Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice

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Somatic hypermutation of Ig genes enables B cells of the germinal center to generate high-affinity immunoglobulin variants. Key intermediates in somatic hypermutation are deoxyuridine lesions, introduced by activation-induced cytidine deaminase. These lesions can be processed further to abasic sites by uracil DNA glycosylase. Mutagenic replication of deoxyuridine, or of its abasic derivative, by translesion synthesis polymerases is hypothesized to underlie somatic hypermutation.

Rev1 is a translesion synthesis polymerase that in vitro incorporates uniquely deoxycytidine opposite deoxyuridine and abasic residues. To investigate a role of Rev1 in mammalian somatic hypermutation we have generated mice deficient for Rev1. Although Rev1−/− mice display transient growth retardation, proliferation of Rev1−/− LPS-stimulated B cells is indistinguishable from wild-type cells. In mutated Ig genes from Rev1−/− mice, C to G transversions were virtually absent in the nontranscribed (coding) strand and reduced in the transcribed strand. This defect is associated with an increase of A to T, C to A, and T to C substitutions. These results indicate that Rev1 incorporates deoxycytidine residues, most likely opposite abasic nucleotides, during somatic hypermutation. In addition, loss of Rev1 causes compensatory increase in mutagenesis by other translesion synthesis polymerases.
by one or more of the TLS polymerases (14). In a second phase of SHM, DNA mismatch repair may induce single-stranded gaps at sites of mispaired deoxuryridine residues, followed by filling the gaps by mutagenic TLS (11, 15). To investigate involvement of the Rev1 TLS polymerase in SHM, we have generated and analyzed Rev1-deficient mice.

RESULTS AND DISCUSSION

Using conventional gene targeting we deleted exon 10 of Rev1 encoding the conserved S(C/I)DE amino acid sequence essential for the catalytic activity of Y family DNA polymerases (Fig. 1, A and B). Rev1+/− chimeric mice were obtained through blastocyst injection of heterozygous embryonic stem cells and crossed to C57BL/6 and 129/OLA mice. Rev1+/− offspring from interbreeding after F1 and F2 backcrosses to both strains was obtained at 63% of the expected Mendelian ratios. Strikingly, Rev1+/− mice were not obtained beyond the F2 backcross into C57BL/6 mice, in contrast to backcrosses to 129/OLA. A similar strain dependence of the phenotype was found for mice deficient for the Rev3 TLS polymerase (16). The milder phenotypes of the 129/OLA mice are not caused by the pol ι defect of 129/OLA mice (17) due to the fact that (pol ι-proficient) Rev1−/− F1 hybrid mice of C57BL/6 and 129/OLA crossings are viable. Rev1−/− mice from all strains displayed a transiently reduced weight in the absence of gross abnormalities (Fig. 2, A and B). Together, these results are consistent with a partially strain-dependent role for Rev1 in TLS of endogenous DNA damage.

Western blot analysis of cell lysates from Rev1−/− mouse embryonic fibroblasts (MEFs), using a COOH-terminal α-Rev1 antiserum, confirmed the absence of protein in Rev1−/− cells (Fig. 1 C). Analysis of Rev1 transcripts from mouse kidney by RT-PCR demonstrated the presence of only shortened transcripts in Rev1−/− cells (Fig. 1 D). Downstream of the deletion of exon 10 all transcripts contained translational frame-shifts (unpublished data). Based on these results we conclude that both the catalytic domain and the domain that interacts with other TLS polymerases and the Rev7 protein (7–10; unpublished data) are absent from our Rev1 mutant. In agreement with a generalized defect in TLS of exogenous DNA damage, Rev1−/− MEFS are sensitive to several genotoxic agents tested, including short-wave UV light (unpublished data).

