*Brief Definitive Report*

# Activation-induced Deaminase (AID)-directed Hypermutation in the Immunoglobulin Sμ Region: Implication of AID Involvement in a Common Step of Class Switch Recombination and Somatic Hypermutation

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## Abstract

Somatic hypermutation (SHM) and class switch recombination (CSR) cause distinct genetic alterations at different regions of immunoglobulin genes in B lymphocytes: point mutations in variable regions and large deletions in S regions, respectively. Yet both depend on activation-induced deaminase (AID), the function of which in the two reactions has been an enigma. Here we report that B cell stimulation which induces CSR but not SHM, leads to AID-dependent accumulation of SHM-like point mutations in the switch μ region, uncoupled with CSR. These findings strongly suggest that AID itself or a single molecule generated by RNA editing function of AID may mediate a common step of SHM and CSR, which is likely to be involved in DNA cleavage.

Key words: B lymphocyte • immunoglobulin gene • heavy chain • DNA cleavage • error-prone repair

## Introduction

*Ig* genes in B lymphocytes undergo three types of genetic alterations during their development, i.e., V(D)J recombination, somatic hypermutation (SHM), and class switch recombination (CSR). V(D)J recombination takes place in developing B lymphocyte precursors and its biochemical mechanism is well characterized (1). In contrast, little is known about the molecular mechanisms of SHM and CSR. Both events occur in activated mature B lymphocytes such as germinal center cells, but outcomes of the events are apparently very different. In SHM, mostly point mutations are introduced in Ig variable (V) region genes, giving rise to Ig with high affinity (2). DNA cleavages are shown to be introduced in the V region during SHM (3–6). On the other hand, in CSR, two switch (S) regions located 5′ to heavy-chain constant ($C_H$) region genes are cleaved and a large DNA fragment between the cleavages is excised out from the chromosome to bring in a downstream Ig $C_H$ region gene to the proximity of a rearranged V gene (7). In addition, neither SHM nor CSR is prerequisite of the other (8, 9). Therefore, it is striking that a defect of AID, a putative RNA editing enzyme, virtually abolishes both SHM and CSR without affecting germinal center formation (10, 11).

To explain this unexpected finding, we have proposed a model that AID edits a precursor mRNA to synthesize an endonuclease essential for generating DNA cleavages in both SHM and CSR reactions (12). However, it remains to be tested whether AID edits separate pre-mRNAs for CSR and SHM, and thus is involved in different steps in the two genetic events. In the present study we provide the evidence that hypermutation takes place in the unrearranged Ig Sμ region under the condition that induces CSR but not SHM in the V region gene. The results imply that CSR and hypermutation may be mediated, at least in part, by the same molecular machinery.

## Materials and Methods

*Mice and B Cell Culture.* Wild-type (wt) and *AID*$^{-/-}$ mice on (CBA × C57BL/6) × C57BL/6 back ground were maintained in our animal facility and used 2–8 mo of age. Spleen B cells were purified by depleting $CD43^+$ cells with the magnetic cell sorting system (MACS; Miltenyi Biotec). The purity checked by B220 staining was 87–95%. Purified B cells were cultured as described (10). After 5–8 d cultivation, live cells were harvested

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and high molecular weight nuclear DNA was extracted with SDS/proteinase K lysis, followed by phenol/chloroform extraction. In some experiments, switched $IgG^+$ cells were enriched (68–90%) or depleted (1.5–6%) by MACS with combination of biotinylated anti-$IgG_1$ and anti–$IgG_3$ antibodies (BD PharMingen) and Streptavidin Microbeads (Miltenyi Biotec). $IgG_1^+$ and $IgG_3^+$ cells constitute the majority of switched population in the culture. Mutation frequencies in each population were determined by sequencing 10,391 and 10,328 bp, respectively, as described in Table I.

