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Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme

Masamichi Muramatsu,* Kazuo Kinoshita,* Sidonia Fagarasan,* Shuichi Yamada,† Yoichi Shinkai,† and Tasuku Honjo*‡
*Department of Medical Chemistry
Graduate School of Medicine
†Department of Cell Biology
Institute for Virus Research
Kyoto University
Kyoto 606-8501
Japan

Summary

Induced overexpression of AID in CH12F3-2 B lymphoma cells augmented class switching from IgM to IgA without cytokine stimulation. AID deficiency caused a complete defect in class switching and showed a hyper-IgM phenotype with enlarged germinal centers containing strongly activated B cells before or after immunization. *AID*$^{-/-}$ spleen cells stimulated in vitro with LPS and cytokines failed to undergo class switch recombination although they expressed germline transcripts. Immunization of *AID*$^{-/-}$ chimera with 4-hydroxy-3-nitrophenylacetyl (NP) chicken γ-globulin induced neither accumulation of mutations in the NP-specific variable region gene nor class switching. These results suggest that AID may be involved in regulation or catalysis of the DNA modification step of both class switching and somatic hypermutation.

Introduction

Alteration of genetic information is essential not only for evolution but also for regulation of various physiological functions in living organisms. Accidental repair-mediated alteration aside, there are several systemic strategies for living organisms to modify their genetic information. Among these, the best characterized and most widely distributed is the homologous recombination, which depends on the sequence homology between target DNA regions, and is known to take place often during meiosis, as well as in somatic cells (Sonoda et al., 1999; reviewed by Haber, 1999). A second type of genetic modification is mediated by site-specific recombination which requires defined sequences recognized by specific recombinases. The immune system utilizes this mechanism to generate antigen recognition diversity. VDJ recombination assembles the variable (V), diversity (D), and joining (J) segments to form a V exon of the antigen receptor genes in the early phase of lymphocyte differentiation and is mediated by two proteins, RAG-1 and RAG-2, which bind to and cleave the specific recognition sequence of DNA. Subsequently, cleaved ends are joined together by the end joining repair system used in general recombination machinery (reviewed by Gellert, 1997; Oettinger, 1999).

‡To whom correspondence should be addressed (e-mail: honjo@mfour.med.kyoto-u.ac.jp).

Third, RNA editing creates new functional mRNAs from the restricted genome in plants and protozoa (reviewed by Scott, 1995). Mammalian cells are also known to edit an increasing number of mRNAs including those for apolipoprotein (apo) B, glutamate receptors, Wilms tumor-1, α-galactosidase, and neurofibromatosis type-1 (reviewed by Smith and Sowden, 1996). A well-documented mammalian RNA editing is apoB mRNA editing by a cytidine deaminase, APOBEC-1, together with its accessory molecule (ACF), which modifies C to U at a specific site, and generates apoB100 and apoB48 proteins from unedited and edited mRNA, respectively, with different physiological functions (Teng et al., 1993; Mehta et al., 2000).

There are two additional types of genetic alteration in the immune system, namely somatic hypermutation and class switch recombination (CSR) of the immunoglobulin (Ig) gene which take place in a highly specialized microenvironment called the germinal center (Jacob et al., 1991; Liu et al., 1996). Somatic hypermutation accumulates massive point mutations in the V exon and gives rise to high-affinity antibodies for a given antigen in a process called affinity maturation, in which B cells expressing high affinity Ig on their surface are selected by limited amounts of antigens. Mutations are introduced at a high frequency (10^{-3}–10^{-4} per base division) at a defined region between the V_H promoter and the intronic enhancer (reviewed by Wagner and Neuberger, 1996). Studies using transgenic mice revealed that hypermutation does not depend on the defined sequence but on transcription of the target locus (Peters and Storb, 1996; Tumas-Brundage and Manser, 1997; Fukita et al., 1998). Although involvement of mismatch repair enzymes has been suggested, there are conflicting interpretations (reviewed by Reynaud et al., 1999; Wabl et al., 1999). Since fairly large deletion was also found in V regions of the stimulated B cells, the double-strand DNA cleavage appears to be involved in hypermutation (Goossens et al., 1998; Wilson et al., 1998).

CSR replaces the Ig heavy chain constant region (C_H) gene to be expressed from Cμ to other C_H genes, resulting in switch of the Ig isotype from IgM to either IgG, IgE, or IgA. Each isotype determines the manner whereby captured antigens are eliminated or the location where Ig is delivered and accumulated. The Ig C_H locus consists of the ordered array of C_H genes, each flanked at its 5′ region by an S region which is composed of tandem repetitive unit sequences with many palindromes (reviewed by Zhang et al., 1995). CSR takes place between two broad areas surrounding S regions, resulting in looped-out deletion of intervening DNA segments (Iwasato et al., 1990; Matsuoka et al., 1990; von Schwedler et al., 1990). Since the Cμ gene is located at the V_H proximal end of the C_H gene cluster, CSR between Sμ and another S region 5′ to a C_H gene brings that particular C_H gene adjacent to the V_H exon. Target specificity of CSR is not determined by the primary sequence of the S region (Kinoshita et al., 1998), but rather by chromatin opening (accessibility) of the target region, which is regulated by cytokine-induced transcription from the specific I promoter located 5′ to each S region (Stavnezer and Sirlin, 1986; Yancopoulos et al., 1986).

Synthesis of so-called germline transcripts by this transcription and subsequent splicing from an I exon to its downstream C_H exons is required to precede CSR (Gu et al., 1993; Jung et al., 1993; Xu et al., 1993; Hein et al., 1998). Since the end-joining repair system is involved in CSR (Rolink et al., 1996; Casellas et al., 1998; Manis et al., 1998), double strand cleavage of S regions appears to be required for CSR. CSR is distinct from homologous recombination because there are generally no homologous sequences at recombination breakpoints (Nikaido et al., 1982; Dunnick et al., 1993; Lee et al., 1998) and Rad54 deficiency does not affect CSR (Essers et al., 1997). Taken together, two critical pieces of information are commonly missing for the molecular mechanism of hypermutation and CSR; DNA cleaving enzyme and its recognition target.

To understand the molecular mechanism of CSR, we have developed an in vitro assay system using artificial DNA constructs and murine lymphoma cells (CH12F3-2), more than 80% of which switch from IgM^+ almost exclusively to IgA^+ in one week after stimulation with CD40L, IL-4, and TGFβ (Nakamura et al., 1996). DNA constructs introduced into CH12F3-2 cells contain Sμ and Sα regions that are constitutively transcribed by separate promoters. CSR takes place in the transgene as well as the endogenous Ig locus only when the transfected CH12F3-2 cells are stimulated with CD40L, IL-4, and TGFβ (Kinoshita et al., 1998). Since the transgene locus is constitutively transcribed and thus accessible to a putative CSR recombinase, we assumed that the stimulation requirement for transgene CSR could be due to induction of a new protein, most likely CSR recombinase. This assumption was partially supported by CSR inhibition with the addition of cycloheximide to culture media of CH12F3-2 cells (Muramatsu et al., 1999). We therefore carried out cDNA subtraction between stimulated and nonstimulated CH12F3-2 cells, and identified a novel member of the RNA editing cytidine deaminase family, activation-induced cytidine deaminase (AID) which is specifically expressed in germinal center B cells (Muramatsu et al., 1999). AID is also induced in spleen B cells by in vitro stimulation with IL-4 and lipopolysaccharide (LPS) or CD40L.

In the present study, we show that induced overexpression of AID in CH12F3-2 cells augmented class switching from IgM to IgA without cytokine stimulation. We also demonstrate that the AID deficiency completely blocked CSR in B cells although they are activated by LPS in vitro as well as antigens in vivo. To our surprise, somatic hypermutation was also abrogated in immunized *AID*$^{-/-}$ B cells, indicating that class switching and hypermutation depend on a common molecular mechanism at least in part.

