The IgH 3’ regulatory region controls somatic hypermutation in germinal center B cells

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During B cell ontogeny and maturation, the IgH locus undergoes successive transcription-coupled remodeling events, including V(D)J recombination, somatic hypermutation (SHM), and Ig class switch recombination (CSR; Henderson and Calame, 1998). RAG-mediated V(D)J recombination occurs during the antigen-independent step of B cell development. Interactions with cognate antigens recruit activated B cells into germinal centers where they undergo somatic hypermutation (SHM) in V(D)J exons for the generation of high-affinity antibodies. The contribution of IgH transcriptional enhancers in SHM is unclear. The Eμ enhancer upstream of Cμ has a marginal role, whereas the influence of the IgH 3’ regulatory region (3’RR) enhancers (hs3a, hs1,2, hs3b, and hs4) is controversial. To clarify the latter issue, we analyzed mice lacking the whole 30-kb extent of the IgH 3’RR. We show that SHM in VH rearranged regions is almost totally abrogated in 3’RR-deficient mice, whereas the simultaneous Ig heavy chain transcription rate is only partially reduced. In contrast, SHM in κ light chain genes remains unaltered, acquitting for any global SHM defect in our model. Beyond class switch recombination, the IgH 3’RR is a central element that controls heavy chain accessibility to activation-induced deaminase modifications including SHM.
RESULTS AND DISCUSSION

SHM at the variable region of the IgH locus is abrogated in IgH 3′RR-deficient mice

3′RR-deficient mice display normal size B cell compartments but a globally decreased Ig secretion, including a partial IgM defect and culminating in a complete blockade of class-switched isotypes, a phenotype involving both decreased Ig secretion in plasma cells and decreased CSR frequency (Vincent-Fabert et al., 2010). Because CSR and SHM are well-known temporally and spatially associated processes, both involving AID-initiated DNA lesions (Neuberger and Ji, 2011), we explored 3′RR-deficient mice lacking the 30-kb extent of the 3′RR, a deletion which we previously characterized as inducing a severe CSR defect (Vincent-Fabert et al., 2010), but with normal V(D)J recombination (Rouaud et al., 2012). To clarify the long-range role of the 3′RR on SHM in the context of the endogenous locus, we analyzed IgH 3′RR-deficient mice lacking the 30-kb extent of the 3′RR, a deletion which we previously characterized as inducing a severe CSR defect (Vincent-Fabert et al., 2010), but with normal V(D)J recombination (Rouaud et al., 2012).

Expression of AID and SHM-related cofactors are unaffected in 3′RR-deficient mice

We investigated the transcripts encoding several proteins involved in SHM (Neuberger and Rada, 2007). Transcripts for AID, POLγ, REV1, UNG, MSH2, and MSH6 were detected in similar amounts (P > 0.1) in Peyrer’s patch B cells from 3′RR-deficient and WT mice, showing that at the RNA level, 3′RR-deficient B cells normally express the SHM trans-acting machinery (not depicted). Thus, lack of SHM in IgH V(D)J segments in 3′RR-deficient mice is not related to a deficiency of AID and related cofactor expression.

SHM at the variable region of the Igκ locus is normal in 3′RR-deficient mice

To further check that the lack of SHM at the heavy chain locus is not related to a global decrease of SHM in 3′RR-deficient mouse B cells, we investigated SHM in the Igκ locus. A similar number of mutated sequences was found in 3′RR-deficient (87%, 33/38) and WT mouse B cells (91%, 44/48; Fig. 1 A). The mutation frequency in 3′RR-deficient mice (255 mutations among 18,282 bp analyzed, 1.39%) was similar to WT (296 mutations among 24,376 bp analyzed, 1.21%). When comparing the number of mutations per sequence, we found in 3′RR-deficient mice a lower frequency (1% compared with 57% in WT, P < 0.0001) of clones carrying multiple mutations (over five mutations in the analyzed region; Fig. 1 B, left). Although the number of mutations in 3′RR sequences is small, we found that the proportion of mutations at C/G did not significantly differ between WT (43.3%) and 3′RR-deficient mice (35.7%) and that A→G transitions represented 50.8% and 41.0% for WT and 3′RR-deficient mice, respectively (not depicted). Thus, deletion of the IgH 3′RR nearly abrogates the SHM process in V_H regions.
IgH transcription is reduced but not abrogated in 3’RR-deficient mice

