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Deoxyuridine Triphosphate Incorporation during Somatic Hypermutation of Mouse VkOx Genes after Immunization with Phenylloxazolone

Benjamin Roche, Aurélie Claeës, and François Rougeon

Somatic hypermutation (SHM) of Ig genes is the result of a two-phase process initiated by activation-induced cytidine deaminase, relying on two different strategies for the introduction of mutations at CG pairs (phase I) and at AT pairs (phase II). To explain the selectivity of phase II, two mechanisms were proposed: AT-selective error-prone DNA-polymerases, deoxyuridine triphosphate (dUTP) incorporation, or both. However, there has been no experimental evidence so far of the possible involvement of the latter. We have developed a ligation-anchored PCR method based on the formation of single-strand breaks at uracils. In this study, we show the presence of uracil in hypermutating VkOx genes in wild type (AID+/+) mice, demonstrating that dUTP incorporation via DNA polymerases could be a major mechanism in SHM. Thus, error-prone DNA polymerases would participate in SHM via low-fidelity replication and incorporation of dUTP. The Journal of Immunology, 2010, 185: 4777–4782.

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Abbreviations used in this paper: AID, activation-induced deaminase; AP, abasic site; C, deoxycytidine; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dUTP, deoxyuridine triphosphate; LA, ligation-anchored; NTS, nontranscribed strand; phOx, 2-phenyl-oxazolone; SHM, somatic hypermutation; TS, transcribed strand; U, deoxyuridine; UNG, uracil-DNA-glycosylase.

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Materials and Methods

Hyperimmunization of mice and extraction of genomic DNA

phOx-chicken serum albumine was prepared as described previously (12), with an estimated 25 molecules of phOx per molecule of chicken serum albumine. Eight-week-old mice were injected i.p. with 50 µl phOx-CSA.
In each assay, 300 ng genomic DNA was digested with 1 U USER enzyme (NEB, Beverly, MA; mix of UDG and endonuclease VIII) in 1X PCR buffer (Taq-CORE kit; Q-Biogene, MP Biomedicals France, Illkirch, France), for 1 h at 37°C in a final volume of 20 μl. The digested DNA was denatured at 5°C at 96°C and then placed on ice; 1 U calf intestinal phosphatase (Roche, Basel, Switzerland) was added in a final volume of 60 μl in the appropriate buffer for 1.5 h at 37°C; 7 μl NaClO3 3M was added, the DNA was denatured at 5°C at 96°C, placed on ice, purified by ethanol precipitation, and resuspended in 15 μl H2O.

LA-PCR

Fifteen microliters of digested and purified genomic DNA was denatured at 5°C at 96°C, placed on ice, and ligated to the anchor oligonucleotide 5'-Phos-ATGCAGGTTTAAAGGGACTGTCGTTGACGGC-2'-3'-dideoxy by 20 U of T4-RNA-ligase (NEB) overnight at room temperature (20°C); 3 μl NaClO3 3M was added to the ligation, which was then purified by ethanol precipitation and resuspended in 30 μl H2O; 10 μl was used in a nested PCR in a volume of 25 μl using the MP Q-Bio TaqCORE kit for 15 cycles (20 s at 96°C, 1 min at 60°C); and 2.5 μl of the reaction was used in a volume of 25 μl for the second step of PCR (30–40 cycles with same temperatures). Oligonucleotides used for LA-PCR were: VkOx-forward, 5'-CAAATGTTCTCACCAGTCTCC-3'; VkOx-forward-nested, 5'-CTCAACCAATGTTCTCACCAGTCTCC-3'; the test donor complementary oligonucleotide complementary to the anchor sequence, 5'-CCTAGCTCAGACGGTTAAGGTCCCTGAT-3'. VkOx-reverse primer was used for verification of the presence of the VkOx genes (5'-CGCAATGTTCTCACCAGTCTCC-3'); and the test donor complementary oligonucleotide used for primer sequences).

Identification of germ-line VkOx genes

We used the VkOx1/3H3 gene sequence (15) in a basic local alignment search tool on the mouse genome to identify germ-line VkOx sequences. We identified 32 sequences (identical to H3 sequence, 85–97%), all of them located in the mouse chromosome 6 (Mus musculus genomic contig: ref|NT_039353.7|NM_39393.37). The 13 other germ-line VkOx sequences described by C. Milstein’s team (16) were present among the 32 sequences we identified. We labeled the sequences VkOx1 to VkOx32 according as references.