Results

Stimulation of Class Switching by AID Induction in CH12F3-2 B Cells

To examine whether AID is involved in CSR, we transfected CH12F3-2 cells with the *AID* cDNA directed by an inducible tetracycline (tet) promoter. Three independent transfectants were examined for stimulation of class switching upon AID induction. We first confirmed that *AID* mRNA was in fact induced in the transfectants by the removal of tet (Figure 1A). Endogenous *AID* mRNA was detectable only by RT-PCR without stimulation (data not shown). The transfectants were stimulated to switch from IgM to IgA with the suboptimal concentrations of CD40L, IL-4, and TGFβ (Figure 1B, right). Under these conditions, AID induction by the removal of tet enhanced class switching of CH12F3-2 cells 2–5 fold. It is worth noting that AID expression alone induced class switching significantly, albeit weakly, in CH12F3-2 cells (Figure 1B, left). These results provide gain-of-function evidence for the involvement of AID in class switching.

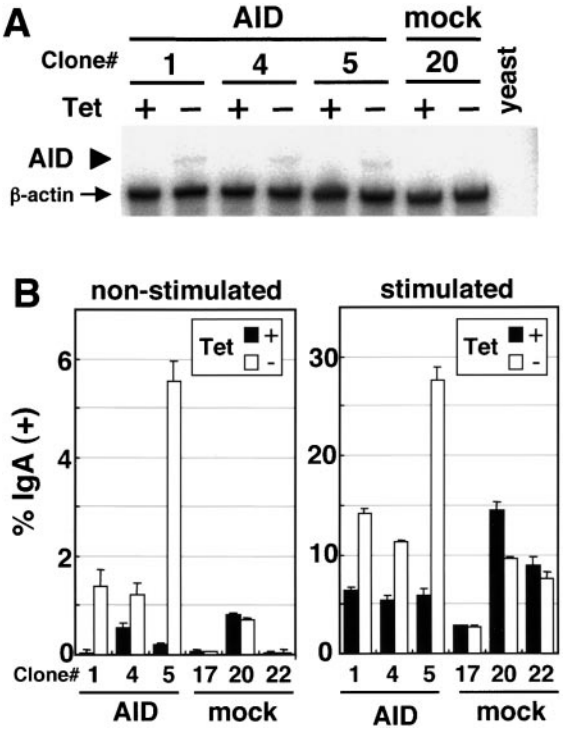

Figure 1. Induced Expression of AID in CH12F3-2 Cells

Clone numbers indicate independent transfectants with AID-expressing vector (1, 4, and 5) or mock vector (17, 20, and 22).

(A) RNase protection assay using 5 μg of total RNA from indicated transfectants cultured in the presence (+) or absence (−) of tet. The same amount of yeast RNA was used as control.

(B) Frequencies of IgA^+ cells were measured by FACS before (non-stimulated) and 24 hr (stimulated) after stimulation. Experiment was performed in triplicate and mean values (bars) with standard deviations are shown. All transfectants expressed similar levels of germline Cα transcripts. Similar results were obtained from at least three independent experiments.

Generation of AID-Deficient Mice

To confirm involvement of AID in class switching in vivo, we have disrupted the gene encoding AID in a TT2 ES cell line using a target vector shown in Figure 2A. The targeting vector was introduced into the ES line, and clones with homologous integration of the construct were selected by PCR screening. Southern blot hybridization confirmed a homologous integration of the target vector in ES cells (Figure 2B), which is expected to replace exon 2 and the 5′ half of exon 3 (encoding the cytidine deaminase motif) of the *AID* gene with the neomycin resistance (Neo^R) gene. The heterozygously

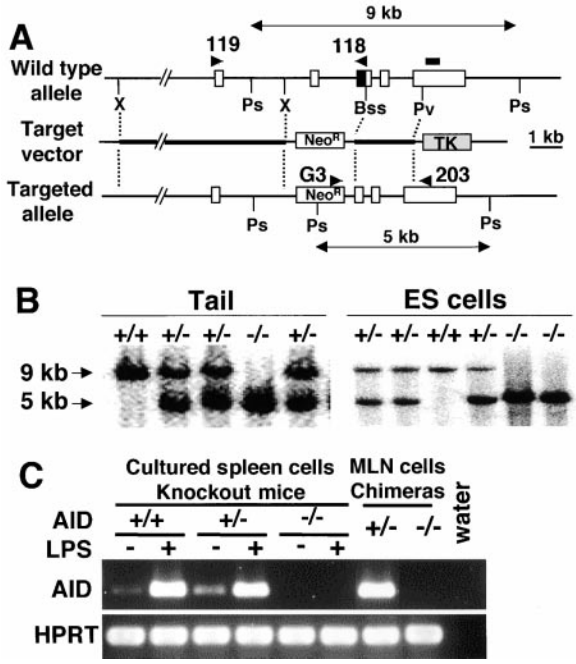

Figure 2. Generation of AID-Deficient Mice and *AID*$^{-/-}$ Chimera

(A) Schematic representation of the wild type and mutant genomic *AID* loci together with the target vector. Restriction enzyme sites (X, XhoI; Ps, PstI; Bss, BssHII; and Pv, PvuI), the probe for Southern blot analysis (solid bar), and primer sites (arrowheads) are indicated. PstI fragments detected by the probe are shown for wild-type and targeted alleles. Exons are represented by open boxes. Cytidine deaminase motif regions in exon 3 are shown by a closed box. TK, thymidine kinase.

(B) Southern blot analysis of PstI-digested DNA prepared from wild-type, single, and double knockout ES cells (right panel) or tails of progenies by mating a heterozygote pair (left side).

(C) Absence of *AID* transcripts in AID-deficient B cells. Spleen cells from *AID*$^{+/+}$, *AID*$^{+/-}$, and *AID*$^{-/-}$ mice were cultured with LPS for two days. Deletion of cytidine deaminase motif sequence in *AID* transcripts was examined by RT-PCR with primer sets (119 and 118). RNAs from MLN of *AID*$^{+/-}$ or *AID*$^{-/-}$ chimera were also examined.

AID-disrupted ES cells were injected into ICR 8-cell embryos. Founder animals were crossed into C57BL/6 background and mated to generate *AID*$^{-/-}$ mice. Figure 2B shows the *AID* genotypes of progenies from one heterozygote mating. Some of the ES cells were cultured in the presence of high concentrations of G418 to facilitate homozygous disruption of the *AID* gene. *AID*$^{-/-}$ ES cells were injected into *RAG-2*$^{-/-}$ 8-cell embryos, giving rise to chimeric animals in which any mature lymphocytes must be derived from the injected ES cells because RAG-2 deficient mice fail to rearrange the antigen receptor genes and thus have no mature T and B cells (Chen et al., 1993). We have confirmed that normal *AID* transcripts are absent in the homozygous mutant of the *AID* gene by RT-PCR (Figure 2C). Spleen cells of *AID*$^{-/-}$ mice stimulated in vitro with LPS and mesenteric lymph node (MLN) cells of *AID*$^{-/-}$ chimera were completely depleted of *AID* mRNA expression whereas *AID*$^{+/-}$ lymphocytes expressed *AID* mRNA as reported previously (Muramatsu et al., 1999). We studied both *AID*$^{-/-}$ chimera and *AID*$^{-/-}$ mice and they showed similar phenotypes as far as examined.

