A Critical Role for REV1 in Regulating the Induction of C:G Transitions and A:T Mutations during Ig Gene Hypermutation

Keiji Masuda, Rika Ouchida, Yingqian Li, Xiang Gao, Hiromi Mori and Ji-Yang Wang

*J Immunol* 2009; 183:1846-1850; Prepublished online 8 July 2009;
doi: 10.4049/jimmunol.0901240
http://www.jimmunol.org/content/183/3/1846

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/07/07/jimmunol.0901240.DC1

References
This article cites 28 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/183/3/1846.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Critical Role for REV1 in Regulating the Induction of C:G Transitions and A:T Mutations during Ig Gene Hypermutation

Keiji Masuda,* Rika Ouchida,* Yingqian Li,† Xiang Gao,† Hiromi Mori,* and Ji-Yang Wang†,*

REV1 is a deoxycytidyl transferase that catalyzes the incorporation of deoxycytidines opposite deoxyguanines and abasic sites. To explore the role of its catalytic activity in Ig gene hypermutation in mammalian cells, we have generated mice expressing a catalytically inactive REV1 (REV1AA). REV1AA mice developed normally and were fertile on a pure C57BL/6 genetic background. B and T cell development and maturation were not affected, and REV1AA B cells underwent normal activation and class switch recombination. Analysis of Ig gene hypermutation in REV1AA mice revealed a great decrease of C to G and G to C transversions, consistent with the disruption of its deoxycytidyl transferase activity. Intriguingly, REV1AA mice also exhibited a significant reduction of C to T and G to A transitions. Moreover, each type of nucleotide substitutions at A:T base pairs was uniformly reduced in REV1AA mice, a phenotype similar to that observed in mice haploinsufficient for Polh. These results reveal an unexpected role for REV1 in the generation of C:G transitions and A:T mutations and suggest that REV1 is involved in multiple mutagenic pathways through functional interaction with other polymerases during the hypermutation process. The Journal of Immunology, 2009, 183: 1846–1850.

The Journal of Immunology

Received for publication April 17, 2009. Accepted for publication June 2, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Ji-Yang Wang, Laboratory for Immune Diversity, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan. E-mail address: oth@rci.riken.jp

2 Abbreviations used in this paper: SHM, somatic hypermutation; BRCT, BRCA1 C-terminal; GC, germinal center; MMS, methyl methanesulfonate; POLH, polymerase η; REV1AA, catalytically inactive REV1; UNG, uracil DNA glycosylase; WT, wild type.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901240

Materials and Methods

Generation of mice expressing REV1AA

The highly conserved aspartate and glutamate residues encoded by exon 10 were mutated to alanines by site-directed mutagenesis (Takara Bio). A fragment containing the mutated exon 10 was used as the 5′-arm of the targeting vector. Transfection and selection of the C57BL/6-derived Bruce4 ES cells were performed as described previously (22). Homologous recombinants were identified by long-range genomic PCR using primers s1 (5′-GCTTTGTCTTTTGGGGATGA-3′) and as1 (5′-TCGCCCTCTTATC GCTTCTT-3′) for 5′ and as1 (5′-TCAGCCACATCCACATCCC-3′) for 3′.
and neos (5'-ATAGCCGAATAGCCTCTCCA-3') for 3' homology regions. PCR was performed at 95°C for 2 min, followed by 30 cycles of amplification at 95°C for 10 s, 57°C for 20 s, and 68°C for 10 min (for 5') or 95°C for 10 s, 63°C for 20 s, and 68°C for 13 min (for 3') using LA-Taq polymerase (Takara Bio). Chimeric mice were bred with C57BL/6 mice to obtain heterozygotes, which were then crossed with CAG-Cre transgenic mice to delete the neo gene. The neo-deleted heterozygous mice were bred to obtain homzygous REV1AA mice. Mouse genotypes were determined by PCR using primers s2 and as2 flanking the loxP site. WT and targeted alleles gave rise to 364- and 400-bp bands, respectively. M, size markers.