Results

User-LA-PCR and KpnI-LA-PCR on hyperimmunized and control mice

The principle of the LA-PCR-based method is to nick the DNA in an uracil-specific manner, using USER enzyme (New England Bio-Labs, Ozyme, Saint-Quentin Yvelines, France), a mix of recombinant uracil-DNA-glycosylase (UNG), and endonuclease VIII. The resulting SSB-containing DNA is denatured and used in a single-stranded ligation reaction (using T4-RNA-ligase) to an oligonucleotide linker (the “anchor”) that is 5’-phosphorylated, 2’-3’-dideoxy to avoid self-ligation. The ligated DNA is used in a nested PCR reaction using forward oligonucleotides specific to the sequence of interest, and a reverse oligonucleotide specific to the linker (Fig. 1A; see Materials and Methods for primer sequences).

We hyperimmunized 25 BALB/c mice with two injections of 2-phenyl-oxazolone (at J0 and J21). The genomic DNA was extracted from the spleens at J24. Using PCR on the genomic DNA, we verified that hyperimmunized mice and control mice have the phox-site at positions 101–106 or 107–112, or codon 35 according to the numbering used by Griffiths et al. (15). When using KpnI-LA-PCR, we could recover two bands of the correct size in both hyperimmunized mice and control mice (Fig. 1B, lanes 4 and 5). In the case of User-LA-PCR, the results differ between hyperimmunized mice and control mice. Whereas there was no amplification in the case of User-LA-PCR, the results differ between hyperimmunized mice and control mice (Fig. 1B, lanes 3 and 1, respectively). The fact that the KpnI-LA-PCR of T4-RNA-ligase inactivation does not occur in the presence of User enzyme (or KpnI digestion of the murine genomic DNA and subsequent single-stranded ligation with the anchor, followed by a nested PCR amplification. 1 and 2, forward primers; 3, reverse primer (see Materials and Methods). B, +, hyperimmunized mice; −, control mice; lane 1, marker; lanes 2 and 3, presence of VkOx genes in mice shown by PCR using VkOx-forward and reverse primers; lanes 4 and 5, KpnI-LA-PCR amplicons are recovered in control and hyperimmunized mice; lanes 6 and 7, User-LA-PCR amplicons are found in hyperimmunized mice only and are of varying length; small-size amplifications in lanes 4–7 are due to the formation of primer dimers during LA-PCR: 1 + 3 (49 bp) and 1/2 + 3 (56 bp).
To verify that this amplification was not attributable to a high background level of dUTP incorporation in the VkOx, owing to its high transcription, we tested three other genes—by User–LA-PCR, namely GAPDH, α-tubulin, and β-actin—that are actively transcribed but do not undergo SHM. No amplification was found on the housekeeping genes. In contrast, we were able to obtain a User–LA-PCR amplification on VHOx genes (data not shown). This result indicates that the DNA-uracil increase is specific to V genes (VkOx and VHOx) and that this effect is above the background level of dUTP incorporation.

Analysis of LA-PCR clones

KpnI– and User–LA-PCR amplicons from five hyperimmunized mice were cloned and subsequently sequenced. For each sequence, we determined the point of ligation, as defined by the nucleotide position where the anchor is ligated to the VkOx sequence. Each sequence was also compared with the 32 VkOx reference sequences for identification.

As a control, 30 KpnI–LA-PCR amplicons were cloned and sequenced; they had a point of ligation at the exactly nucleotide position of the KpnI site (i.e., 5¢-ACT-GGTAC-ATCGAG . . .-3¢; VkOx sequence-KpnI site-Anchor.

![FIGURE 2. Points of ligation of User-LA-PCR amplicons. Boldface indicates position of the dU removed by UNG. A, VkOx sequence showing the positions corresponding to the points of ligation of User–LA-PCR amplicons (CDR1, codons 24–34; CDR2, codons 50–56; CDR3, codons 89–102). B, Table showing the sequence contexts for each point of ligation (identified by the codon and nucleotide position in first column), number of sequences recovered that yield each point of ligation, deduced U:G or U:A substitutions, and percentage of sequences recovered for each point of ligation, deduced U:G or U:A substitutions. Substitutions are shown in Table I and Fig. 3).