*PCR and Sequencing.* PCR were performed with the primers shown below using *Pyrobest* DNA polymerase (TaKaRa) that has the 3′ exonuclease activity and high fidelity. After purification, the PCR fragments were digested with EcoRI or SpeI and ligated into pBluescript vector. The ligation mixture was used for transformation and the library was plated without preculturing to avoid amplification of sister clones. No more than 21 clones were sequenced from a single PCR reaction for the Sμ. Clonality of the V region clones were checked by their CDR3 sequences. Nucleotide sequences were determined with ABI PRISM 3100 genetic analyser (PerkinElmer). The Sμ region germline sequence of CBA and C57BL/6 were determined and compared. 6 and 5 bp polymorphic differences were found in the Sμ and $J_{H4}$ downstream regions of our interest, respectively, and excluded from mutations. Primers used for Sμ PCR are: 5′-GGAATTCATTCCACACAAAGACTCTGGACC-3′; 5′-GGAATTCCAGTCCAGTGTAGGCAGTAGA-3′ (A and D in Fig. 1 A, respectively) with 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min. Primers used for sequencing in addition to common primers for pBluescript are: 5′-GGAATTCG-TAAGGAGGGACCCAGGCTAAG-3′; 5′-GGAATTCTTC-CAGAATCCCAGGATTGCC-3′ (B and C in Fig. 1 A, respectively). The 3′ subregions of nonswitched alleles were amplified by nested PCR as follows: the first step, 20 cycles of 98°C for 10 s, 68°C for 7 min, with the primer A and 3′-AGCCCATGCT-AGCTCAGCCTCACATAA-5′ (3′ of the Sμ core); the second step, 15 cycles of 98°C for 10 s, 68°C for 80 s, with the B and D primers. For $V_{J558}$-$J_{H4}$ downstream region PCR, primers described (13) were used with 35 cycles of 98°C for 10 s, 68°C for 80 s.

*Retrovirus Infection.* Recombinant retrovirus constructs (*pMX-AID-IRES-GFP*) to express AID or $AID^{m-1}$, inactive mutant of AID, and preparation and infection of retroviruses were described before (14). $GFP^+$ cells were sorted by FACSVantage™ (Becton Dickinson).

## Results and Discussion

We reasoned that CSR target *S* regions might receive extensive sequence alterations like SHM even without actual CSR upon stimulation of B cells if SHM and CSR share a common mechanism for DNA cleavage. To assess this possibility, we examined DNA sequences of the Sμ region in splenic B lymphocytes stimulated with LPS and IL-4. To avoid PCR artifacts due to highly G rich repetitive sequences in the Sμ core region (15), we chose to analyze the upstream flanking region to the Sμ core sequence (hereafter called the Sμ region), in which practically frequent CSR can take place (16, 17). We also analyzed such Sμ regions that are located on nonswitched alleles, using nested PCR. We found a significant number of mutations accumulating in the Sμ region of spleen B cells stimulated with LPS and IL-4 but not of unstimulated B cells (Table I). The mutations are located mainly in the 3′ subregion of the Sμ region (Fig. 1 A) in agreement with distribution of CSR junctions (17). The muta-

**Table I.** *Induction of Hypermutation in the Sμ Region upon CSR Stimulation*

| B cells | Stimulation with LPS/IL-4 | Mutations in Sμ region: 5′ subregion | Mutations in Sμ region: 3′ subregion [NS allele] | V region |
|---|---|---|---|---|
| wt | − | 2/22,723 (2/48)[a] | 1/27,071 (1/47)[b] [0/12,718 (0/21)][d] | 4/13,499 (1/27)[e] |
| | + | 6/40,571 (5/87)[a] | 21/47,191 (17/81)[b, c] [10/21,111 (8/35)][d] | 11/18,500 (3/37)[e] |
| *AID*$^{-/-}$ | − | 0/21,244 (0/45) | 0/26,542 (0/46) | 0/3,500 (0/7) |
| | + | 0/27,760 (0/58) | 0/34,036 (0/59)[c] | 0/5,000 (0/10) |

DNA was extracted from purified spleen B cells cultured with or without LPS and IL-4. The Sμ region (1.1 kb) composed of the 5′ and 3′ subregions (0.5 and 0.6 kb, respectively) and $V_{J558}$-$J_{H4}$ downstream region (1.2 kb but 0.5 kb of the $J_{H4}$ 3′ flanking sequence was examined) were amplified, subcloned, and then sequenced. Results of six independent culture experiments are shown together. The switch efficiency to $IgG_1$ and $IgG_3$ in the LPS/IL-4 cultured wt cells was 29–56%. Numbers indicate mutated bases per total bases sequenced in each category. Numbers in the parentheses indicate mutated clones among total clones examined. NS, non-switched. Statistical significance was evaluated by Fisher's exact tests for indicated sets of data. Statistic tests were done with SAS version 2000 software (SAS Institute Inc.).

