preparation—UNG-deficient mice were provided by R. Jaenisch, Massachusetts Institute of Technology, Boston, MA (29). MSH2-deficient mice were provided by K. Tanaka (Osaka University, Osaka). MSH2 and UNG heterozygotes were intercrossed to yield double-knockout mice. Ung−/−, msh2−/−, and aicda−/− mice from among the littermates of the double-knockout mice were used as the UNG-deficient mice. Wild-type (C57BL/6) and AID−/− mice were used as positive and negative controls, respectively. The mice were maintained at the Institute of Laboratory Animals of Kyoto University, under the specific pathogen-free condition.

Generation of Hybridomas—Splenocytes were obtained from 7- to 10-week-old mice and cultured at a concentration of 1.0 × 10^6 cells/ml in complete RPMI medium containing 7.5 ng/ml recombinant mouse IL-4 and 25 μg/ml LPS. On day 4 of culture, the switching efficiency was monitored, and the activated B cells were fused to the NS-1 myeloma cells. IgM-secreting clones were screened by enzyme-linked immunosorbent assay using goat anti-mouse IgM (Southern Biotech), and the allotype of the cytoplasmic IgMs was then examined by FACS, using anti-mouse IgMa (DS-1, BD Pharmingen) and IgMb (AF6–78, BD Pharmingen). Single allotype-expressing hybridomas were expanded.

Southern-blotting hybridization was carried out by standard

FIGURE 1. Internal deletions in the Sμ region of IgM+ hybridomas. Southern blot analysis of the Sμ regions in IgM-secreting hybridomas stimulated to undergo CSR. A, genomic organization of the 5' Cμ region of the IgH locus, including the location of the intrinsic IgH enhancer (Eμ) and Sμ regions. The double-headed arrow indicates germ line EcoRI and BamHI restriction fragment sizes for C57BL/6 and BALB/c DNA. Black bars represent the hybridization probes used for Southern blotting analyses. Hybridoma DNA was digested with EcoRI and BamHI restriction enzymes, separated by electrophoresis, blotted, and probed sequentially with 3' Sμ (B) and 5' Sμ probes (C). G, chromosomes with germ line size restriction fragments. D, chromosomes with restriction fragments different from germ line. Ga and Gb, germ line fragments from BALB/c and C57BL/6, respectively. NS1, fragment derived from fusion partner NS1. D, table shows the result of Southern blot analysis of all the hybridomas screened, from wild-type, ung−/−, ung−/−msh2−/−, and aicda−/− mice. Deletion frequencies in ung−/−, ung−/−msh2−/−, and wild-type B cells were statistically indistinguishable, whereas those in aicda−/− were significantly different from the others (p < 0.01). Statistical analyses were performed with the Fisher’s exact test.
procedures, after digestion of the hybridoma DNA with EcoRI and BamHI.

**Probes**—S\textsubscript{H}9262 probes were designed as previously reported (30, 31). The 5\textsubscript{H}11032 and 3\textsubscript{H}11032 S\textsubscript{H}9262 probes were synthesized by PCR using Pyrobest DNA polymerase (TaKaRa) with genomic DNA as the template. The primers were: 5\textsubscript{H}11032 forward, 5'-CGAAAC-CAGGCACCGCAAATG-3' and reverse, 5'-AGCCGGACTA-AACTATGCTG-3'; 3\textsubscript{H}11032 forward, 5'-CCCATTGCCACCA-ATTCTTATAG-3'.

The PCR amplification products were poly-A-tailed and purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI). The purified fragments were cloned into the plasmid pGEM-T easy vector (Promega). Plasmid DNA was digested with EcoRI and separated by electrophoresis on an agarose gel, and the DNA was isolated from the gel bands. The purified fragments were used as probes for Southern blots. Probe labeling was performed by the random-priming procedure, using DIG DNA Labeling mix (Roche Applied Science). Hybridized probes were revealed using the DIG detection Starter Kit (Roche Applied Science).