![FIGURE 3. 3¢ terminus nucleotide substitution in User-LA-PCR. The abasic site subsequent to UNG-excision of the uracil is hydrolyzed at its 3¢ side by endonuclease VIII (β-elimination). After anchor ligation and during LA-PCR the abasic site is replicated, explaining the mutation spectrum of the 3¢ terminus nucleotide (~T > C > A). The nucleotide position corresponding to the initial uracil is the position of the 3¢ terminus nucleotide of the VkOx sequence (i.e., at the position of the nucleotide just 5¢ to the anchor).]
whether the uracil was in a CG or AT pair. When the nucleotide was a C in the reference sequence, the uracil was in a U:G context and thus was caused by cytidine deamination. When the nucleotide was a T, the uracil was in a U:A context and thus was caused by incorporation of dUTP during polymerization.

The sequence contexts for each point of ligation are presented in Fig. 2B, along with the number of sequences that yielded this point of ligation. Twenty-four percent of the amplicons yielded the VkOx22 gene (Supplemental Data 4), which is the main VkOx gene used in the antiphenyloxazolone response (11). It is interesting to note that the relative distribution of uracil excision corresponds closely to the previously described hypermutation distribution on the VkOx gene (15, 19).

Sixty-five percent of the positions corresponded to a U:G context, and 35% corresponded to a U:A context (73 and 27%, respectively, in terms of number of amplicons sequenced). Not surprisingly, there were no cuts at purine residues in the reference sequence, indicating that there were no U:C or U:T mismatches, nor AP:C or AP:T APs, which could have been formed by excision of 8-oxoguanine and hypoxanthine, respectively, or by depurination.

It is known that during SHM there are “hotspot” nucleotide sequences at which phase I and phase II mutations are preferentially introduced. For GC mutations, the hotspot sequence is GYW/WRC (RGYW/WRCY and DGYW/WRCH) were also proposed; D = A/G/T; H = T/C/A) and WA/TW for AT mutations. It is noteworthy that in our assay, the U:G-corresponding positions that were the most mutated (codons 34 [102] and 36 [108]). Likewise, three of the five most mutated U:A positions were in a TW hotspot (codons 30 [89], 34 [100], 36 [106]).

Interestingly, some WR hotspots (phase I) were never mutated in the sequence, whereas some SY coldspots were occasionally found, which supports evidence for the important role of epigenetic targeting by AID and SHM.

Kpl– and User–LA-PCR on AID-deficient mice

To confirm that the incorporation of dUTP in VkOx genes is indeed attributable to somatic hypermutation, we repeated the experiment on four AID-deficient mice. Two were immunized by one injection of phenyloxazolone at J0, the genomic DNA being extracted from spleens at J10, and the other two were used as controls. First, we verified by PCR that the VkOx genes were present in the genomic DNA (Fig. 4, lanes 2 and 3). An amplicon of the correct size was obtained by Kpl–LA-PCR (lanes 4 and 5). However, in both immunized and control mice, no User–LA-PCR amplification was observed (lanes 6 and 7), indicating that in the case of AID-deficient mice, which are SHM-deficient, no presence of uracil was observed in VkOx genes in immunized mice. These results support the view that dUTP incorporation is AID-dependent (Fig. 5).

Discussion

We have shown that the uracil is present in VkOx genes of hyperimmunized mice, but not control mice or AID-deficient mice. In particular, we established that in one third of the positions analyzed, the uracil is in a U:A context (i.e., resulting from dUTP incorporation). Our result therefore provides experimental ground agreeing with a proposal of the involvement of dUTP incorporation in SHM (20).

It is important to note that we observed a snapshot of hypermutating cells, not the result of hypermutation; thus, the precise extent at which uracil at U:G and U:A pairs will yield mutations is not known. It has been proposed that during SHM up to half of AID-induced U:G mismatches could be repaired in an error-free manner (21). Furthermore, the respective T1/2 of U:G mismatches and U:A base pairs is likely to be different as well; this makes it difficult to precisely evaluate their relative contribution to SHM phase I and phase II.

However, the results demonstrate that dUTP incorporation could play a crucial role in SHM. In this mechanism, phase II mutations at AT pairs would be introduced subsequently to the incorporation of dUTP by DNA-polymerase η in the mismatch repair tract, when the uracil in a U:A context is excised from DNA by a uracil-DNA-glycosylase and the subsequent abasic site replicated erroneously, in a manner similar to phase Ib mutations (Fig. 5). Therefore, AT mutations would depend on mismatch repair proteins and the availability of a uracil-DNA-glycosylase, as is phase Ib.