FIGURE 1. Generation of REV1AA mice. A, Targeting strategy. The targeting vector was designed to replace exon 10 (E10) with a mutated E10 (indicated by an “x”) in which the highly conserved aspartate and glutamate residues were substituted with alanines. The positions of PCR primers (s1, as1, s2, as2, s3, as3, neos, and neos) are indicated. B, Long-range PCR analysis to detect the targeted allele, using primers s1 and neos for the 5'-arm and as1 and neos for the 3'-arm. The predicted sizes for the 5'- and 3'-PCR were 4.8- and 6.3-kb, respectively. M, size markers. C, The neo gene was deleted by crossing the mutant mice with CAG-Cre transgenic mice, leaving a single ~35-bp loxP site in the intron between exons 10 and 11. Mouse genotypes were screened by PCR using primers s2 and as2 flanking the loxP site. WT and targeted alleles gave rise to 364- and 400-bp bands, respectively. M, size markers. D, Northern blot analysis for Rev1 gene expression in WT and REV1AA spleen B cells (upper panel). A 1.6-kb Rev1 cDNA fragment encompassing exons 12–22 (corresponding to cDNA sequence 2101–3695) was used as a probe. Lower panel, β-actin expression as a control. E, A genomic fragment containing exon 10 was amplified using primers s3 and as3 from WT, heterozygous, and homozygous mice and analyzed by direct sequencing.

Sensitivity to DNA damaging agents

Purified spleen B cells were seeded in 12-well plates in duplicate at 5 × 10^5/ml (1 ml/well) in the presence of 10 μg/ml LPS. The cells were then exposed to different doses of methyl methanesulfonate (MMS) or cisplatin or irradiated with UV light and cultured for 2 days. The cells were then stained with Annexin V–FITC and propidium iodide (PI) to detect apoptotic and dead cells, respectively. The percentages of live cells (Annexin V–PI–) were determined by FACS.

Results

Generation of REV1AA mice

We mutated the highly conserved aspartate and glutamate residues encoded by exon 10 to alanines by gene targeting (Fig. 1, A–C). Northern blot analysis revealed a similar transcript level of Rev1 gene in WT and REV1AA B cells (Fig. 1D), indicating that the targeted mutations did not affect Rev1 gene transcription. Sequence analysis of a fragment containing exon 10 further verified the designated mutations (Fig. 1E). Attempts to detect WT and mutant REV1 protein by immunoblot have been unsuccessful due to the poor specificity of the available Abs. However, it has been shown previously that the corresponding mutations in human REV1 did not affect the global protein structure, and the mutant
REV1 could be successfully purified like the WT protein (15). These observations collectively suggest that REV1AA mice express normal levels of REV1AA.

Normal B cell development, maturation, and activation in REV1AA mice

REV1AA mice were derived from Bruce4 ES cells and were therefore on a pure C57BL/6 genetic background. The homozygous mice were born at the expected ratio and developed normally with no obvious abnormalities by appearance. FACS analysis of BM cells of WT and REV1AA mice revealed no obvious differences in the percentages of B220<sup>+</sup>/H<sup>+</sup>CD43<sup>+</sup>/H<sup>+</sup>IgM<sup>-</sup>/H<sup>-</sup>progenitor, B220<sup>+</sup>/H<sup>+</sup>CD43<sup>-</sup>/H<sup>-</sup>IgM<sup>-</sup>/H<sup>-</sup>precursor, B220<sup>dull</sup>/H<sup>+</sup>IgM<sup>-</sup>/H<sup>-</sup>immature, and B220<sup>high</sup>/H<sup>+</sup>IgM<sup>-</sup>/H<sup>-</sup>recirculating B cells (Fig. 2 A, upper and middle panels). The proportion of CD23<sup>high</sup>/CD21<sup>dull</sup> follicular and CD23<sup>dull</sup>/CD21<sup>high</sup> marginal zone B cells in the spleen was also similar between WT and REV1AA mice (Fig. 2 A, lower panels). T cell development and differentiation were not affected in REV1AA mice as judged by CD4 and CD8 profiles in the thymus and spleen (Fig. 2 B). In addition, REV1AA B cells exhibited normal proliferative responses to anti-IgM Abs, CD40L and LPS (Fig. 2 C), and switched normally from IgM to IgG1 upon in vitro stimulation (Fig. 2 D). Representative results of three independent experiments are shown.