**Mutation Analysis of Switch Recombination Junctions and the Germ Line S\textsubscript{H}9262 Region**—Spleen cells from 2- to 8-month-old mice were cultured for 4 days in the presence of LPS (25 μg/ml) with or without IL-4 (7.5 ng/ml) to stimulate the B cells (2), and the DNA was extracted. To analyze the germ line S\textsubscript{H}9262 region of non-switched B cells, the B220\textsuperscript{+} IgG\textsubscript{1} cell population was sorted by FACSVantage\textsuperscript{TM} using anti-IgG\textsubscript{1} and IgG\textsubscript{3} antibodies (BD Biosciences) after the cells were stimulated. The LPS plus IL-4 stimulation generated 19% and 4.6% IgG\textsubscript{1} cells among the wild-type and UNG-deficient B cells, respectively. LPS stimulation alone produced 7.2% and 0.47% IgG\textsubscript{3} cells among the wild-type and UNG-deficient B cells, respectively.

Splenocytes were digested with proteinase K, and their genomic DNA was isolated. The S\textsubscript{H}9262-S\textsubscript{H}9253\textsubscript{1}, S\textsubscript{H}9262-S\textsubscript{H}9253\textsubscript{3} junctions and germ line S\textsubscript{H}9262 region were amplified using Pyrobest DNA polymerase (TaKaRa). The primers used for the first round of the nested PCR for switch recombination junctions were S\textsubscript{H}9262\textsubscript{1} (5'-TAGTAAACCGAGGTCTTAAAAGCAT-3') and either S\textsubscript{H}9253\textsubscript{1.1} (5'-CTGTAACCCTACCCAGGACC-3') or S\textsubscript{H}9253\textsubscript{3.1} (5'-CTATTGCAGTCTGCCCACG-3') (32). The PCR conditions were: 6 cycles of 93°C (40 s), 64°C (40 s), and 72°C (120 s), followed by a further 24 cycles with the annealing performed at 55°C.

The second round of nested PCR was performed using the primers S\textsubscript{H}2 (5'-ATCGAATTCTCGGTACG-3').
GTAGACT-3’ and either Sy1.2 (5’-GTCGAATTCCCCCATCCTGTACCTATA-3’) or Sy3.2 (5’-CCGGAATTCTTGA-CCTGGTACCCCTAGC-3’) (32). The PCR conditions were 24 cycles of 93 °C (40 s), 55 °C (40 s), and 72 °C (120 s). To amplify the germ line Sμ region, the primer pair Sμ1 and γμ1 (5’-TCACCAGAGTCTCATCCAGTTTAC-3’) was used for the
first-round PCR, and the Sµ2 and γSµ2 (5'-CCGGAATTCC-TACCCCTAGCTAGCCAGGAG-3') pair for the second-round, nested PCR. The same PCR conditions were used as for the analysis of switch recombination junctions.

The PCR products were digested with EcoRI, purified with the Wizard SV Gel and PCR Clean-Up System (Promega), cloned into the pBluescript vector, and sequenced. The sequences were determined using an ABI Prism 3100 genetic analyzer (PerkinElmer Life Sciences). Polymorphic replacements were carefully differentiated from mutations.

**Mutagenesis of UNG Constructs**—Site-specific mutagenesis of the 231WXXF234 motif of mouse UNG was performed using the Quick-Change procedure (Stratagene). Primer sequences for the various mutants were: W231A (5'-AAGGAGGGGGCCGACGTTCAGG-3'), W231K (5'-AAGGAGGGGGCAAGGACGTTCAGG-3'), F234G (5'-AGGGGAGAGGCGGACGTTCAGG-3'), F234Q (5'-AGGGGAGAGGCGGACGTTCAGG-3'), and W231A/F234G (5'-AAGGAGGGGGCAAGGACGTTCAGG-3').

All the constructs were subcloned into the EcoRI and SalI sites of a retroviral vector, pFB-IRES-GFP. To monitor expression in live cells, an additional set of constructs was prepared by fusing the coding sequence for EGFP to the N terminus of each mutant and cloning the tagged mutants into the pFB retroviral vector (Stratagene). Various N-terminal mutants of UNG (serially truncated, exogenous NLS-bearing, and proliferating cell nuclear antigen (PCNA) binding-defective forms) were also produced by Pfu-PCR using appropriately designed primers and subcloned into the pFB retroviral vector.