The Absence of Class Switching in AID-Deficient B Cells

The most obvious sign of class switching defect would appear in the serum Ig levels even without active immunization. Serum levels of IgM, IgG, and IgA in *AID*$^{-/-}$ mice were quantitated at 10 weeks of age. As shown in Figure 3A, there were no detectable levels of IgG3, IgG2b, and IgA in *AID*$^{-/-}$. Serum IgG1 and IgG2a levels in these mice were less than one tenth of those in *AID*$^{+/-}$. Similar levels of IgG1 and IgG2a are also found in AID$^{-/-}$ chimera and these residual IgGs are most likely to be derived from maternal blood but not from *AID*$^{-/-}$ B cells because (1) IgG1 levels in *AID*$^{-/-}$ chimera decreased to one-tenth in a 5 week interval between 7 and 12 weeks and (2) IgG1 molecules detected in *AID*$^{-/-}$ chimera and its ICR mother have the same allotype a, while IgG1 from wild-type TT2 ES cell-derived B cells have a/b allotypes as detected by allotype-specific monoclonal antibodies (data not shown). On the other hand, serum IgM levels of *AID*$^{-/-}$ were slightly (2–3 fold) elevated as compared with those of *AID*$^{+/-}$ mice.

To examine whether AID deficiency in lymphocytes causes defects of Ig production upon antigen stimulation, we immunized *AID*$^{-/-}$ chimera by two intraperitoneal injections of sheep red blood cells (SRBC) with a 14 day interval. Serum Ig levels specific to SRBC were measured by ELISA at various time points. As shown in Figure 3B, *AID*$^{-/-}$ chimera showed no IgG1 antibody production against SRBC, whereas significant amounts of SRBC-specific IgG1 antibodies were produced in *AID*$^{+/-}$ chimera. In contrast, SRBC specific IgM levels were comparable between *AID*$^{-/-}$ and *AID*$^{+/-}$ chimeras. These results suggest that *AID*$^{-/-}$ B cells have a severe defect in class switching although they can produce antigen-specific IgM in response to T-dependent antigens (SRBC).

Normal Development and Hyperactivation of AID-Deficient IgM$^+$ B Cells

FACS analysis of bone marrow, thymus, and peritoneal cavity of *AID*$^{-/-}$ mice as well as *AID*$^{-/-}$ chimera showed no obvious difference of B, T, and other hematopoietic cells as compared with *AID*$^{+/-}$ mice (data not shown). Peripheral, mature *AID*$^{-/-}$ B cells expressed both IgM and IgD (data not shown). When lymphocytes from Peyer's patches, MLN, and spleen were stained for surface Ig expression, we found no IgG$^+$ B cells in *AID*$^{-/-}$ chimera before or after immunization, while the number of IgG$^+$ B cells increased in *AID*$^{+/-}$ chimera after SRBC immunization (Figure 4 and data not shown). By contrast, surface expression of IgM was higher in *AID*$^{-/-}$ than *AID*$^{+/-}$ chimera, particularly in Peyer's patches and MLN either before or after immunization (Figure 4). Moreover, the surface expression of several activation markers, such as MHC class II, CD69, or CD86 was higher in *AID*$^{-/-}$ than *AID*$^{+/-}$ chimera regardless of SRBC immunization (Figure 4 and data not shown). Similarly, Peyer's patches, MLN, and spleen of *AID*$^{-/-}$ chimera contained more B cells expressing peanut agglutinin (PNA) receptor, another activation marker specific to germinal centers, before and after SRBC immunization. In contrast, PNA$^+$ B cells were very few in inguinal lymph nodes (ILN) of *AID*$^{-/-}$ and *AID*$^{+/-}$ chimera even after immunization (Figure 4). These results indicate that *AID*$^{-/-}$ IgM$^+$ B cells develop normally, are activated more strongly, and accumulate more after antigenic stimulation than *AID*$^{+/-}$

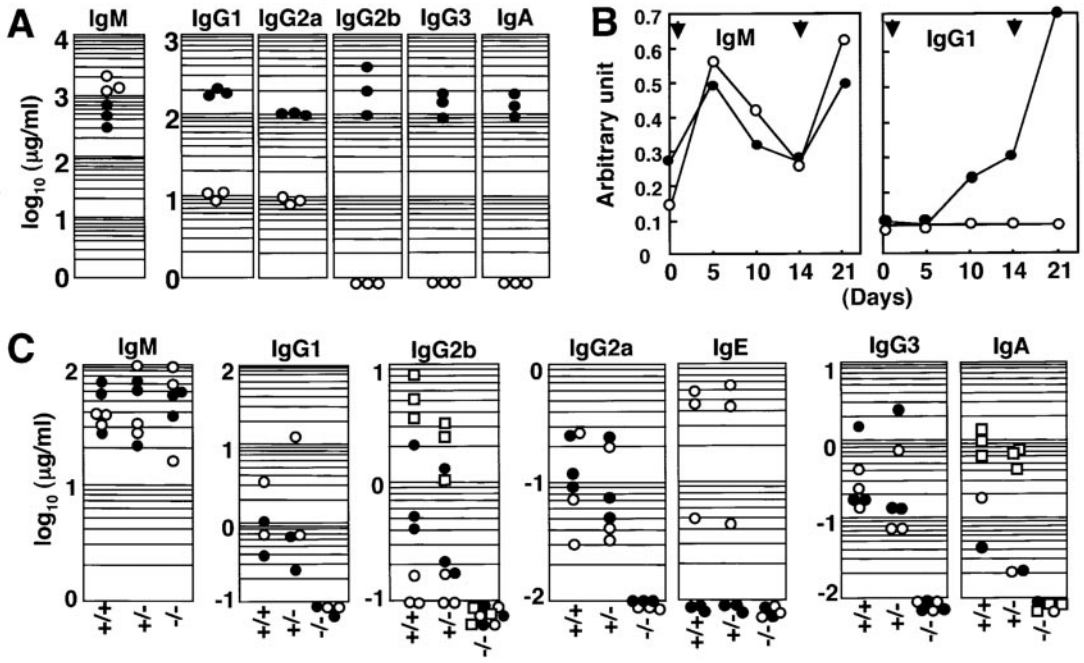

Figure 3. Absence of Class Switching in AID-Deficient B Cells

(A) Sera from 10-week old *AID*$^{-/-}$ (open circle) and age-matched *AID*$^{+/-}$ (closed circle) mice were collected and their Ig levels were determined by ELISA. Ig levels less than 0.01 μg/ml were plotted below the base lines.

(B) *AID*$^{-/-}$ (open circle) or *AID*$^{+/-}$ (closed circle) chimeras were immunized with 1 × 10^8 SRBC on day 0 and 14 (arrows). Anti-SRBC IgM or IgG1 serum levels were measured at day 0, 5, 10, 14, and 21. Arbitrary unit is absorbance at OD_{405}nm. Similar results were obtained from two independent experiments.

(C) Ig levels of culture supernatants of spleen cells from 5-week old littermates (*AID*$^{+/+}$, *AID*$^{+/-}$, and *AID*$^{-/-}$) mice were measured after culture for 7 days in the presence of LPS (closed circle), LPS and IL-4 (open circle), or LPS and TGFβ (open square). Ig levels less than 0.01 μg/ml were plotted below the base lines.

IgM$^+$ B cells, although AID-deficient B cells have the defect in class switching.

Immunohistochemical analyses of spleen in nonimmunized or immunized *AID*$^{-/-}$ chimera indicate the presence of large numbers of IgM$^+$PNA$^+$-activated B cells, widely spread in lymphoid follicles, which themselves expand enormously (Figures 5A–5F). The formation of germinal centers was clear and the structure appeared to be retained with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressing marginal sinus; follicular dendritic cells in *AID*$^{-/-}$ chimera spleen were diffusely spread in lymphoid follicles in parallel with enlargement of germinal centers (Figures 5G, 5H, 5K, and 5L). As expected, IgG$^+$ B cells or plasma cells were not detected in spleen of *AID*$^{-/-}$ chimera, whereas those of *AID*$^{+/-}$ chimera were clearly observed (Figures 5I and 5J). These immunohistochemical examinations together with FACS analysis showed enhanced germinal center formation and hyperactivated features of IgM$^+$ B cells in spleen of nonimmunized as well as immunized *AID*$^{-/-}$ chimeras consistent with their elevated serum IgM levels.