SHM correlates with transcription (Fukita et al., 1998). Alterations of the SHM rate have been reported using BAC transgenes with large deletion of the 3’RR, although transcription of these transgenes was variably affected by the deletion (Dunnick et al., 2009). In contrast, the partial IgH transcription defect observed in hs3b/4-deficient mouse resting B cells did...
not lead to any significant SHM decrease in germinal center B cells (Morvan et al., 2003). The situation is quite different in 3’RR-deficient mice, which showed a nearly complete V(D)J SHM blockade (98% of reduction) along with only a partial decrease (40%) of IgH primary transcripts in B220+ PNA<sup>hi</sup>Fas+ Peyer’s patch B cells (Fig. 2 A). In contrast, deletion of the 3’RR did not affect SHM (Fig. 1 C) and transcription at the κ light chain locus (Fig. 2 B). Our model indicates that Ig heavy chain primary transcription is maintained at a fairly high level in germinal center B cells devoid of 3’RR, although SHM is almost completely abrogated. This suggests that such a strong SHM defect cannot solely result from

Figure 2. IgH transcription and AID-ChIP in 3’RR-deficient and WT mice. (A and B) Analysis by real-time PCR of IgH (A) and κ (B) primary transcripts in B220+PNA<sup>hi</sup>Fas+ cells from Peyer’s patches of immunized 3’RR-deficient and WT mice. Transcript levels were normalized to GAPDH transcripts. Results are the mean ± SEM of six WT and six 3’RR-deficient mice. (C) Accessibility of IgH locus downstream of J<sub>H4</sub> to AID. ChIP assays were performed with splenic B cells isolated from immunized AID-deficient, 3’RR-deficient, and WT mice. For each sample, AID-Chip values were normalized to the input control, and AID-Chip signal in WT B cells was assigned an arbitrary value of 1. Data presented are from one primer pair and error bars corresponding to technical replicates. One representative experiment out of two is shown. (D and E) Analysis by real-time PCR of IgH (D) and κ (E) primary transcripts in B220-PNA<sup>hi</sup> splenic B cells of immunized 3’RR-deficient and WT mice. Results are the mean ± SEM of four WT and five 3’RR-deficient mice. (F) Accessibility of IgH locus to RNA polymerase II (Pol II). ChIP assays were performed with 3-d LPS-stimulated splenic B cells isolated from 3’RR-deficient and WT mice. The relative enrichments (percent input) were analyzed by real-time PCR (same primers as for analysis of IgH primary transcripts) and compared with those of negative controls obtained without antibody (mock). Results are the mean ± SEM of three 3’RR-deficient and three WT mice. ns, not significant. (G) ChIP analysis for AID occupancy at the S<sub>E</sub> region in B splenocytes of immunized WT, 3’RR-deficient, and AID-deficient mice. For each sample, AID-Chip values (mean ± SD) were normalized to the input control, and AID-Chip signal in WT B cells was assigned an arbitrary value of 1. Data represent results obtained using two different primer pairs (error bars are indicative of the variation between the two PCRs). One representative experiment out of two is shown. *, P < 0.05; Mann–Whitney U test.
decreased transcription. Rather, our data suggest that the 3’RR could be directly essential for the recruitment of AID onto the IgH locus, a hypothesis which fits well with recent results showing that the IgH 3’RR is itself an AID target (Péron et al., 2012). To determine whether AID recruitment to the JH region is dependent on the 3’RR, we performed chromatin immunoprecipitation (ChIP) analysis using an anti-AID antibody on chromatin prepared from B splenocytes of WT, 3’RR-deficient, and AID-deficient mice (immunized orally with sheep red blood cells for 2 wk and intraperitoneally with 10 µg LPS for 3 d). We found that AID occupancy at the JH region was dramatically reduced in 3’RR-deficient mice as compared with WT (Fig. 2 C). Only background levels of immunoprecipitation were found both for 3’RR-deficient and AID-deficient mice, leading to the conclusion that AID enrichment to the IgH JH region is drastically impaired in 3’RR-deficient mice. Confirming results obtained with Peyer’s patches, deletion of the 3’RR lead only to a partial decrease (50%) of IgH primary transcripts in B220+PNA+ high splenic B cells (Fig. 2 D) without significant effect on κ primary transcripts (Fig. 2 E). The significant binding of RNA polymerase II on the IgH allele of 3’RR-deficient mice (after 3-d LPS stimulation of splenic B cells in vitro) confirms that transcription is maintained (Fig. 2 F). This work provides the first conclusive evidence that, first, AID-mediated SHM is targeted to the IgH locus by the cis-acting 3’RR and, second, that SHM within Ig genes does not imply parallel transcription. These results are reminiscent of those reported for 3’ regulatory elements located in the chicken Ig light chain (Kothapalli et al., 2008; Blagodatski et al., 2009). Similarly to the IgH 3’RR, the DIVAC (diversification activator) cis-acting element is required for SHM at the chicken Ig light chain gene (Blagodatski et al., 2009) but does not function by increasing transcription (Kohler et al., 2012). Although high levels of transcription are not sufficient for robust SHM in IgL locus (Yang et al., 2006), separated mutational and transcriptional enhancers were recently reported for IgL genes (Kothapalli et al., 2011). The mechanism of how cis-regulatory sequences activate SHM in neighboring transcription units remains speculative. As hypothesized for DIVAC (Blagodatski et al., 2009), the IgH 3’RR might promote the formation of protein complexes that first bind AID and then hand it over to the neighboring transcription initiation complex.