[a] $P = 0.720$.

[b] $P = 1.22 \times 10^{-3}$.

[c] $P = 1.62 \times 10^{-5}$.

[d] $P = 0.017$.

[e] $P = 0.299$.

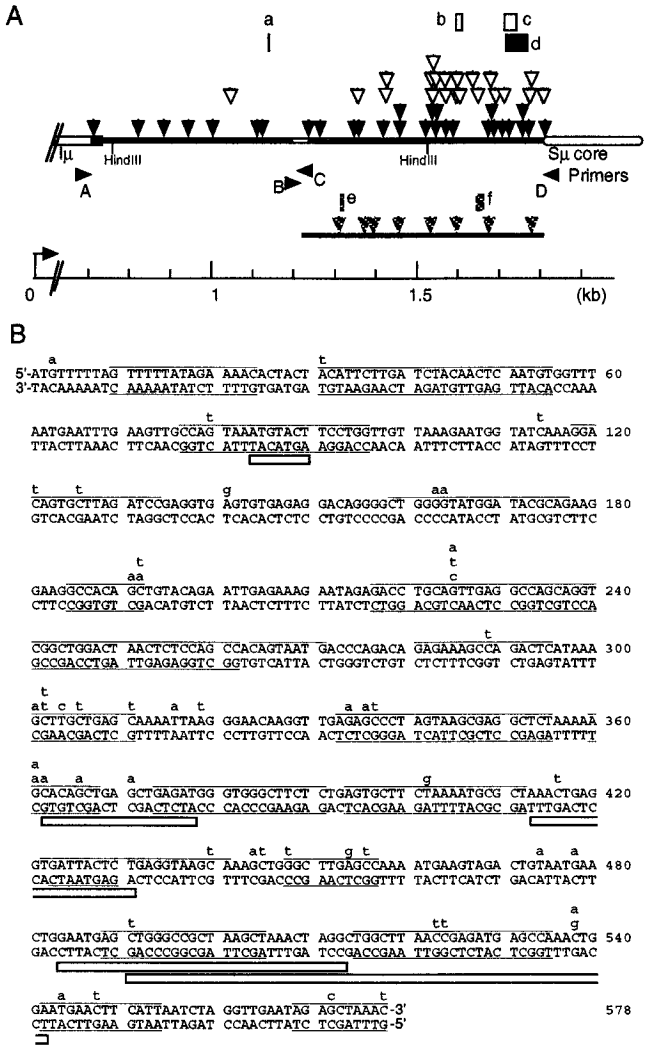


**Figure 1.** Distribution of mutations in the Sμ region. (A) A total of 58 point mutations and 6 deletions shown in Table I (closed and hatched symbols) and Table II (open symbols) are mapped by triangles and rectangles, respectively, on the Sμ region (bar). Hatched symbols represent mutations on the non-switched allele. Nucleotide sequences were determined for closed regions. *Iμ* exon and the Sμ core region are indicated by thicker bars. Position from the transcription start site (arrow) is indicated by a scale below. Primers A–D were used for PCR or sequencing of the Sμ region. Lengths (bp) of deletions (a–f) are: a, one; b, 17; c, 30; d, 52; e, 7; f, 19. (B) Mutations in the 3′ subregion. The Sμ nucleotide sequence of the C57BL/6 mouse tail DNA is shown by upper case letters. 51 point mutations are indicated in lower case letters. The position of five deletions are shown by open bars. Sequences that are predicted to form S/L structures are underlined (references 12 and 34). Conditions used for the S/L prediction were: the ionic conditions (mM), $[Na^+] = 150$, $[Mg^{2+}] = 0.5$; the folding temperature, 37°C; the maximum distance between paired bases, 25.