Defect of Class Switch Recombination in AID-Deficient B Cells Stimulated In Vitro

To further confirm that the absence of Ig class switching in AID-deficient B cells is due to the defect of the CSR machinery and not B cell activation, we analyzed spleen cells of *AID*$^{-/-}$ mice after stimulation with LPS and cytokines. First, culture supernatants of *AID*$^{-/-}$ spleen cells stimulated with LPS in the presence or absence of IL-4 were analyzed for their contents of Ig by ELISA. *AID*$^{-/-}$ supernatants contained comparable amounts of IgM with those of littermates (*AID*$^{+/+}$ and *AID*$^{+/-}$) but did not contain any other isotypes of Ig including IgG1, IgG2a, IgG2b, IgG3, IgE, and IgA (Figure 3C). The absence of class switching to IgG2b and IgA persisted even when the cytokine was changed to TGFβ. On the other hand, supernatants of *AID*$^{+/+}$ and *AID*$^{+/-}$ spleen cells stimulated under the same condition contained comparable levels of IgM, IgG, IgE, and IgA, which varied depending on the presence or absence of cytokines. The absence of class switching was also confirmed by FACS analysis (data not shown).

The defect of CSR was also demonstrated by the absence of class-specific mRNA as well as DNA rearrangement of the Ig locus. Since the Iμ promoter is active in all mature B cells, including those after class switching, transcripts composed of the Iμ exon spliced onto the 5′ exon of a C_H gene other than Cμ are generated after switching to that particular C_H gene (Li et al., 1994). As shown in Figure 6A, *AID*$^{-/-}$ spleen cells stimulated with LPS in the presence or absence of IL-4 or TGFβ did not express any of Iμ-Cγ_1, Iμ-Cγ_{2a}, Iμ-Cγ_{2b}, Iμ-Cγ_3, Iμ-Cε, or Iμ-Cα transcripts, demonstrating that AID deficiency resulted in the complete absence of class switching. Control spleen cells of *AID*$^{+/+}$ or *AID*$^{+/-}$ mice generated expected classes of Iμ-C_H transcripts, depending on the presence or absence of the cytokines.

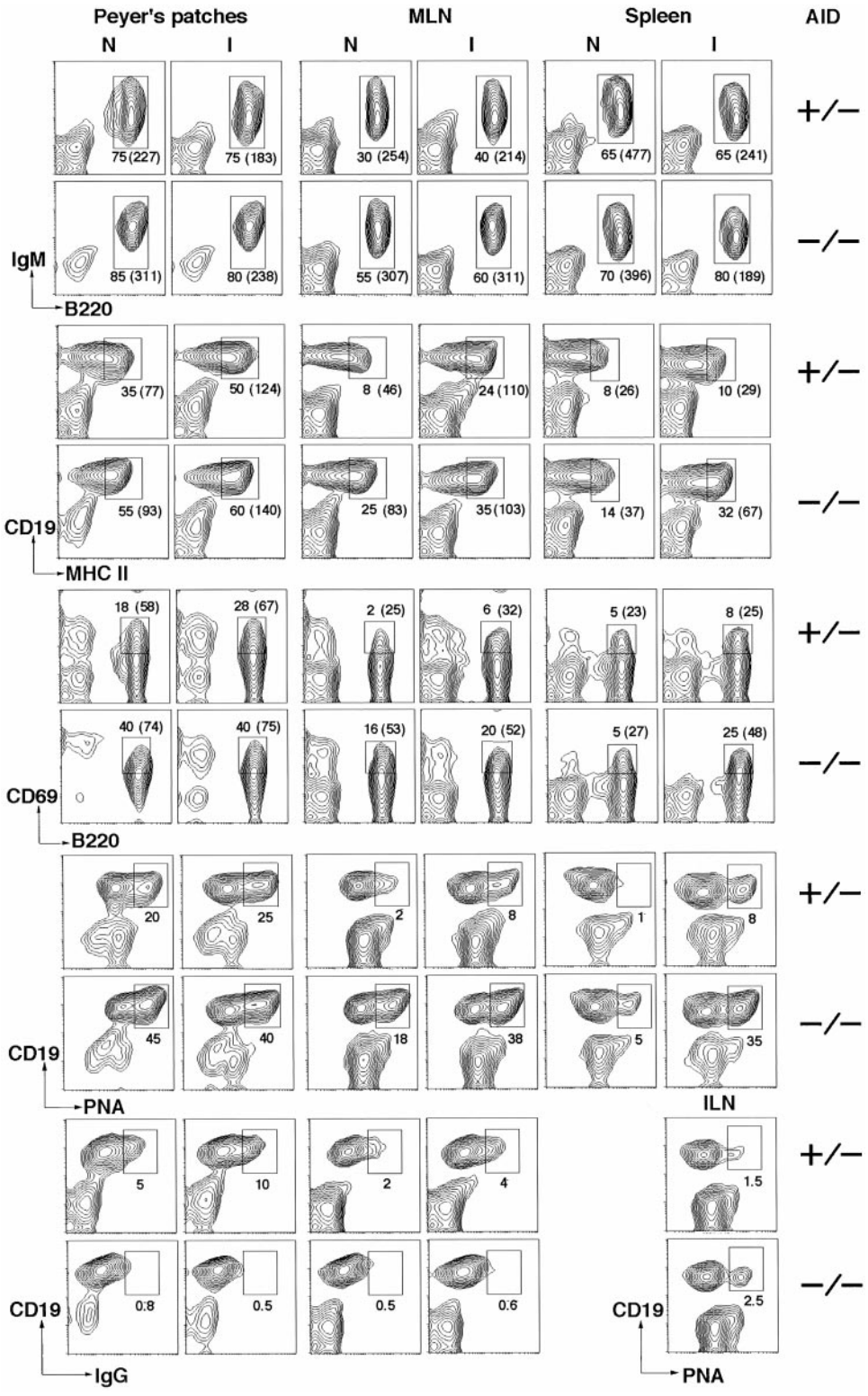

Figure 4. FACS Analysis of *AID*$^{-/-}$ Chimera

Flow cytometric analysis of Peyer's patch, MLN, ILN, and spleen cells derived from nonimmunized (N) and immunized (I) *AID*$^{-/-}$ and *AID*$^{+/-}$ chimeras. Immunization was done as described in Figure 3B. 2×10^4 cells were analyzed for expression of B220 or CD19 in combination with IgM, IgG, CD69, MHC class II, or PNA. Percentages of gated cells are shown. Mean fluorescence intensities (MFIs) of IgM, CD69, and MHC class II of total B cells are shown in parentheses.