Mutation in Sµ is severely compromised in 3’RR-deficient mice

Studies reported that Sµ targets SHM; mutational frequency peaked 5’ of Sµ and decreased thereafter (Maul and Gearhart, 2010). We investigated whether deletion of the 3’RR abrogated this pattern of SHM. Because of high GC content and the repetitive nature of the Sµ sequence (Fig. S1), accurate PCR amplification across the region is known to be problematic. We thus cloned the entire Lµ–Cµ sequence (location of PCR primers in Fig. S1) and sequenced both the 5’ and the 3’ part of the PCR product encompassing Sµ. Experiments were performed with B splenocytes of WT and 3’RR-deficient mice immunized orally with sheep red blood cells and intraperitoneally with LPS. A dramatic decrease (P < 0.0001, Mann–Whitney U test) of mutated sequences 5’ to Sµ was found in 3’RR-deficient mice (14%, 5/36) as compared with WT (65%, 20/31; Fig. 1 A). The mutation frequency in 3’RR-deficient mice (7 mutations among 19,078 bp analyzed, 0.036%) was compared with WT (92 mutations among 20,934 bp analyzed, 0.44%) and showed a >90% reduction (Fig. 1, A and D). In agreement with previous results (Maul and Gearhart, 2010), no mutations were documented 3’ to Sµ in WT nor in 3’RR-deficient mice (not depicted). ChIP experiments indicated that AID occupancy at the Sµ region was markedly reduced in 3’RR-deficient splenocytes as compared with WT (Fig. 2 G). Only background levels of immunoprecipitation were found both for 3’RR-deficient mice and AID-deficient mice, leading to the conclusion that AID binding to Sµ is totally impaired in 3’RR-deficient mice.

Concluding remarks

There have been several previous genomic alterations of the IgH 3’RR, notably through individual enhancer deletions (Manis et al., 1998; Pinaud et al., 2001; Vincent-Fabert et al., 2009; Bébin et al., 2010). Partial functional redundancies of the four 3’RR enhancers have long kept their role quite elusive, especially with regard to SHM. The complete deletion now clarifies that the 3’RR is mandatory for SHM. This major SHM defect paralleled a partly decreased IgH transcription, suggesting that elements beyond primary transcription accessibility are missing for recruitment of the SHM machinery onto the 3’RR-deficient IgH locus. The very low residual SHM suggests that no additional elements cooperate with the 3’RR for SHM. Although transcription is a prerequisite for several regulatory mechanisms controlling key stages in the B cell physiology, it is clearly not sufficient to ensure Ig locus accessibility to AID. The 3’RR-deficient mice will facilitate further elucidation of how the 3’RR “enhanceosome” or the 3’RR-dependent IgH locus 3D-conformation might regulate AID accessibility beyond transcriptional regulation.