Reduced frequency of C:G transitions and A:T mutations in REV1AA mice

To address the role of the catalytic activity of REV1 in Ig gene SHM, we analyzed the J<sub>B</sub> intronic region of GC B cells isolated from spleens of immunized mice. We have analyzed four WT and five REV1AA mice, and the results are summarized in Table I (see supplemental Table I for detailed results of individual WT and REV1AA mice). Consistent with our previous studies (24), the overall mutation frequency in the J<sub>B</sub> intronic region was ~1% in WT mice with roughly half of the mutations occurring at C:G and A:T. The overall mutation frequency was reduced by 35% in REV1AA mice (0.651 vs 0.996% in WT mice). Mutations at C:G and A:T were decreased by 37 and 33%, respectively, compared with WT mice (Table I). To determine more precisely how SHM

---

**Table I. Mutation frequency in WT and REV1AA mice**

<table>
<thead>
<tr>
<th>J&lt;sub&gt;B&lt;/sub&gt; Intron (509 bp)</th>
<th>WT (four mice)</th>
<th>REV1AA (five mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sequences</td>
<td>494</td>
<td>570</td>
</tr>
<tr>
<td>Mutated sequences (%)</td>
<td>372 (75.3%)</td>
<td>400 (70.2%)</td>
</tr>
<tr>
<td>Total length of mutated sequences</td>
<td>189.5k</td>
<td>203k</td>
</tr>
<tr>
<td>Overall mutation frequency (%)</td>
<td>0.996</td>
<td>0.651*</td>
</tr>
<tr>
<td>Mutation frequency at C:G (%)</td>
<td>0.460</td>
<td>0.291</td>
</tr>
<tr>
<td>Mutation frequency at A:T (%)</td>
<td>0.536</td>
<td>0.360</td>
</tr>
<tr>
<td>% mutation at C:G : A:T</td>
<td>46.2:53.8</td>
<td>44.7:55.3</td>
</tr>
</tbody>
</table>

*The values in bold type indicate significant differences from WT mice (p < 0.05, unpaired t test).
was affected in REV1AA mice, we calculated the absolute frequency of each type of nucleotide substitutions. For this purpose, we included data of WT mice from our previous studies (24), allowing the analysis of a large number of each type of mutations for comparison. As expected, C to G and G to C transversions were greatly decreased (75 and 71% reduction, respectively) in REV1AA mice, with a significant increase of C to A substitution (Fig. 3 and supplemental Table II). Intriguingly, C to T and G to A transitions were also significantly decreased (47 and 49% reduction, respectively) in REV1AA mice as compared with WT mice. Moreover, we found a uniform reduction of each type of nucleotide substitutions at A:T base pairs in REV1AA mice (Fig. 3 and supplemental Table II), a phenotype resembling that observed in Polh−/− mice (24).

**Normal sensitivity to DNA damaging agents in REV1AA B cells**

REV1 is known to play a critical noncatalytic role in tolerance to DNA damage in yeast and chicken DT40 cells presumably by interacting with other polymerases and coordinating their activities during translesion DNA synthesis (25, 26). Consistently, WT and REV1AA B cells exhibited similar sensitivities to MMS, cisplatin, and UV light (Fig. 4). In addition, cell cycle analysis revealed no differences in the relative proportion of cells at G1, S, and G2-M phases between WT and REV1AA B cells treated with these agents (data not shown). These results demonstrate that REV1 catalytic activity per se is not essential for tolerance to DNA damage in mammalian B cells.

**Discussion**

The great reduction of C to G and G to C transversions in REV1AA mice is in agreement with the results of REV1-deficient mice (21). In addition, REV1-deficient chicken DT40 cells reconstituted with a catalytically inactive human REV1 (D570A/E571A) also exhibited a dramatic reduction of C to G and G to C transversions (27). These observations are consistent with the catalytic property of REV1 and suggest that REV1 participates in Ig gene SHM by incorporating C opposite UNG-mediated abasic sites. However, unlike REV1-deficient mice in which C to G transversions were virtually absent while G to C transversions were reduced, we found a similar reduction of C to G and G to C transversions in REV1AA mice. It is unclear at this point why REV1-deficient but not REV1AA mice exhibit a strand-biased defect in C/G transversions.