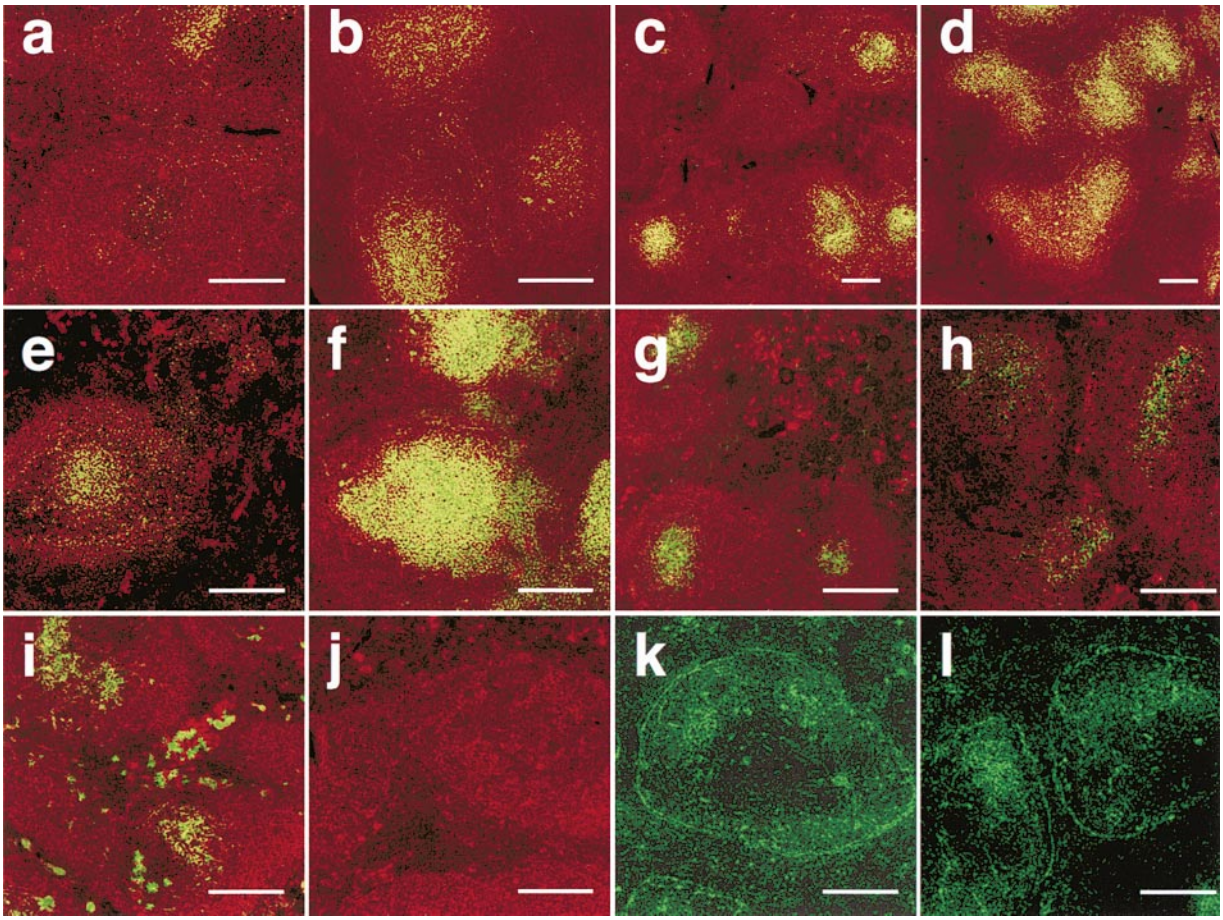

Figure 5. Immunohistochemical Analysis of *AID*$^{-/-}$ Chimera

Serial sections of spleens were prepared from nonimmunized (A and B) and SRBC-immunized (day 21, C–L) mice; *AID*$^{-/-}$ (B, D, F, H, J, and L) and *AID* $^{+/-}$ (A, C, E, G, I, and K). Immunofluorescence microscopic study was performed to detect germinal centers (A–F; PNA-FITC, anti-IgM-Texas Red), development of follicular dendritic cell networks (G and H; FDC-M1-FITC, anti-IgM-Texas Red), marginal zone lining cells (K and L; MAdCAM-1-FITC) and IgG plasma cells (I and J; anti-IgG-FITC, anti-IgM-Texas Red). Scale bars, 200 μm.

To directly examine DNA rearrangement in the Ig locus, we carried out digestion-circularization (DC) PCR of DNA obtained from spleen cells stimulated with LPS and IL-4 or TGFβ (Chu et al., 1992). As shown in Figure 6C, μ−γ$_1$ and μ−γ2b DC-PCR products were clearly amplified in stimulated spleen cell DNA of *AID*$^{+/+}$ and *AID*$^{+/-}$ but not of *AID*$^{-/-}$ mice. The results show that the AID deficiency abolished the CSR activity of B cells.

We have also examined the expression of germline transcripts directed by the I promoter of each isotype, which is required to precede CSR upon B cell stimulation. *AID*$^{-/-}$ spleen cells expressed germline transcripts of all the isotypes in response to LPS with and without cytokines in a similar manner to *AID*$^{+/+}$ and *AID*$^{+/-}$ spleen cells (Figure 6B). The germline transcripts were of expected sizes for appropriately spliced forms, and their amounts were similar among all *AID* genotypes as assessed by serial 3-fold dilution of cDNA (data not shown). These results taken together indicate that AID deficiency causes defect in the CSR machinery per se that plays a role after target S regions become accessible by germline transcription from I promoters.

The Defect of Hypermutation in AID-Deficient B Cells

Since CSR is often but not always coupled with hypermutation in the V$_H$ exon after antigen stimulation, we examined whether the AID deficiency affects the efficiency of hypermutation. *AID*$^{-/-}$ chimeras were immunized with NP-conjugated chicken γ-globulin (CGG). After two intraperitoneal injections of the antigen with a 3 week interval, the NP-specific IgM antibody titer was slightly higher in sera of *AID*$^{-/-}$ chimera than those of *AID*$^{+/-}$ chimera. However, no anti-NP IgG1 was produced by *AID*$^{-/-}$ chimera (Figure 7A). MLN RNA was extracted from *AID*$^{+/-}$ and *AID*$^{-/-}$ chimeras and V$_H$186.2-Cμ (or Cγ1) cDNA segments were amplified by RT-PCR to determine their nucleotide sequences. *AID*$^{+/-}$-derived V$_H$186.2 sequences of the μ and γ1 chains carried comparable levels of mutation in the total and framework regions (Table 1). However, CDR of the *AID*$^{+/-}$ γ1 chain contained a 60% higher frequency of mutations than that of the *AID*$^{+/-}$ μ chain. R/S ratios were higher in CDR than framework region of both μ and γ1 chains of *AID*$^{+/-}$ V$_H$186.2 sequences. Furthermore, *AID*$^{+/-}$ V$_H$186.2 sequences of the γ1 chain contained, more frequently than those of the μ chain, the characteristic mutation at ^{33}Trp (TGG) to ^{33}Leu (TTG), which is known to be responsible for high-affinity binding to the NP hapten (Figure 7B and data not shown) (Allen et al., 1988). By contrast, *AID*$^{-/-}$ μ chains contained 10 times fewer mutations than *AID*$^{+/-}$ μ chain (Table 1). There were no mutations in CDR 1 and CDR 2 of the *AID*$^{-/-}$ V$_H$186.2 μ chain. The