MATERIALS AND METHODS

Mice. Our research has been approved by the ethics committee review board of our university (Limoges, France) and hospital (Comité Régional d’Ethique sur l’Expérimentation Animale du Limousin, CHU Dupuytren, Limoges, France). Generation of 3’RR-deficient mice has been previously reported (Vincent-Fabert et al., 2010) and was performed on a C57BL/6 background. Mice were bred and maintained under specific pathogen-free conditions. Age-matched littermates (8–12 wk old) were used in all experiments. C57BL/6 mice were used as WT mice.

Cell cytometry and sorting procedures for SHM experiments. Mouse immunizations were performed orally with sheep red blood cells for 2 wk and intraperitoneally with 10 µg LPS for 3 d. Single-cell suspensions from Peyer’s patches were labeled with B220–APC–, PNA–FITC–, and Fas–PE–conjugated antibodies. Purification of B220+PNA+highFas+ cells was realized on a FACSVantage (BD). These cells were used for SHM analysis of V(D)J-rearranged fragments and Igκ light chain VJ-rearranged fragments and analysis of IgH and κ primary transcripts. Single-cell suspensions from

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splenocytes were labeled with B220-APC– and PNA-FITC–conjugated antibodies for purification of B220+PNAhi B splenocytes. These cells were used for SHM analysis of S.<ref>

DNA extraction and amplification for SHM experiments. Genomic DNA was extracted from sorted B220+PNAhi cells. IgH V(D)J-rearranged fragments were amplified by PCR using the following primers and multistep programs: the forward primer 5'-GGCAGGCTTARCTGGCTG- RCTTCACTGAG-3', complementary to the VHJ588 segment, and the backward primer 5'-AGGCTCTGATCCCTCTTACAGC-3', corresponding to a sequence 171 bp downstream of the JH4 segment using 1 cycle at 98°C for 30 s, 33 cycles (98°C for 10 s, 67°C for 30 s, and 72°C for 90 s), and 1 cycle at 72°C for 10 min. Igk light chain VJ-rearranged fragments were amplified by PCR as previously reported (Xiang and Garrard, 2008) using the following primers and multistep programs: the forward primer 5'-GGCTG- CAGSTTCAGTGGCAGTGGRTCWGGRAC-3' and the backward primer 5'-GGCTGACTTCTGCTCCGTTTGGTG-3'.

Online supplemental material. Fig. S1 shows S<sub>h</sub> sequence and location of primers for S<sub>h</sub> PCR experiments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130072/DC1.

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ChIP. Mice immunizations were performed orally with sheep red blood cells for 2 wk and intraperitoneally with 10 µg LPS for 3 d. CD19 magnetic cell-sorted splenic B cells were used for AID-Chip experiments as previously described (Jeevan-Raj et al., 2011). For S<sub>h</sub> experiments, two primers (UPL 3'J<sub>h</sub> forward, 5'-AGGCGACTTGGGCTCTATG-3' and UPL 3'J<sub>h</sub> reverse, 5'-CTCCAATACGGCCCACACT-3') have been designed and were used in combination with UPL probe#19 and Roche LightCycler 480 Probes Master mix. Splenic B cells were purified by CD43 magnetic cell sorting from nonimmunized mice and cultured in vitro with 10 µg/ml LPS for 3 d and used for Pol II–ChIP experiments as previously described (Tinguely et al., 2012). PCR primers were those used for analysis of IgH primary transcripts (probe located in the intron between the last J<sub>h</sub> and the intronic E<sub>h</sub> enhancer; see sequences above).

These primers were used with Quantitect SYBR Green mix from QIAGEN. For J<sub>h</sub> experiments, two primers (UPL 3'J<sub>h</sub> forward, 5'-AGGCGACTTGGGCTCTATG-3' and UPL 3'J<sub>h</sub> reverse, 5'-CTCCAATACGGCCCACACT-3') have been designed and were used in combination with UPL probe#19 and Roche LightCycler 480 Probes Master mix. Splenic B cells were purified by CD43 magnetic cell sorting from nonimmunized mice and cultured in vitro with 10 µg/ml LPS for 3 d and used for Pol II–ChIP experiments as previously described (Tinguely et al., 2012). PCR primers were those used for analysis of IgH primary transcripts (probe located in the intron between the last J<sub>h</sub> and the intronic E<sub>h</sub> enhancer; see sequences above).

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