Activation-induced Deaminase (AID)-directed
Hypermutation in the Immunoglobulin S\(\mu\) 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-
directed 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-depen-
dent accumulation of SHM-like point mutations in the switch \(\mu\)/H9262 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 recombi-
nation, somatic hypermutation (SHM), and class switch re-
combination (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 lympho-
cytes 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 lo-
cated 5’ to heavy-chain constant (CH) region genes are
cleaved and a large DNA fragment between the cleavages is
excised out from the chromosome to bring in a down-
stream Ig CH region gene to the proximity of a rearranged
V gene (7). In addition, neither SHM nor CSR is prerequi-
site of the other (8, 9). Therefore, it is striking that a defect
of AID, a putative RNA editing enzyme, virtually abol-
ishes both SHM and CSR without affecting germinal cen-
ter 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 S\(\mu\) 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 \(\times\) C57BL/6) \(\times\) C57BL/6 back ground were main-
tained 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
and high molecular weight nuclear DNA was extracted with SDS/protease 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, and anti-IgG, antibodies (BD PharMingen) and Streptavidin Microbeads (Miltenyi Biotec). IgG1+ and IgG2+ cells constitute the majority of switched population in the culture. Mutation frequencies in each population were determined and excluded from mutations. Primers used for S region were sequenced from a single PCR reaction for the S region. Nucleotide sequences were determined with ABI 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 3' end of C57BL/6 germline sequence of CBA and C57BL/6 were determined and excluded from mutations. Primers used for Sμ PCR are: 5'-GGAATTCATTCCACACAAAGACTCTGGACC-3' and 5'-J558 downstream region (1 kb but 0.5 kb of the J558 flanking sequence was examined) were amplified, supplemented, and then sequenced. Results of six independent culture experiments are shown together. The switch efficiency to IgG 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.).

### 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          | 3' subregion [NS allele] | V region |
| wt      | −                         | 2/22,723 (2/48)       | 1/27,071 (1/47)         | 4/13,499 (1/27) |
|         |                           | [0/12,718 (0/21)]     | [11/18,500 (3/37)]     |         |
| +       | 6/40,571 (5/87)           | 21/47,191 (17/81)     | 11/18,500 (3/37)       |         |
|         |                           | [10/21,111 (8/35)]    | [10/21,111 (8/35)]     |         |
| 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)        | 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 Vμ downstream region (1.2 kb but 0.5 kb of the Jμ flanking sequence was examined) were amplified, subcloned, and then sequenced. Results of six independent culture experiments are shown together. The switch efficiency to IgG1 and IgG3 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.).

$^aP = 0.720.$

$^bP = 1.22 \times 10^{-3}.$

$^cP = 1.62 \times 10^{-5}.$

$^dP = 0.017.$

$^eP = 0.299.$

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Fraction 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 non-specific 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 VJ558-JH4 downstream regions (Table I). The VJ588 family is shown to constitute the major VH population in C57BL mice and VJ588-JH4 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 Sγ) 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-
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. 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 IgG1, whereas only a background level of IgG1 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 (AIDm-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 IgG1 cells or GFP− cells by cell sorting. Clearly, only AID−virus transfected cells mutated their Sμ region while AIDm-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−/−/AIDm-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).
developed by Zuker as described and shown in Fig. 1 B. Accordingly, the present level of the S region during CSR (10) is likely to be involved in cleavage of the S region during CSR. 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).

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 proximity of 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 (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).

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

| Association with | RGYW/WRCY | Stem and loop* |
|------------------|-----------|----------------|
| +                | -         | +              | -               |

| Mutated sequences | 32b       | 19b          | 73c          | 29c       |
|-------------------|-----------|--------------|--------------|-----------|
| Total sequences   | 212       | 366          | 618          | 538       |

*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 \( P = 4.26 \times 10^{-4} \) (χ² test).

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.

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 is 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 VJ558-JH4 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.

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).

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