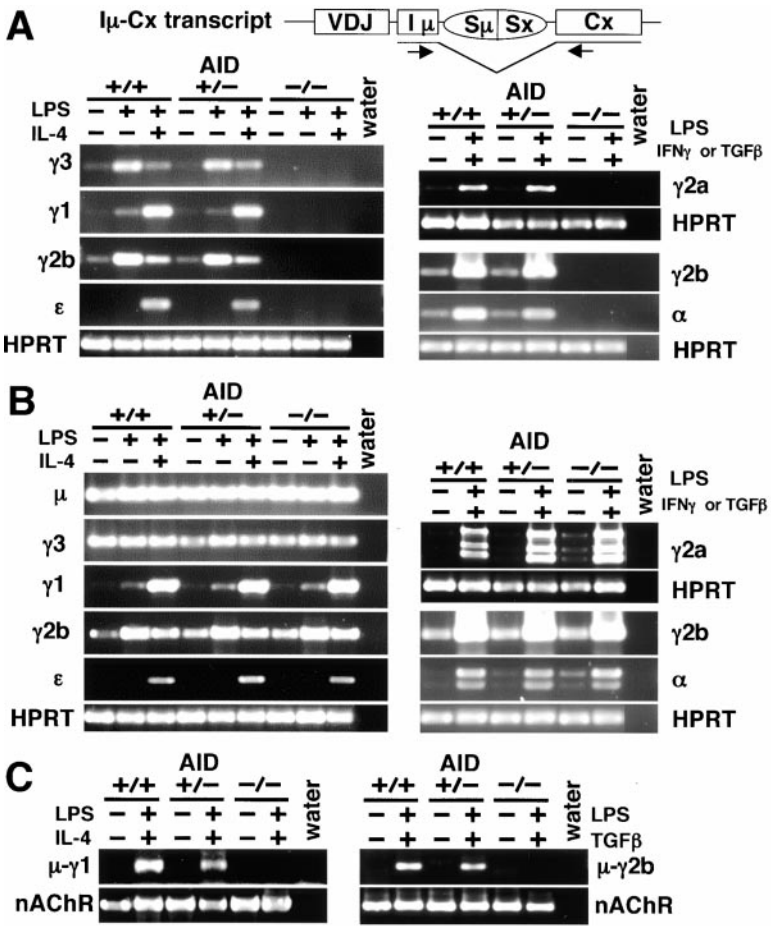

Figure 6. The Absence of CSR in AID-Deficient B Cells Stimulated In Vitro

Spleen B cells from 5-week old *AID*$^{+/+}$, *AID*$^{+/-}$, and *AID*$^{-/-}$ mice were stimulated in vitro with LPS alone, LPS and IL-4, and LPS and interferon-γ (γ2a), or LPS and TGFβ (γ2b, α) for 2 (B) or 4 (A and C) days. Controls without stimulation were harvested at day 0. Water, without cDNAs. HPRT, hypoxanthine phosphoribosyltransferase, cDNA control.

(A) RT-PCR of postswitch transcripts containing Iμ and each of C_H exons. Schematic representation of Iμ-Cx transcripts is shown at the top.

(B) RT-PCR of germline transcripts containing I and C_H exons of the same isotype. Among three kinds of alternatively spliced forms of α germline transcripts, only 497 and 357 bp species are shown.

(C) DC-PCR assay of genomic DNA from stimulated B cells.

mutation frequency (1.5×10^{-3}) of *AID*$^{-/-}$ V_H186.2 sequences was comparable to that due to PCR by Taq polymerase (manufacturer's data). These results indicate that *AID*$^{-/-}$ B cells are defective, not only in CSR, but also in hypermutation.

Discussion

AID was isolated by cDNA subtraction from CH12F3-2 cells which can be induced to switch efficiently from IgM to IgA by stimulation with CD40L, IL-4, and TGFβ (Muramatsu et al., 1999). AID is induced not only in stimulated CH12F3-2 cells but also in LPS-stimulated spleen B cells, and expressed in germinal centers of antigen-stimulated spleen and Peyer's patches. We have shown that AID induction by the tet-inducible promoter in CH12F3-2 cells augmented class switching even in the absence of cytokine stimulation (Figure 1). AID deficiency causes the complete absence of class switching in mice before or after immunization with T-dependent antigens (Figures 3 and 7). In addition, *AID*$^{-/-}$ spleen B cells stimulated in vitro with LPS and cytokines can not undergo CSR (Figure 6). Surprisingly, AID deficiency also abrogates hypermutation of the specific V_H gene in response to NP-conjugated, T-dependent antigen immunization (Figure 7 and Table 1). In the accompanying paper (Revy et al., 2000 [this issue of *Cell*]), we report that an essentially similar conclusion is drawn by the analysis of a human hyper-IgM syndrome (HIGM2), in which not only was class switching completely blocked, but also that hypermutation was extremely low in IgM antibodies. We found that all the patients of HIGM2 have mutations at the various positions in the *AID* gene (Revy et al., 2000 [this issue of *Cell*]), indicating that *AID*$^{-/-}$ mouse phenotypes described here are not due to artifacts of the neomycin gene integration.

CSR in B cells can be affected by the defects of either extracellular stimulation, intracellular signal transduction, or nuclear events. The AID deficiency does not appear to affect maturation of B cells and other cell types that regulate B cell activation, as shown by FACS and immunohistochemical studies (Figures 4 and 5). Signal transduction from the B cell antigen receptor appears to be functional in *AID*$^{-/-}$ mice because there are many signs of activation of B cells before or after immunization. In addition, AID deficient mice can produce antigen specific IgM in response to the T-dependent antigens (Figures 3 and 7). These phenotypes are in a contrast to those of X-linked hyper-IgM syndrome patients, in which the CD40L mutation causes the loss of germinal center formation and Ig production in response to T-dependent antigens. This mutation, however, does not affect class switching by in vitro culture of B cells with appropriate stimulation (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthauer et al., 1993). In fact, AID-deficient mice is the first case in which class switching is completely blocked despite intact germinal center formation. These results indicate that AID

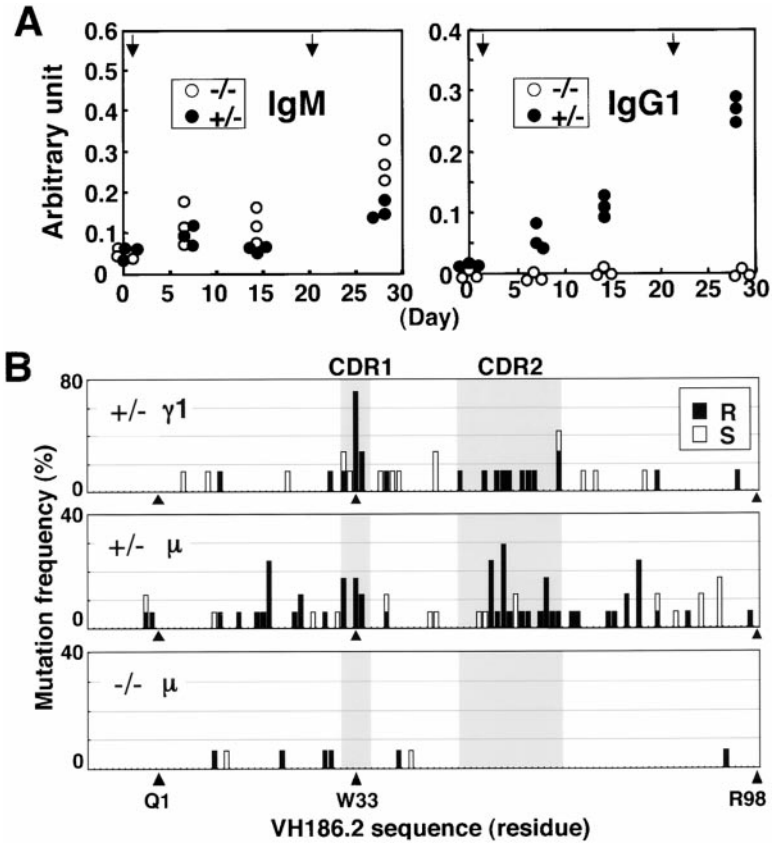

Figure 7. Defect of Hypermutation in *AID*$^{-/-}$ Chimera

(A) Primary and secondary responses against NP in *AID*$^{-/-}$ chimera. Anti-NP IgM and IgG1 levels were measured 0, 7, 14, and 28 days after *AID*$^{-/-}$ (open circle) or *AID*$^{+/-}$ (closed circle) chimeras were immunized with NP-CGG intraperitonealy on day 0 and 21 (arrows). Each circle represents an arbitrary unit (absorbance at OD_{405} nm) of a single mouse.