To investigate lymphopoiesis of Rev1−/− mice, we analyzed the cellularity and the composition of B and T cell subsets of primary and secondary lymphatic organs like thymus, spleen, lymph nodes, and bone marrow. Consistent with the moderately reduced size of 7-wk-old Rev1−/− mice (Fig. 2), a proportional reduction in the cellularity of lymphoid organs was observed (Fig. 3 A). The normal frequency of all tested lymphoid subsets suggests that qualitative aspects of lymphocyte development are not impaired by the absence of Rev1.
The moderate reduction in cellularity likely is related to the reduced size of spontaneous apoptosis in the chicken DT40 B cell line. Rev1 deficiency causes a slower expansion rate and increased activity of pol η (21, 22) and/or other TLS polymerases. This result indicates that the interaction between Rev1 and other

Table 1. Mutation frequencies of hypermutated VA1 gene segments in Rev1−/− memory B cells

|                      | WT   | Rev1 |
|----------------------|------|------|
| Cells analyzed       | 368  | 372  |
| Cells mutated        | 41%  | 40%  |
| Number of mutations  | 508  | 587  |
| Mutation frequency   | 0.46%| 0.53%|
| Actual mutation frequencya | 1.1% | 1.3% |

a Only mutated VA1 sequences are taken into account.

To address the role of Rev1 in mammalian SHM, single class-switched (IgM−IgD−) memory B cells expressing a IgA light chain were isolated from mice derived from intercrosses of F2 backcrosses to C57BL/6. PCR amplification and direct sequencing of the rearranged VA1 gene segment from single cells was performed to determine the distribution, frequency, and base exchange pattern of somatic mutations as well as the frequency of memory B cells carrying a mutated VA1 gene (18). SHM, corrected for clonality of the mutations, occurs at a normal frequency in Rev1−/− B cells. Thus, regardless of the genotype, 40–41% of all analyzed class-switched B cells contained a mutated rearranged VA1 gene segment. Taking only the mutated VA1 sequences into account, the mutation frequency is 1.1% for wild-type and 1.3% for Rev1−/− B cells. Likewise the distribution of mutations along the VA1 segments, with a concentration at mutation hot spots within the complementarity-determining regions (CDR 1, 2, and 3), is similar in wild-type and Rev1−/− B cells (Fig. 4 A). In contrast, highly significant differences in mutation spectra are found (Fig. 4, B and C; Table S2, available at http://www.jem.org/cgi/content/full/ jem.20052227/DC1). In the coding (nontranscribed) strand we find an almost complete absence of C to G transversions as well as a moderate decrease in G to C transversions (Fig. 4, B and C). These results indicate a catalytic role of Rev1 in incorporating dCMP during SHM in mammals, in agreement with recent data demonstrating a role of the Rev1 catalytic domain in SHM in chicken DT40 cells (19). The strand-specific mutational defect at GC basepairs mimics that of Ung−/− B cells in which AID-induced deoxyuridine residues cannot be converted into abasic sites (15, 20). This result suggests that abasic sites, generated by subsequent AID and UNG activities on deoxycytidine residues, are the substrates for Rev1 in vivo. Because mice doubly deficient for the mismatch repair gene Msh2 and for Ung have lost all transversion mutations at GC basepaitn during SHM (15), the residual G to C transversions in Rev1−/− B cells are likely induced during the second, mismatch repair–dependent phase of SHM. Compensating the strand-specific defect in transversions at GC basepairs, the frequencies of A to T and C to A transversions along the Vλ1 segments, with a concentration at mutation hot spots within the complementarity-determining regions (CDR 1, 2, and 3), is similar in wild-type and Rev1−/− B cells (Table I).
TLS polymerases, including pol η, is dispensable for their activity in SHM. In addition, deletion of the BRCT domain of Rev1 that regulates TLS of short-wave UV light–induced photoproducts by other TLS polymerases, including pol η, does not affect SHM (6). This further supports the specific involvement of the catalytic activity of the Rev1 protein in transversion mutagenesis at GC basepairs.

In conclusion, we have demonstrated an important role of mammalian Rev1 in determining the mutation spectrum of hypermutating Ig genes, in a strand-biased fashion. This likely is achieved by the direct incorporation of deoxycytidine opposite abasic sites, generated at cytidines via AID-mediated deamination and UNG activity, respectively.