tion frequency observed was $4.5 \times 10^{-4}$/bp and the fraction of mutated clones reached 21.6% of sequenced clones. These mutations in the Sμ region are independent of CSR because (a) the mutation frequency in Sμ regions on nonswitched alleles is as high as that of total Sμ (Table I), (b) the mutation frequency was not significantly changed between switched and unswitched B cells ($4.8 \times 10^{-4}$/bp and $2.9 \times 10^{-4}$/bp, respectively; $P = 0.58$, Fisher's exact test), and (c) no CSR junctions were included in the Sμ region sequenced although frequent mutations are found in the proximity (most often within 10 bp) of CSR junctions (18, 19). Furthermore, no mutations were found in a non-Ig gene, *c-myc* (total 9,430 bp sequences of 26 clones), excluding the possibility of nonspecific genomewide hypermutation due to DNA damage and repair.

Most importantly, these mutations are not a part of SHM in the V region because LPS and IL-4 stimulation could not induce hypermutation in $V_{J558}$-$J_{H4}$ downstream regions (Table I). The $V_{J558}$ family is shown to constitute the major $V_H$ population in C57BL mice and $V_{J558}$-$J_{H4}$ downstream regions are known to accumulate mutations in in vivo activated B cells (13). The absence of SHM induction by LPS and IL-4 stimulation in vitro is consistent with the previous reports (20–22). A few heavily mutated clones exist in both before and after the stimulation in wt samples, which are likely due to memory cells. Direct comparison of the mutation rate in the Sμ region to that in the V region is not straightforward because unlike SHM this mutation frequency represents unselected clones in in vitro primary cultures. Nonetheless, the V region of a human B lymphoma cell line, Ramos spontaneously accumulates $2.3 \times 10^{-3}$/bp mutations during 2 wk (3), the frequency of which is slightly higher than but comparable to the present data. Occasionally we identified small deletions (Fig. 1 A), which is in agreement with previous reports that internal deletions of S (Sμ and $S\gamma_1$) regions can occur upon CSR induction (23, 24). These data indicate that CSR stimulation of B cells induces wide spread cleavage in Sμ DNA, which can cause point mutations as well as deletions.

To determine if the hypermutation in the Sμ region also depends on AID, we analyzed spleen B cells from $AID^{-/-}$ mice (10) in parallel with those of wt mice. Strik-

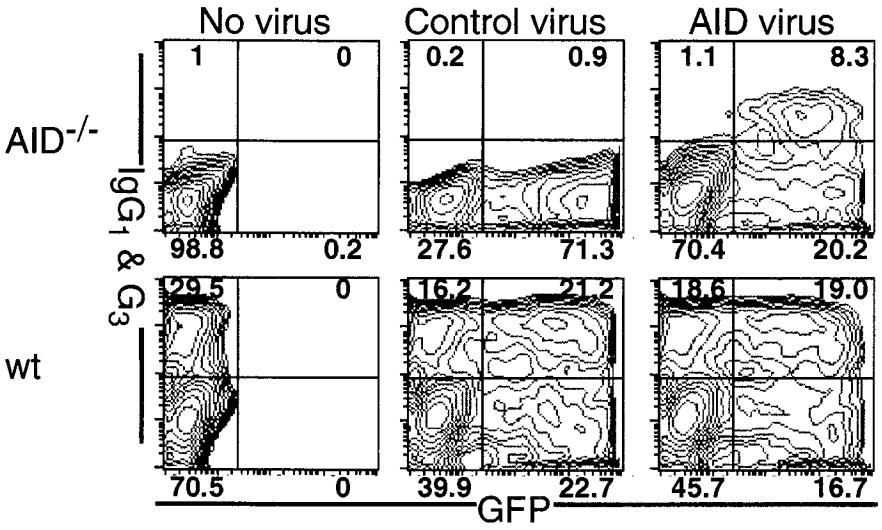


**Figure 2.** Rescue of CSR ability in $AID^{-/-}$ B cells by retrovirus-mediated AID transfection. Purified spleen B cells from $AID^{-/-}$ (reference 10) and wt mice were stimulated with LPS (25 μg/ml) and IL-4 (75 μ/ml). On day 1, the culture were split into three, and two of them were infected with either $AID^{m-1}$ or AID expressing virus. On day 6, cells were harvested and stained with anti-B220, anti-$IgG_1$, anti-$IgG_3$ (BD PharMingen), and propidium iodide (PI). Data were acquired by FACSCalibur™ and analyzed by CELLQuest™ software (Becton Dickinson). $B220^+$ and PI-negative cells were electronically gated and their IgG and GFP expressions are shown. Percentages of the cells in each quadrant are indicated for each panel.

ingly, no mutations were found in both unstimulated and stimulated $AID^{-/-}$ B cells. Altogether the 109.5 kb sequences of the Sμ region were mutation free in $AID^{-/-}$ B cells (Table I). We conclude that the recombination-uncoupled hypermutation in the Sμ region is mediated by the function of AID.

