Molecular mechanism for generation of antibody memory

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Activation-induced cytidine deaminase (AID) is the essential enzyme inducing the DNA cleavage required for both somatic hypermutation and class switch recombination (CSR) of the immunoglobulin gene. We originally proposed the RNA-editing model for the mechanism of DNA cleavage by AID. We obtained evidence that fulfils three requirements for CSR by this model, namely (i) AID shuttling between nucleus and cytoplasm, (ii) de novo protein synthesis for CSR, and (iii) AID–RNA complex formation. The alternative hypothesis, designated as the DNA-deamination model, assumes that the *in vitro* DNA deamination activity of AID is representative of its physiological function *in vivo*. Furthermore, the resulting dU was removed by uracil DNA glycosylase (UNG) to generate a basic site, followed by phosphodiester bond cleavage by AP endonuclease. We critically examined each of these provisional steps. We identified a cluster of mutants (H48A, L49A, R50A and N51A) that had particularly higher CSR activities than expected from their DNA deamination activities. The most striking was the N51A mutant that had no ability to deaminate DNA *in vitro* but retained approximately 50 per cent of the wild-type level of CSR activity. We also provide further evidence that UNG plays a non-canonical role in CSR, namely in the repair step of the DNA breaks. Taking these results together, we favour the RNA-editing model for the function of AID in CSR.

Keywords: activation-induced cytidine deaminase; class switch; RNA editing; uracil DNA glycosylase; DNA deamination; APOBEC1

1. INTRODUCTION

A large repertoire of natural antibodies is produced by B cells in which the RAG1/2 complex has completed V(D)J recombination of the variable region gene segments in both heavy and light chain gene loci. However, successful vaccination depends on the generation of antibody memory that is mediated by two additional types of genetic alterations: somatic hypermutation (SHM) and class switch recombination (CSR). Mature B cells are stimulated by exposure to antigen and induced to initiate further gene alterations (SHM and CSR) in the Ig loci. In CSR, B cells replace their C μ region gene by one of the other downstream constant region genes by means of excisional deletion so that different isotypes of antibodies can be generated to facilitate antigen elimination (Honjo *et al.* 2002).

When we proposed the allelic deletion model, we envisaged the role of a specific endonuclease to cause DNA cleavage at specific regions between C H loci (Honjo & Kataoka 1978). Subsequently, the identification of the switch (S) regions (Davis *et al.* 1980;

Dunnick *et al.* 1980; Takahashi *et al.* 1980; Kataoka *et al.* 1981; Obata *et al.* 1981) and their requirement for germ line transcription (Stavnezer-Nordgren & Sirlin 1986; Stavnezer *et al.* 1988) yielded further insights into the mechanism of CSR but the key enzymes to trigger this process remained obscure. The mystery was solved when we found both CSR and SHM totally abolished in mice deficient for a new member of the cytidine deaminase family called activation-induced cytidine deaminase (AID; Muramatsu *et al.* 1999, 2000). Furthermore, collaboration with French colleagues revealed that patients with hyper-IgM syndrome type 2 had mutations in the coding regions in both AID alleles (Revy *et al.* 2000). These striking confirmations of the key role of a small cytidine deaminating enzyme, AID, in CSR and SHM (and subsequently in gene conversion in chicken B cells; Arakawa *et al.* 2002; Harris *et al.* 2002b) led to extensive investigation of the mechanisms through which AID triggers the genome modification processes in B cells. Being a member of the family of cytidine deaminases, AID possesses a cytidine (C) deamination domain that is indispensable for its physiological functions. It is, however, of long-standing dispute whether AID deaminates C to uridine (U) in the context of DNA (DNA-deamination model; Petersen-Mahrt *et al.* 2002;

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Figure 1. (a) RNA-editing and (b) DNA-deamination models. Mechanisms that induce DNA cleavage or point mutation are shown. Critical requirements for each model are shown in the boxes. See text for detailed explanation.

Chaudhuri *et al.* 2007) or in RNA (RNA-editing model; Muramatsu *et al.* 2000, 2007; Honjo *et al.* 2005).

(a) Two models for AID function

Based on structural homology with APOBEC1, a well-established RNA-editing cytidine deaminase, AID was first proposed by our group to edit some mRNA(s), giving rise to putative endonucleases or their cofactors (Muramatsu *et al.* 2000; figure 1a). The DNA-deamination model postulates that transcription in the V(D)J or S regions leads to formation of R-loops from the non-templated DNA strand that is exposed to the enzymatic action of AID, converting dC to dU to create a dU:dG mismatch. That mismatch can be recognized by uracil DNA glycosylase (UNG) and the abasic site after U removal should be cleaved by AP endonuclease, resulting in single- and double-strand DNA breaks required for CSR and SHM (Chaudhuri *et al.* 2007; Di Noia & Neuberger 2007; figure 1b). In the past few years, we undertook extensive experimental studies that, in our opinion, could critically judge the validity of either of the models described above.