Which uracil-DNA-glycosylases could be implied? UNG-deficient mice have been described as having a strongly inhibited phase Ib with a strong bias toward transitions at CG pairs. There

![FIGURE 4. Kpl– and User–LA-PCR on AID-deficient mice. +, hyperimmunized mice; −, control mice; lane 1, marker; lanes 2 and 3, presence of VkOx genes in AID-deficient mice as shown by PCR; lanes 4 and 5, Kpl–LA-PCR amplicons are recovered both in control and hyperimmunized mice; lanes 6 and 7, in AID-deficient mice, no User–LA-PCR amplification is found, with no difference between control and hyperimmunized mice.](http://www.jimmunol.org/6)

![FIGURE 5. Molecular mechanism of SHM phase II mutations by dUTP incorporation. The AID-induced mismatch is recognized by the MSH2-MSH6 heterodimer. After nicking and partial digestion of the strand by EXO1, dUTP is incorporated by DNA-polymerase η during gap-filling synthesis. The uracil in U:A base pairs subsequent to the incorporation of dUTP will be excised by a uracil-DNA-glycosylase, forming an AP that can be erroneously copied, generating AT-specific mutations.](http://www.jimmunol.org/7)
is no bias for AT pairs, which are mutated at a lower frequency (22). The most efficient way to introduce a mutation at a U:A pair is by replicating the AP, resulting from uracil excision, whereas replication before uracil excision is not mutagenic because it will be replicated with an adenine. Thus, inhibiting UNG cannot create a transition–transversion bias for AT pairs, but it will make more U:A pairs to be replicated before the uracil is excised, which is error free. This finding could partly explain the lower frequency of AT mutations during SHM of such mice, and it suggests that UNG2 participates in phase II. In addition, UNG2 has been shown recently to stimulate up to two thirds of AT mutations (23).

The slight decrease of AT mutations in UNG−/− mice suggests that the uracil in the U:A pairs could be excised by other DNA-glycosylases, such as SMUG1 (24). As AID deaminates cytidines on ssDNA, uracil at U:G positions will be mostly formed in a single-stranded context on which UNG2 has more activity (25), while dUTP is incorporated in double-strand DNA on which SMUG1 has more activity when associated to AP-endonuclease 1 (26). It would be interesting to know whether SMUG1 could back up phase II mutations more efficiently than phase Ib mutations in UNG−/− mice.

It is important to note that this mechanism for explaining phase II mutations does not contradict the DNA-polymerase-based model, because dUTP is incorporated via a DNA polymerase. In fact, the involvement of dUTP incorporation could shed new light on some of the puzzles of SHM, in particular the fact that DNA polymerase η is not AT specific (27), and it has been estimated to introduce up to 26% of mutations at GC bases in vitro on a murine κ L chain transgene (28). Moreover, whereas phase II hypermutation targets TW hotspots with a similar efficiency, DNA-polymerase η preferentially mutates TA over TT (29). Furthermore, although MSH2−/− mice have a residual 10% of AT mutations (30), and MSH2−/− POLH+ mice make no more AT mutations, showing that residual AT mutations in MSH2−/− mice are caused by polymerase η (31), the mutation spectrum of AT mutations in some MSH2−/− murine B cells does not correspond to DNA-polymerase η mutation spectrum, leading the authors to suggest that an alternative pathway exists, which would depend on DNA-polymerase η, but independent of its mutational spectrum (32). dUTP incorporation via DNA-polymerasers, rather than their low fidelity, would explain the strict AT specificity of phase II mutations.

Another important issue in the current view of phase II of SHM is that it does not fully explain why AT mutations are strand biased, with adenosine being targeted on the nontranscribed strand (NTS) twice as frequently as thymine. It has been proposed that a mechanism of transcription-coupled repair may account for the strand polarity of SHM phase II, in which the transcribed strand (TS) is preferentially repaired (33). We propose that dUTP would be preferentially incorporated facing NTS adenosine. Replication of the subsequent AP on the TS will introduce a mutation on the NTS, in place of the adenine. This strand bias could be further enhanced by DNA-polymerase η low-fidelity synthesis, because it mutates thymidine preferentially (28).

In conclusion, all these results support the view that dUTP incorporation could be the major mechanism insuring mutations at AT pairs during SHM phase II. Although we cannot exclude that phase II mutations are also introduced by error-prone DNA polymerasers, the mechanism presented in this study explains more conveniently the selectivity for AT pairs without excluding the participation of these DNA polymerasers in the process. It is remarkable that the immune system has diverted one of the oldest nucleotide metabolic pathways to introduce replication-indepen- dent mutations during somatic hypermutation to generate the high-affinity Abs of the secondary repertoire.

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Disclosures

The authors have no financial conflicts of interest.

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