To exclude the possibility that an apparent induction of the hypermutation in the Sμ region represents the outgrowth of the population already with mutations, most likely memory B cells, we transfected $AID^{-/-}$ primary B cells with AID-expressing retroviruses. As $AID^{-/-}$ B cells have no background mutations, population changes in the cell culture would not affect the result. Splenic B cells that had been stimulated with LPS and IL-4 1 d before virus infection were harvested for analysis 5 d after infection. 29% of infected $AID^{-/-}$ cells, which were distinguished by green fluorescent protein (GFP) coexpressed bicistronically with AID, switched to $IgG^+$ whereas only a background level of $IgG^+$ cells was found in uninfected cells (Fig. 2). The switch efficiency of rescued $AID^{-/-}$ cells is comparable to that (29.5%) of wt cells without AID virus infection. By contrast, a control virus carrying a deletion mutant *AID* ($AID^{m-1}$) did not rescue CSR ability of $AID^{-/-}$ cells. Thus, the exogenous AID almost completely rescued CSR ability of $AID^{-/-}$ B cells.

We then analyzed the sequence of the Sμ region in $AID^{-/-}$ B cells infected with AID virus. Virus-uninfected cells were removed by enrichment of $IgG^+$ cells or $GFP^+$ cells by cell sorting. Clearly, only *AID*-virus transfected cells mutated their Sμ region while $AID^{m-1}$-virus

**Table II.** *Retrovirus-mediated AID Transfection Rescues the Hypermutation Phenotype in $AID^{-/-}$ B Cells*

| Cell/virus | Infected cell (%) | Mutation in the Sμ region | | |
|---|---|---|---|---|
| | | 5′ subregion | 3′ subregion | V region |
| $AID^{-/-}$/*AID* | | | | |
| IgG enriched | 76 | 1/3,843 (1/8) | 6/4,624 (3/8)[a] | 1/9,500 (1/19)[c] |
| $GFP^+$ sorted | 91 | 0/7,954 (0/16) | 11/9,206 (6/16)[b] | 0/9,500 (0/19) |
| $AID^{-/-}$/$AID^{m-1}$ | | | | |
| Total | 72 | 0/3,828 (0/8) | 0/4,624 (0/8)[a] | 0/12,500 (0/25)[c] |
| $GFP^+$ sorted | 92 | 0/6,366 (0/13) | 0/7,514 (0/13)[b] | 0/4,000 (0/8) |
| *wt*/*AID* | | | | |
| $GFP^+$ sorted | 88 | 0/3,493 (0/7) | 5/4,046 (3/7) | 2/3,500 (1/7) |

Cells were infected and selected as indicated. The percentage of virus-infected cells within the selected populations was measured by expression of GFP coexpressed with AID. Data were obtained and presented as in Table I.
[a] $P = 0.031$ (Fisher's exact test).
[b] $P = 0.00156$ (Fisher's exact test).
[c] $P = 0.432$ (Fisher's exact test).

**Table III.** *Mutation Bias to RGYW/WRCY and S/L Structure*

| | Association with | | | |
|---|---|---|---|---|
| | RGYW/WRCY | | Stem and loop[a] | |
| | + | − | + | − |
| Mutated sequences | 32[b] | 19[b] | 73[c] | 29[c] |
| Total sequences | 212 | 366 | 618 | 538 |

Mutations in the 3′ subregion were analyzed for association with sequence and structural motifs. Mutations which associate or do not associate with the RGYW/WRCY motif were counted as (+) and (−), respectively. S/L structure was predicted by a computer program that is developed by Zuker as described and shown in Fig. 1 B.