Both REV1-deficient and REV1AA mice exhibited a significant increase of C to A and a moderate increase of G to T transversions. These observations suggest that induction of these mutations not only does not require REV1 but also is not inhibited by the presence of an inactive REV1. Therefore, these mutations are generated by a REV1-independent pathway. The increased C to A and, to a lesser extent, G to T transversions are likely due to compensatory activation of this mutagenic pathway in the absence of a functional REV1.

The reduction of C to T and G to A transitions and A:T mutations observed in REV1AA mice is unexpected because REV1 does not possess the catalytic property to directly introduce these mutations. According to the DNA deamination model of Ig gene SHM, C:G transitions can be generated by replication of either activation-induced cytidine deaminase-triggered U:G lesion or UNG-mediated abasic site (1). Replication of the U:G lesion is thought to be conducted by replicative polymerases, and it is thus unlikely that REV1 participates in this process. It is more likely that REV1 is involved in the generation of C:G transitions during replication of the abasic site. Mammalian REV1 has been shown to interact with Y-family polymerases, raising the possibility that REV1 may regulate the generation of C:G transitions by recruiting other polymerases. This possibility, however, is unlikely because the interaction of REV1 with Y-family polymerases is mediated by the C-terminal region of REV1, which is intact in REV1AA mice. A more likely possibility is that the REV1AA might be stuck or stabilized at the abasic site, thereby preventing the switching between REV1 and other polymerases involved in the generation of C/G transitions. A similar mechanism may account for the reduction of A:T mutations in REV1AA mice. The induction of A:T mutations is highly dependent on POLH (8–13), and REV1AA might inhibit the access of POLH to the site of DNA lesions. We have recently found that Polh heterozygous mice exhibit a uniform reduction of each type of nucleotide substitutions at A:T pairs (24). A similar uniform reduction of A:T mutations in REV1AA mice is consistent with the hypothesis that POLH function is compromised in the presence of an inactive REV1. Regardless of the precise mechanism, our results suggest that REV1 and other polymerases, including POLH, do not function independently, and the interaction and switching between these polymerases are important for efficient induction of somatic mutations in Ig genes. Recently, REV1-POLH interaction was shown to be required for the formation of REV1 foci and for suppression of spontaneous mutations in human cells (28). It would be interesting to generate mice expressing a mutant REV1 that has normal catalytic activity but cannot interact with POLH and other Y-family polymerases.

The reduction of C:G transitions and A:T mutations in REV1AA mice was not observed in REV1-deficient mice. If the reduction of these mutations is indeed due to the stabilization of REV1AA at the site of DNA lesions, it is not surprising that the absence of REV1 did not affect the generation of these mutations. Conversely, the increased A to T and T to C substitutions were observed in REV1-deficient but not REV1AA mice. In this case, the absence of REV1 might have resulted in increased access of POLH to the abasic site, leading to the increase of certain A:T mutations in REV1AA mice.
mutations. The differences in the mutation patterns between REV1AA and REV1-deficient mice might also be due to the different methods used to analyze SHM. We have analyzed the J_{H4} intronic region of GC B cells isolated from immunized mice, and the mutations are induced within 2 wk by acute Ag stimulation. In contrast, V_{J} genes of memory B cells isolated from 3- to 5-mo-old mice were analyzed for Rev1-deficient mice, and these memory B cells had likely undergone relatively long-term selection by endogenous Ags. Further studies are required to completely reveal how REV1 participates in Ig gene SHM. In conclusion, our results suggest that REV1 is involved in multiple mutagenic pathways and contributes to the generation of a significant portion of both C:G and A:T mutations during Ig gene hypermutation.

Acknowledgments

We thank C. Stewart for providing the Bruce4 ES cells, Y. Masuda for helpful discussions, the Animal Facility for breeding and maintaining the mice, and the FACS Laboratory for cell sorting.

Disclosures

The authors have no financial conflict of interest.

References