**CSR and UDG Assays**—To infect B cells from the spleen, the retrovirus-containing supernatant was prepared from platE cells following a standard protocol. Splenic B cells from UNG-deficient mice were pre-activated by exposure to LPS and IL-4 for 36–48 h before they were infected with the various UNG constructs, and the surface IgG1 expression was measured 48 h after the retroviral infection. A biotin-conjugated anti-IgG1 primary antibody in combination with a streptavidin-APC secondary reagent was used for the surface staining. The percentage of IgG1-positive cells among the infected cells was calculated (GFP+ in the live gate).

For the immunoblotting and enzyme assays, spleen cell lysates were prepared by brief sonication in 25 mM HEPES, pH 7.8, 5 mM EDTA, 1 mM dithiothreitol, and 5% glycerol (HED buffer) (33) supplemented with protease inhibitors. Crude lysate was cleared by high speed centrifugation, and 60 µg of soluble supernatant was subjected to Western detection.

The uracil DNA glycosylase assay was carried out using a 5'-fluorescein isothiocyanate-labeled oligonucleotide (a 30-
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**A**

mUNG2: MIGQKTLSSFSPSPTGRTRTSSPEP-VPDGSGFA...AEGGDAVSAPK
hUNG2: MIGQKTLSSFSPSPTGRTRTSSPEP-VPDGSGFA...AEGGDAVSAPK

PCNA & RPA2

NLS 77 86

mUNG2: ARVEQNEG...QPLSAGEILVRCVKNAAARLRAHAVNPAGGES...

hUNG2: APMQREGRGTPSSPLSAGLPGLDCMRNAAARLRAHAVNPAGGES...

PCNA binding consensus: QxxLxxFF
PCNA binding site of UNG2: MIGQxTLYxFP
PCNA mutant: MIGQxTLYxA

**B**

|          | Vector | Wt UNG | PCNA mutant | Δ28 UNG |
|----------|--------|--------|-------------|---------|
| Δ77 UNG  | 2.4    | 15.8   | 19.8        | 21.5    |
| Δ86 UNG  | 21.0   | 22.0   | 20.6        | 3.0     |
| NLS-Δ86 UNG | 2.4 | 4.3 | 3.0 | 1.5 |
| Δ144 UNG | 18.0   | 22.0   | 20.6        | 3.0     |

**C**

![Graph showing % IgG in GFP gate](image)

**D**

|          | W231A | F234G | Vector |
|----------|-------|-------|--------|
| Δ28      | 2.7   | 2.4   | 3.0    |
| Δ77      | 3.1   | 2.2   | 18.0   |

**E**

![Graph showing % IgG in GFP gate](image)

**F**

|          | Control | W231A | W231K | F234G | F234Q | W231AF234G | W231N | W231N/F234G |
|----------|---------|-------|-------|-------|-------|------------|-------|-------------|
| Anti-GFP |         |       |       |       |       |            |       |             |
| Anti-Tubulin |       |       |       |       |       |            |       |             |

**G**

UDG Assay

- Substrate
- Cleaved
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Internal Deletions in the Sμ Region of UNG-deficient IgM+ B Cells—To re-evaluate the dispensability of UNG for the DNA cleavage step of CSR, we examined partial deletions in the Sμ region of IgM+ B cells from ung−/− mice that had been stimulated to undergo CSR.

IgM+ hybridomas were generated from stimulated wild-type, ung−/−, ung−/−msh2−/−, and aicda−/− B cells, and the hybridoma DNAs were analyzed by Southern blot using the S′ and 3′ Sμ probes (Fig. 1, A–C). We classified the hybridomas into three groups: (a) germ line on both alleles (G/G), (b) deletion on one allele (G/D), and (c) deletion on both alleles (D/D). The deletion frequency was calculated per chromosone (Fig. 1D).