(B) Mutation frequencies in the V_H186.2 transcripts from MLN of NP-immunized *AID*$^{-/-}$ and *AID*$^{+/-}$ chimeras. V_H186.2 transcripts of μ and γ1 isotypes at day 28 were amplified by RT-PCR and cloned into a plasmid vector before sequencing. Six clones of γ1 isotype from *AID*$^{+/-}$ chimera, and 17 and 16 clones of μ isotype from *AID*$^{+/-}$ and *AID*$^{-/-}$ chimeras, respectively, were sequenced. Mutation frequency of clones that have amino acid replacements (closed bar) or silent mutation (open bar) at specific position was plotted along residue number from −11 to 98.

is involved in neither development nor activation of B cells, but more specifically in a step of CSR. AID appears to play a role in DNA cleavage because (1) AID deficiency does not affect germline transcription of target DNA (Figure 6), (2) AID induction augments class switching of CH12F3-2 cells, which are known to express germline transcripts even without stimulation (Nakamura et al., 1996) and (3) AID deficiency does not affect VDJ recombination, which includes the end joining repair system.

It is unexpected that a single gene mutation of *AID* abolishes both CSR and hypermutation. It has been shown that the V_H region of IgM can also contain hypermutation, indicating that class switching is not required for hypermutation (Mantovani et al., 1993; Sohn et al., 1993). However, this observation does not exclude the possibility that a single molecule is involved in both reactions. Although our knowledge about other molecules involved in hypermutation is very limited, there are several candidates, such as the error-prone repair system (Ni et al., 1999), the low-fidelity DNA polymerase family (reviewed by Friedberg et al., 2000), and mutations associated with double-strand break repair (Holbeck and Strathern, 1997).

FACS and immunohistochemistry data suggest accumulation of *AID*$^{-/-}$ B cells upon stimulation. Increase in activated IgM$^+$ B cells in *AID*$^{-/-}$ mice is probably not due to the cell autonomous defect of cell cycle regulation because (1) the fraction of PNA$^+$ B cells is very low in ILN of *AID*$^{-/-}$ chimera in which B cells are exposed to much less antigen injected into the peritoneal cavity than those in MLN, and (2) *AID*$^{-/-}$ and *AID*$^{+/-}$ spleen cells proliferate comparably when cultured with or without LPS in vitro (see Supplementary Data). Apparent hyperproliferation of IgM$^+$ B cells was also seen in HIGM2 patients (Revy et al., 2000 [this issue of *Cell*]). It is possible that class switching or hypermutation is

Table 1. Somatic Mutations in Rearranged V_H186.2 Sequences in AID$^{-/-}$ Chimeras[a]

H chain (*AID*)	Total number of Chains			Mutation × 10^3 per base[b]											
				Total (327 bp)				CDR1+2 (66 bp)				Framework (228 bp)			
	Sequenced	Mutated	Bases	Total	R[c]	S[c]	R/S[c]	Total	R	S	R/S	Total	R	S	R/S
α1(+/−)	7	6	2289	16	10	6.1	1.6	46	39	6.5	6.0	10	3.1	6.9	0.5
μ(+/−)	17	11	5559	13	9.9	3.2	3.1	28	24	3.6	6.8	11	7.2	3.6	2.0
μ(−/−)	16	6	5232	1.5	1.1	0.4	3.0	0.0	0.0	0.0	—	2.2	1.6	0.5	3.0

[a] Nucleotide exchange frequency in the V_HDJ_H186.2 segments of γ1 and μ transcripts. Nucleotide replacements at the V_HD_H junctions were not scored as mutations. Dinucleotide replacements in a codon were counted as one.

[b] Mutation frequency was calculated by dividing the accumulated number of mutations in a given region with the total number of nucleotides sequenced for the region, length of which is shown in parentheses.

[c] R and S stand for replacement and silent mutations, respectively. R/S indicates ratio of replacement to silent mutations.

coupled with negative feedback signal for stimulation-induced proliferation of B cells so that activated B cells can differentiate.

AID is not only structurally related to APOBEC-1, RNA editing cytidine deaminase, but also functionally similar in its cytidine deaminase activity (Muramatsu et al., 1999). *AID* was mapped to the locus close to that of *APOBEC-1* on human chromosome 12p13, suggesting that these two genes were generated by recent duplication (Muto et al., 2000). The most straightforward possibility for the function of AID is, therefore, an RNA editing enzyme with a substrate specificity determined by an additional co-factor like ACF for APOBEC-1 (Mehta et al., 2000). In that case, mRNA encoding an unknown protein might be converted to that for CSR recombinase and hypermutator by the function of AID and its cofactor. In fact, CSR and hypermutation may share a part of the genetic alteration machinery because frequent mutations are reported in the vicinity of CSR breakpoints (Dunnick et al., 1993; Lee et al., 1998), and frequent large deletions have been reported in the V region (Goossens et al., 1998; Wilson et al., 1998). Obvious candidates of the AID target may be the DNA repair enzymes, DNases, and DNA polymerases discussed above. It is also possible that AID associates with different accessory molecules to recognize different target mRNAs; one for CSR and the other for hypermutation. Although less likely, AID may edit DNA directly or edit RNA to generate a ribozyme that can alter genetic information of the Ig locus.

CSR has several unique properties as compared to oher known recombination systems: (1) CSR target sequences are not clear except that repetitive palindromic S regions are required (Kinoshita et al., 1998), (2) germline transcription and splicing are essential (Gu et al., 1993; Jung et al., 1993; Xu et al., 1993; Hein et al., 1998), and (3) transcription orientation of two S regions determines recombination products, i.e., deletion and inversion by the same and opposite orientations, respectively (Kinoshita et al., 1999; reviewed by Kinoshita and Honjo, 2000). The present study suggests that RNA editing may be involved in CSR and hypermutation either directly or indirectly, adding yet another unique and complex feature to CSR and hypermutation. This would be the first case in which two different genetic alteration systems (RNA editing and CSR or hypermutation) cooperate for a given biological function. Although we have provided the evidence that AID is the essential component for both CSR and hypermutation, their molecular mechanisms still remain to be solved.

Experimental Procedures

Induced Expression of AID in CH12F3-2 Cells

An AID expression vector with tet-responsive promoter (GIBCO BRL) and IRES-EGFP segment (Clontech) was introduced to CH12F3-2 cells that had been previously transfected with a construct expressing tet-responsive transactivator (tTAk, GIBCO BRL) driven by tet promoter. To suppress AID expression, 0.5 μg/ml of tet was used. Transfectants that upregulate EGFP one day after removal of tet were stimulated with the half dose of CD40L, IL-4, and TGFβ required for the optimal induction of IgA switching (Nakamura et al., 1996). Stimulated cells are analyzed 24 hr later by FACS after staining with phycoerythrin (PE)-conjugated goat anti-mouse IgA antibody (Southern Biotechnology). At the same time, total RNA was extracted from cells and amount of *AID* transcripts was analyzed by RNase protection assay using the RPA II kit (Ambion). The template of RNA probe was prepared by PCR and contains a 196 bp region surrounding the termination codon of AID. Mouse β-actin probe (157 bp) was also prepared by PCR.