[a]The two strands were analyzed separately for association with the S/L structure and the results are combined.

[b]$P = 2.5 \times 10^{-4}$.

[c]$P = 4.26 \times 10^{-4}$ ($\chi^2$ test).

infected cells did not (Table II). The mutation frequencies of $IgG^+$ and $GFP^+$ cells were $1.2 \times 10^{-3}$ and $1.3 \times 10^{-3}$ per bp, respectively, which are almost the same as the frequency in wt cells infected with AID virus ($1.2 \times 10^{-3}$/bp). Almost all mutations are found in the 3′ subregion in consistence with virus non-infected wt cells (Table I, Fig. 1 A). Again, practically no mutations were observed in $V_{J558}$-$J_{H4}$ downstream regions in *AID*$^{-/-}$ or wt B cells infected with AID virus (Table II). These results indicate that the mutations of the Sμ region are introduced de novo upon CSR stimulation without SHM in the V region, and absolutely dependent on AID, implicating that both recombination and hypermutation in S regions may be catalyzed by a certain common reaction regulated by AID.

If so, the Sμ hypermutation may be biased to stem and loop (S/L) structures as shown for CSR cleavage sites (12, 18, 25). Therefore, we mapped the hypermutation sites to the predicted S/L structures on both strands of the Sμ region, and found significant bias of the mutation to the S/L structures (Table III, Fig. 1 B). Note that the majority of mutations coupled with CSR are located in the proximity (within 10 bp) of the CSR junctions but do not necessarily coincide with cleavage sites (18). This is because mutations are probably introduced during error prone DNA synthesis to repair cleavages. Accordingly, the present level of the coincidence between Sμ mutations and S/L structures well supports the assumption that both CSR and Sμ mutations may be mediated by an enzyme recognizing S/L structures.

Sμ region hypermutation shares important features with V region SHM: AID dependency, high frequency point mutations with occasional deletions, and mutations biased to transition (64%) and to the RGYW/WRCY motif (R, purine; Y, pyrimidine; W, A/T; Table III) (2). It is therefore reasonable to assume that either a similar mechanism activated by AID or AID itself is responsible for hypermutations in the Sμ as well as V region. It has been shown that the defect in the non-homologous end-joining (NHEJ) system affects CSR but not SHM (26–29), and that *AID*$^{-/-}$ mice show no obvious abnormality in the NHEJ system (10), indicating that CSR and SHM differ in the repair mechanism. These results taken together suggest that a common step of CSR and SHM, most likely at DNA cleavage rather than DNA repair, may be regulated by AID. It is well established that CSR and SHM are independent events in B cells (8, 9). In fact, under the present conditions both CSR and hypermutation in *S* regions occur concurrently without SHM in the V region. There must be another level of regulation to distinguish the targets of the AID-activated system.

As AID deficiency does not affect germline transcription and NHEJ repair, AID is likely to be involved in cleavage of the S region during CSR (10). This conclusion is confirmed by the recent finding that accumulation of γH2AX and Nbs1 at double strand breakages in the IgH locus during CSR is dependent on AID (30). The present finding suggests that CSR and SHM are likely to be mediated by the same enzyme. It is therefore less likely that AID edits separate pre-mRNAs for CSR and SHM. These results taken together argue for, but do not prove, the hypothesis that AID edits a precursor mRNA to synthesize an endonuclease essential for generating DNA cleavage in both SHM and CSR reactions (12). Alternatively, AID itself may have DNA attaching activity although we consider it less likely because (a) AID does not bind to double-stranded or single-stranded Sμ sequences (data not shown) and (b) AID is not associated with the Sμ core region by chromatin immunoprecipitation assay of CSR-induced B cell chromatin (data not shown).

We have further proposed that the endonuclease introduces nicks by recognizing secondary structures such as stem and S/L in S and V regions, which are formed transiently by transcription-promoted strand separation (12). Nicking in the V region will be repaired by exonuclease and error-prone DNA synthesis (31–33), followed by mismatch repair and ligation. By contrast, in S regions frequent nicking generates staggered cleavages (18) which are also repaired by exonuclease and error-prone DNA synthesis, followed by the NHEJ repair system (26–28). Identification of AID target will be able to discriminate these possibilities.

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