11 of the 86 Sμ loci (12.8%) in wild-type B cell-derived IgM+ hybridomas contained deletions, which is in agreement with previous reports (15, 31). Strikingly, both the ung−/− and ung−/−msh2−/− B cell-derived IgM+ hybridomas had deletions in the Sμ region at frequencies statistically indistinguishable from the wild-type ones (20.7 and 9.6%, respectively). By contrast, the aicda−/− B cells showed a deletion frequency that was scarcely above the background level (15), indicating that the Sμ deletions in IgM+ hybridomas are AID-dependent and thus mediated by DNA cleavage. It is therefore likely that both UNG and Msh2 are dispensable for DNA cleavage in CSR.

Increased Mutations in the Germ Line Sμ Region of UNG-deficient B Cells—To confirm the UNG dispensability for the DNA cleavage in CSR, we compared the mutation frequency in the germ line S regions between wild-type and ung−/− B cells. We analyzed the nucleotide sequences of the 200-bp region S′ to the Sμ core sequence in 50 clones each from UNG-deficient and wild-type splenic IgG− B cells, after stimulation with LPS and IL-4 or with LPS alone (Fig. 2A). Although the CSR frequency in the ung−/− B cells was reduced to 6–24% (variable between isotypes) of the frequency in the wild-type B cells, the mutation frequency in the germ line Sμ region in ung−/− B cells increased nearly 3- to 4-fold for cells stimulated with either LPS plus IL-4 or LPS alone (Fig. 3, A and B, left panels). The increase in mutation frequency in the Sμ region of the UNG-deficient B cells correlated well with the number of mutations per mutated clone. The results indicate that the processivity of mutations per targeted site increased in the UNG-deficient cells. It is therefore likely that UNG somehow inhibits processive mutations in the Sμ region during repair.

The mutations were biased to a GC transition in the UNG-deficient B cells (Fig. 3, A and B, right panel), as is found with SHM in the Ig V genes (34). The locations of the mutations were not skewed compared with the locations of mutations induced by junctional breakpoints identified by the same PCR primer in switched B cells, supporting the assumption that germ line Sμ mutations are generated through abortive DNA cleavage in CSR (Fig. 2A). The mutation frequencies in the germ line Sy1 and Sy3 were too low to be evaluated quantitatively, as shown previously (35).

Increased Mutations in Rearranged S Regions of UNG-deficient B Cells—We next examined the effects of UNG deficiency on the mutation frequency in the S regions surrounding CSR breakpoints. We first scored mutations in the Sμ region immediately upstream of Sμ-Sy1 or Sμ-Sy3 recombination junctions generated by stimulation with LPS plus IL-4 or LPS alone, respectively (Fig. 4, A and B, left panel). The distribution of the recombination breakpoints in the Sμ, Sy1, and Sy3 regions was generally similar between the wild-type and ung−/− B cells (Fig. 2, A–C).
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However, the mutation frequency in the recombed S\mu region of the UNG-deficient B cells was ~4-fold higher in the S\mu-S\gamma1 junctions and 6-fold higher in the S\mu-S\gamma3 junctions than in wild-type cells, which is in sharp contrast to the drastic reduction in CSR in these UNG-deficient cells. Both the average number of mutations per mutated clone and the frequency of mutated clones were higher in the UNG-deficient B cells (Fig. 4, left panels).

The mutation frequencies in the rearranged S\gamma regions were also higher than in their germ line counterparts. The mutation frequency and the number of mutations per mutated clone in the rearranged S\gamma1 region were 2- to 3-fold greater in the UNG-deficient B cells than in the wild-type cells (Fig. 4A, left panel).

The rearranged S\gamma3 region showed a modest increase in mutation frequency in the UNG-deficient B cells (Fig. 4B, left panel). The base specificity of the mutations was similarly skewed to the GC transition in all the rearranged S regions of the UNG-deficient B cells compared with wild-type B cells (Fig. 4, right panels).