Target Vector, Generation of AID-Deficient Mouse, and *RAG-2*$^{-/-}$ Blastocyst Complementation

Genomic DNA containing the *AID* locus was isolated from a 129 SvJ genomic library (Stratagene). Ten kb XhoI fragment and 1.8 kb BssHII/PvuI fragment of *AID* gene were ligated to SalI and XhoI sites of pLNTK (Gorman et al., 1996), respectively. The linearized target vector was transfected into TT2 ES cells (C57BL/6 × CBA F1 origin) as previously described (Yagi et al., 1993). Five clones with homologous recombination were isolated by PCR screening using G3 and 203 primers, and confirmed by Southern blot analysis using the 3′ external probe (Figure 2). Founder mice generated by injection of *AID*$^{+/-}$ ES clones into 8-cell embryos of ICR mice were mated with C57BL/6 mice and their germline transmission was confirmed by Southern blot analysis as shown in Figure 1B. Double knockout ES cells were isolated in almost the same way as described elsewhere (Cogne et al., 1994). *AID*$^{+/+}$, *AID*$^{+/-}$, and *AID*$^{-/-}$ TT2 clones were injected into 8-cell embryos of RAG-2 deficient mouse (C57BL/6 background) and transplanted to foster mothers (ICR) (Yagi et al., 1993). All of the *AID*$^{+/-}$ and *AID*$^{-/-}$ chimeras used in this study showed almost 100% ES contribution as determined by their coat color. Mice were bred in specific pathogen free conditions.

Immunization and Determination of Ig Titers

Eight- to twenty-week old mice were immunized by intraperitoneal injection of 1×10^8 SRBC or 100 μg NP-CGG (23:1) (Biosearch Technologies) mixed with alum. Levels of anti-SRBC and anti-NP antibodies were determined by ELISA using soluble SRBC (Kelly et al., 1979) and NP-BSA (23:1) (Biosearch Technologies), respectively, as a capture agent. To measure secreted Ig, red blood cell–depleted spleen cells (5×10^5/ml) were cultured for 3 days in 2 ml of medium (Burstein et al., 1991) containing 50 μg/ml LPS (Sigma), LPS + 10 ng/ml murine IL-4 (Life Technology), LPS + 10 U/ml mouse interferon-γ (Genzyme), or LPS + 1 ng/ml human TGFβ (R&D). After serial dilution of samples to find the linear range, Ig levels were determined by ELISA as described previously (Nishimura et al., 1998) except that rat anti-mouse ε chain monoclonal antibody (PharMingen) and goat anti-mouse μ chain specific antibody (Southern Biotechnology) were used to determine IgE and IgM respectively.

Flow Cytometry Analysis

The following monoclonal antibodies were used. Fluorescein isothiocyanate (FITC)-conjugated antibodies; anti-B220 (RA3-6B2) and anti I-A^b (Aβ^b) (AF6-120.1) (PharMingen). PE-conjugated antibodies; anti-CD19 (1D3) and anti-CD69 (H1.2F3) (PharMingen). Polyclonal antibodies; FITC-conjugated; anti-IgG (Southern Biotechnology). PE-conjugated; anti-IgM (Southern Biotechnology). FITC-conjugated PNA (Vector). All analyses were performed on a FACSCaliburÆ (Becton Dickinson). Data were obtained on 2×10^4 viable cells, as determined by forward light scatter intensity and propidium iodide gatings.

Immunohistochemical Examination

Tissue samples from spleen of nonimmunized or immunized mice were frozen in tissue-tek O.C.T. compound (Sakura Finetechnical). Tissue sections (6 μm thick) were prepared and fixed in a fresh solution of 4% paraformaldehyde for 15 min. Cells were stained using the following reagents: anti-IgM-Biotin (Cappel), anti-PNA-FITC (Vector), anti-FDC-M1 (rat anti-mouse, gift from Dr. M. Kosco-Vilbois), anti-IgG-FITC (Southern Biotechnology), anti-MAdCAM-1 (rat anti-mouse MECA-367; PharMingen), streptavidin-Texas Red (Gibco BRL), and goat anti-rat IgG-FITC (Cappel). The sections were mounted in Immunon (Shandon). Slides were analyzed with a Bio-Rad confocal laser scanning microscope (model MRC-1024).

RT-PCR

Total RNA was extracted from spleen cells of *AID*$^{-/-}$ mice cultured in vitro and MLN of *AID*$^{-/-}$ chimeras using TRIzol (Gibco BRL) according to manufacturer's instructions. For amplification of *AID* transcripts, 119 and 118 primer pairs were used in 30 cycles of PCR. For PCR of germline transcripts, the following primers were used

to obtain indicated sizes of products: (μ) ImF and CmR, 245 bp; (γ3) Ig3F and Cg3R, 323 bp; (γ1) Ig1 and Cg1R, 429 bp; (γ2b) Ig2bF and Cg2bR, 371 bp; (γ2a) Ig2aF and Cg2aR, 913, 678, and 560 bp; (ε) IeF and CeR, 392 bp; and (α) IaF and CaR, 497, 357, and 95 bp. Postswitch transcripts were amplified using the following primer pairs: (γ3) ImF and Cg3R, 323 bp; (γ1) ImF and Cg1R, 353 bp; (γ2b) ImF and Cg2bR, 422 bp; (γ2a) ImF and Cg2aR, 472 bp; (ε) ImF and CeR, 285 bp; and (α) ImF and CaR, 267 bp. Germline and postswitch transcripts were amplified by 30 cycles of PCR except ε germline transcripts (34 cycles).

DC-PCR

DC-PCR analysis was carried out as described (Chu et al., 1992). Genomic DNA was isolated from spleen cells in *AID*$^{-/-}$ mice before or after culture in vitro for 4 days with LPS and either IL-4 or TGFβ. After EcoRI digestion, genomic DNA was purified and self-ligated. Ligated DNA was purified again and served as a template for PCR using primers as reported previously for μ-γ1 (Chu et al., 1992) and μ-γ2b (Ballantyne et al., 1998).

Somatic Hypermutation Assay

V_H186.2 transcripts were amplified from MLN cDNA from NP-CGG immunized mice. V_H186.2F-CmR and V_H186.2F-Cg1R primer pairs were used for amplification of μ and γ1 transcripts, respectively. After 30 cycles of reaction using LA Taq polymerase (Takara Shuzo), PCR products were cloned into pGEM-T vector (Promega) before sequencing.

PCR Primers

118: 5′-GGCTGAGGTTAGGGTTCCATCTCAG-3′; 119: 5′-GAGGG AGTCAAGAAAGTCACGCTGGA-3′; ImF: 5′-CTCTGGCCCTGCTTAT TGTTG-3′; Ig3F: 5′-TGGGCAAGTGGATCTGAACA-3′; Ig1: 5′-GGC CCTTCCAGATCTTTGAG-3′; Ig2bF: 5′-CACTGGGCCTTTCCAGAA CTA-3′; Ig2aF: 5′-GGCTGTTAGAAGCACAGTGACAAAG-3′; IeF: 5′-TGGGATCAGACGATGGAGAATAG-3′; IaF: 5′-CCTGGCTGTTCC CCTATGAA-3′; CmR: 5′-GAAGACATTTGGGAAGGACTGACT-3′; Cg3R: 5′-CTCAGGGAAGTAGCCTTTGACA-3′; Cg1R: 5′-GGATCC AGAGTTCCAGGTCACT-3′; Cg2bR: 5′-CACTGAGCTGCTCATAGT GTAGAGTC-3′; Cg2aR: 5′-GCCACATTGCAGGTGATGGA-3′; CeR: 5′-CCAGGGTCATGGAAGCAGTG-3′; CaR: 5′-GAGCTGGTGGGAGT GTCAGTG-3′; V_H186.2F: 5′-TTCTTGGCAGCAACAGCTACA-3′; G3: 5′-GGGCCAGCTCATTCCTCCACTC-3′; 203: 5′-ATGCCACTACCT CTCTGGCCTTCA-3′.

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