MATERIALS AND METHODS
Generation of Rev1-deficient mice. Replacement of exon 10 of Rev1 in the 129/OLA-derived embryonic stem cell line E14 with a pPGK-hygromycin cassette (Fig. 1 A) was performed according to established procedures. Gene targeting was analyzed by Southern blotting of KpnI-digested DNA and hybridization with probes A (Fig. 1 B) and B. Rev1−/− cells were used to generate C57BL/6-129/OLA chimeric mice that were crossed to 129/OLA and C57BL/6 mice. Tail DNA of progeny was genotyped in a multiplex PCR reaction (35 cycles at 94° C for 60 s, 53° C for 60 s, and 72° C for 90 s), using primers SCDEKA1 (5′-ATTGATAGTCTCTAGCTTGTG-3′) and SCDEKA2 (5′-GCTGATTTGAAATTCTAGGG-3′) amplifying the wild-type allele, and primers SCDEKA1 and PGKPR1 (5′-GCTTCCATTGCT-CAGCGTG-3′) amplifying the mutant allele. All required permits were obtained (government permit number VVM/BD 00.235 D06 for generating the transgenic animals and University Animal Ethical Committee permit numbers DEC 01058 and DEC 03139).

Characterization of Rev1 expression. Rev1 cDNA was generated from kidney-derived RNA as described (6). Primers p13 (5′-GAGCTGAGCAGACTT-3′) and p14 (5′-GAGCTAAGGAGCCGTGACTT-3′) amplify a PCR fragment containing exons 8–15. Primers pSCDE (5′-GCTCAGCTGTGAT-GAACACT-3′) and p14 amplify a product encompassing exons 10–15 (Fig. 1 D). PCR products were cloned and sequenced. Rev1 protein in lysates from immortalized MEFs was analyzed by immunoblotting as described (6).
Flow cytometry. Single-cell suspensions from thymus, spleen, lymph nodes, and bone marrow were stained with specific antibodies conjugated to FITC, PE, or APC or biotin; biotinylated monoclonal antibodies were revealed with Streptavidin-PerCP or Streptavidin-APC. Antibodies were purchased from BD Biosciences unless mentioned otherwise: CD98-FITC (53–5.8), CD19–FITC (1D3), IgD–FITC (11–26c.2a), IgM–Fluos (331.12, self-conjugated), CD3–PE (17A2), CD8–PE (53–6.7), CD19–PE (1D3), Vγ–PE (LS136, self-conjugated), CD4–APC (RM4–5), CD19–APC (1D3), cKit–APC (CD117, clone 2B8), Thy1–APC (CD90, clone 53–2.1), biotinylated TCRγδ (GL3), biotinylated IgM (II/41), and streptavidin-APC. Analysis was performed on a FACScalibur.

Proliferation of LPS-stimulated B cells. Isolated splenocytes were labeled in serum-free IMDM medium for 10 min at 37°C with 5 μM carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). Next, the cells were cultured for 3 d in the presence of 25 μM fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). Next, the cells were cultured for 3 d in the presence of 25 μM fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes).

Analysis of somatic hypermutation. Single live IgD+ and IgM+ (FITC−) and CD19+ (APC+) and Vλ1+ (PE+) B cells were isolated from spleens of 3–5-mo-old mice. Analysis of SHM in the rearranged Vλ1 gene segment by single-cell PCR was performed as described (18).

Mutation analysis. Multiple, nonimmunized, wild-type (n = 7) and Rev−/− (n = 5) mice were analyzed, minimizing the incidence of clonally related sequences (Table S2). Data represent mutations between codons 13 and 97 of Vλ1 (23) after exclusion of putatively clonally related mutants.

Online supplemental material. Table S1 is a composition of B and T cell subsets of primary and secondary lymphatic organs of wild-type and Rev−/− mice. No qualitative differences are found between the genotypes. Table S2 shows a complete mutational spectrum at the Vλ1 gene segment of wild-type and Rev−/− mice. All mutated Rev1-deficient and proficient clones used for analysis are shown. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052227/DC1.

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