Of note, the mutations surrounding each recombination junction were spread over a larger region in ung−/− B cells than in the wild-type B cells (Fig. 3, C and D). These results again suggest that the increased mutations in UNG-deficient B cells are probably due to the enhanced processivity of the mutations associated with DNA repair. All these changes in the frequency and processivity of mutations in the rearranged S regions of the UNG-deficient B cells are in parallel with those seen in the germ line S\mu region. The increased mutations in S regions are consistent with the above conclusion that UNG deficiency inhibits CSR by the repair defect rather than the cleavage defect although another interpretation is possible as described under “Discussion.”

Replication-coupling Motifs of UNG Are Dispensable for CSR—The role of UNG in DNA repair during chromosomal replication is well established (36–38). The N-terminal domain of UNG contains a PCNA-binding motif (QXCLXXFF) and two replication protein A (RPA)-binding motifs (Fig. 5A); the PCNA and both RPAs are essential for the association of UNG with the replication fork.

We examined whether these N-terminal motifs are required for CSR by generating a series of truncation and point mutants. A defective mutant of the PCNA-binding motif (QXCLXXAA) (39) completely rescued CSR activity in the ung−/− B cells (Fig. 5, B and C). N-terminal truncations of up to residue 86 (Δ28, Δ77, and Δ86) did not affect the UNG CSR activity, but further deletions (Δ97 and Δ144) abrogated it, and the N-terminal fragments alone (N86 and N144) did not show any CSR activity either.

We also found that E. coli UNG, which lacks the N-terminal domain, was as active as the wild-type form (data not shown). These results indicate that the N-terminal 86 residues of UNG are not required for CSR activity, implying that the roles played by UNG in CSR and replication are distinct.

CSR Requires the UNG Vpr Interaction Motif—UNG is required for HIV propagation in mammalian cells, although its precise function is unknown (40, 41). Host UNG is recruited to HIV particles by interaction with a virally encoded peptide, Vpr, which is mediated through the WXXF motif of UNG (27, 28, 42).

We examined whether the WXXF motif is also required for CSR activity by introducing mutations (W231A, W231K, F234G, F234Q, and W231A/F234G) known to abolish the interaction of UNG with the Vpr peptide (27). These WXXF mutants (whether or not they were fusion proteins with tag) had almost no CSR activity in the UNG-deficient B cells (Fig. 5, D and E), although they were expressed efficiently (Fig. 5F). All the WXXF motif mutants retained the U-removal activity of UNG, although for some the activity was slightly reduced (Fig. 5G). The results indicate that the WXXF motif of UNG is required for CSR.

The WXXF motif is highly conserved among the UNG orthologues from various species, including Escherichia coli. These results further imply that an unknown cellular protein interacts with the UNG WXXF motif and that this interaction is critical to the role of UNG in CSR.

To test this possibility, we used a retroviral vector to express the Vpr protein as a competitor with a putative cellular UNG-interacting protein. If the exogenous Vpr protein successfully competed for UNG, we expected to see a dominant-negative condition reflected in the CSR activity.

As shown in Fig. 6, strong Vpr expression drastically decreased the IgA class switching in stimulated CH12F-3 cells. However, Vpr mutants (W54R and H33R) known to be UNG interaction-defective (1.7 and 7% of wild type, respectively) (43), had much weaker dominant-negative effects on CSR.

Another Vpr mutant (R90K) that can interact with UNG but does not affect cell-cycle regulation showed a dominant-negative effect similar to that of wild-type Vpr. Importantly, this effect was dose-dependent, with infectants that expressed less Vpr-GFP showing weaker effects on CSR (Fig. 6A, R2 versus R3).

These results strongly indicate that Vpr competes with an unknown host factor for binding to UNG. It is therefore likely that UNG is involved in CSR through an interaction with a Vpr-like host protein that may recruit UNG to the CSR machinery complex.

DISCUSSION

The involvement of UNG in DNA cleavage was examined by two types of DNA cleavage footprints in CSR: deletions and mutations in the S region. First, we found that internal deletions in the S\mu region of stimulated IgM+ B cells from ung−/− and ung−/−msh2−/− mice were not significantly lower than in wild-type mice.

The deletion of DNA segments is generally caused by the DNA cleavage except for DNA polymerase slippage during replication. DNA cleavage is repaired by either gap filling or various types of recombination. Inversely, DNA recombination cannot occur without DNA cleavage. Because the internal S\mu deletion in CSR-stimulated B cells is AID-dependent and thus associated with DNA cleavage, the S\mu deletion we measured here should reflect DNA cleavage-dependent recombination. Taken together, the above results clearly indicate that neither UNG nor Msh2 is responsible for DNA cleavage.
DSB in the S region can result in several different outcomes: (a) repair without deletion but with replacement mutations, (b) internal deletion, (c) recombination between S regions, i.e., CSR, and (d) recombination between other chromosomes, i.e., chromosomal translocation. UNG is required for deletions but not for mutations (a) and for recombination between distant targets (c and d) (44), suggesting that UNG may be needed for synapsis formation between cleaved ends located far apart. It is totally unknown how synapsis is formed for CSR or chromosomal translocation involving the S regions. The dispensability of UNG for deletion in the Sμ region of activated B cell DNA suggests that the joining mechanism for two DSB within the Sμ region may be different from the NHEJ used for CSR (31, 45–48). It is possible that two ends within the Sμ region are repaired by illegitimate recombination through microhomology between repeated sequences in the Sμ region (49, 50).

Second, DNA sequence analyses of S regions revealed that UNG deficiency introduces more frequent mutations in the Sμ and Sγ1 regions. These observations agree well with the previous results showing the dispensability of UNG in DNA cleavage during CSR and SHM (24, 51). However, the increased mutations in the S region in the absence of UNG can be also explained by the DNA deamination model, because U generated by DNA deamination by AID remains without repair, resulting in the increase of G/C → A/T transition mutation. Nonetheless, there are three observations that are difficult to explain by the DNA deamination model: (a) SHM frequency is not augmented despite increase in CSR-associated mutations in the S region in UNG-deficient B cells; (b) S region mutations always cluster immediately adjacent to the recombination junction and diminish sharply as it goes away from the junction; and (c) the mutation frequencies are higher in the recom- bined S region than the germ line S region. On the other hand, all these observations can be easily explained by the assumption that S region mutations are introduced during the repair phase of recombination that is induced by UNG-independent DNA cleavage as described below.

**FIGURE 6. Dominant-negative effect of Vpr expression on CSR.** A, surface IgA-staining FACS profiles of CH12F3-2 cells expressing wild-type Vpr or its mutants that had variable ability to interact with UNG. Mutant R90K binds UNG, W54R does not bind it, and H33R interacts with it only weakly (43). An SSC versus GFP dot plot shows that CH12F3-2 cells infected with Vpr constructs could be divided into low and high expressing populations. Histograms show the IgA expression profile of the VprR1 (R1), VprR2 (R2), and VprR3 (R3) cell populations. Cells were stimulated with the CD40 ligand, IL-4, and transforming growth factor-β in culture and harvested to determine the surface IgA examination after 24 h in culture with stimulation. B, plots show the kinetic profile of the effect on CSR efficiency of various of Vpr mutants in CH12F3-2 cells over time. The values represent the mean from three to four experiments, and the error bars represent ± S.D.
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It is well established that the repair phase of CSR is mediated by the NHEJ mechanism (6–9). However, UNG is unlikely to be involved in NHEJ per se because UNG is not required for VDJ recombination, which depends on NHEJ. The major difference between VDJ recombination and CSR is the mode of cleavage: staggered nick cleavage in CSR and blunt-end cleavage in VDJ. It seems therefore likely that UNG is involved in the repair of staggered nick ends containing 5' or 3' overhangs to create the blunt ends that are required for NHEJ. This process necessitates either DNA synthesis, exonuclease cleavage, or both.

The strikingly higher mutation frequencies in the immediate proximity of recombination breakpoints in the rearranged Sμ and Sγ regions suggested that the majority of mutations in the rearranged S regions are introduced by errors during junctional repair. Current understanding of DNA repair indicates that mutations are most likely to be introduced by error-prone polymerases (52, 53). A positive correlation between the increase in the total mutation frequency and the mutation number per mutated clone in the UNG-deficient B cells suggests that CSR-associated mutagenesis in the S regions becomes more processive in the absence of UNG.

One possible explanation for this phenotype is that the UNG deficiency alters the type of error-prone polymerases recruited, given that the mutation frequency, mutation base specificity, and processivity depend on the fidelity of the error-prone polymerases. Other repair enzymes, such as MSH2, may recruit different polymerases. In fact, MSH2 associates with the error-prone polymerase η (54, 55).

Processive mutations may also be associated with excessive exonucleic activities. In fact, the mutations surrounding the recombination breakpoints were distributed over a larger stretch of DNA in the UNG-deficient B cells than in wild-type B cells. It is also possible that UNG may inhibit the excessive digestion of cleaved ends by exonucleases. Taking all of the evidence together, it is easiest to explain our results in terms of error-prone DNA polymerases and/or excessive exonucleic cleavage for the repair of staggered nick ends.

Several enzymes involved in DNA repair are required for CSR and SHM (56–59). A deficiency of the mismatch repair enzymes MSH2, MSH6, PMS2, or MLH1 reduces the CSR efficiency to between 15 and 90% of wild-type (32, 60–64). Interestingly, B cells from msh2−/− and mlh1−/−, but not msh6−/−, mice cause a 2- to 3-fold increase in the mutation frequency in the recombined Sμ and Sγ regions (35, 63–65). The mutated bases in the Sμ and Sγ regions did not show a significant skew with the deficiency of any of these mismatch repair enzymes.

To explain the residual CSR activity in ung−/− B cells, Msh2 was proposed to compensate for the U-removal function of UNG (34). However, the present study showed that both UNG and Msh2 were dispensable for DNA cleavage in the Sμ region in stimulated IgM+ B cells. The CSR efficiency in the double-knock-out msh2−/−mlh1−/− mice is not much reduced compared with its efficiency when only one of the two genes is knocked out (62). However, CSR in msh2−/−ung−/− B cells is greatly reduced, to 1% of the wild-type level (34). These genetic data suggest that MSH2 and MLH1 function in a similar step, and that MSH2 and UNG are involved in separate steps in CSR (66).

Because the N-terminal motifs for PCNA and RPA binding are dispensable for the CSR activity of UNG, UNG may have an additional function in the base excision repair system that is associated with DNA replication, through PCNA and RPA. Previously, we showed that the U-removal activity of UNG was not required for CSR (23). Although one interpretation offered was that the retained CSR activity in loss-of-catalysis mouse UNG mutants is due to a trace residual activity (0.04%) identified in similar mutants of E. coli UNG (67, 68), the CSR activities of the wild-type UNG and its mutants are equivalent, strongly arguing against this idea (69).

In fact, UNG plays a role different from U-removal in viral DNA replication. Viral UNG is required for the replication of vaccinia virus, but viral UNG mutants lacking their U-removal activity can replace the wild-type viral UNG in vaccinia viral replication (70). The role of UNG in the HIV particle may also be different from U-removal, although UNG is required for
HIV propagation (71). Together, the CSR requirement for the highly conserved WXXF motif of UNG and the dominant-negative effect of the exogenous Vpr peptide protein indicates that UNG is recruited to the CSR machinery complex by a host protein for a novel non-canonical function.

In summary, we propose that a non-canonical function of UNG is required in a step between DSB and NHEJ in CSR (Fig. 7). This step is responsible for the repair of staggered nick cleavage ends with 5' or 3' overhangs to form blunt ends and probably depends on the function of exonucleases and/or error-prone polymerases. Although it is not clear how UNG is involved in this repair phase, the WXXF motif and its interaction with a host protein are essential to CSR. It is therefore likely that UNG forms a complex with the DNA repair machinery. These results are consistent with our previous proposal (23) that UNG serves as a scaffold for exonucleases and/or DNA polymerases that function after DNA cleavage but before NHEJ.

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