(b) Evidence for the RNA-editing hypothesis

The RNA-editing hypothesis predicts that recombinase and mutator (or their guiding factors) are synthesized from the mRNA after editing by AID. Therefore, experiments to assess the requirement for de novo protein synthesis after AID activation were considered important. Because AID is also newly synthesized upon B cell activation (Muramatsu *et al.* 1999), simple addition of a protein synthesis inhibitor cannot be applied. A new trick for inducible activation of AID independent of protein synthesis was developed by fusing AID and the human oestrogen receptor (ER) hormone-binding domain (Doi *et al.* 2003). This AID-ER protein can be overexpressed in an inactive form and then activated by adding 4-hydroxytamoxifen (OHT), an oestrogen analogue. Doi *et al.* introduced AID-ER protein into AID-deficient B cells that were stimulated by lipopolysaccharide (LPS) and interleukin-4 (IL-4), and cycloheximide (CHX) or puromycin was added before or after the addition of OHT. Inhibition of de novo protein synthesis by addition of these chemicals severely impaired CSR when added 1 hour before but not after OHT treatment (Doi *et al.* 2003).

Figure 2. Evidence for the RNA-editing hypothesis. Inhibition of de novo protein synthesis by addition of cycloheximide (CHX) severely impaired CSR when added 1 hour before 4-OHT treatment that activates AID-ER (Doi *et al.* 2003). Mutations at NES of AID inhibit both shuttling to cytoplasm and CSR activity (T. Doi & T. Honjo 2008, unpublished data). AID-mRNA complex formation was shown by UV cross-linkage (T. Nonaka, T. Doi, T. Honjo & K. Kinoshita 2008, unpublished data).

However, the possibility cannot be excluded that the synthesis of factors required for DNA repair was inhibited. Therefore, Begum *et al.* (2004b) examined whether CSR inhibition occurs before or after the DNA cleavage step. To monitor the formation of DNA double strand breaks (DSBs) at the S region of the IgH gene, γ H2AX foci formation (a specific marker for DSBs) was detected by chromatin immunoprecipitation analysis. By addition of CHX before OHT, γ H2AX focus formation in the IgH locus was severely impaired, indicating that de novo protein synthesis is required for the DNA cleavage step of CSR after AID activation (Begum *et al.* 2004b). APOBEC1 shares a strong homology with AID. APOBEC1 shuttles between the nucleus and the cytoplasm by association with importin α at its N-terminal nuclear localization signal (NLS) and with the nuclear export machinery at its C-terminal nuclear export signal (NES; Chester *et al.* 2003). Likewise, AID was shown to shuttle between the nucleus and the cytoplasm (Ito *et al.* 2004; McBride *et al.* 2004). A NES (residues 189–198) and potential NLS (residues 8–25) exist at the C- and N-termini of AID, respectively (Brar *et al.* 2004; Ito *et al.* 2004; McBride *et al.* 2004). This biological similarity between AID and APOBEC1 supports functional homology, i.e. RNA editing, but obviously does not prove it.

Since the NLS and the NES partially overlap the SHM- and CSR-specific domains, respectively (Ta *et al.* 2003; Shinkura *et al.* 2004), correlation between AID cellular localization and activity was expected. AID mutants lacking the 16 C-terminal amino acid residues (including the NES) lose the shuttling ability and accumulate in the nucleus (Ito *et al.* 2004). These mutants are inactive or severely impaired for CSR but active for SHM, suggesting that efficient export of AID from the nucleus is important for CSR but not for SHM induction. In addition, CSR is blocked in the presence of leptomycin B (LMB), an inhibitor of exportin-1-dependent nuclear export (T. Doi & T. Honjo 2008, unpublished data).

Although, the presumable catalytic activity of AID on RNA has not been demonstrated, there is accumulating evidence that AID forms a complex with RNA. ssDNA deamination activity of AID requires RNase treatment (Pham *et al.* 2003) and AID purified from bacterial cells can be cross-linked to non-specific RNA (Dickerson *et al.* 2003). Recently, UV cross-linkage of B cells allowed us to demonstrate that AID forms a complex with RNA *in vivo* (T. Nonaka, T. Doi, T. Honjo & K. Kinoshita 2008, unpublished data). The three findings (shuttling, translation and RNA complex) satisfy the requirement of the RNA-editing model as shown schematically in figure 2, although they are not a direct proof.

(c) Evidence for the DNA-deamination model

A large body of observations such as the AID-induced mutator phenotype in *Escherichia coli* (Petersen-Mahrt *et al.* 2002; Ramiro *et al.* 2003), *in vitro* dC deamination to dU on ssDNA (Branstetter *et al.* 2003; Chaudhuri *et al.* 2003, 2004; Dickerson *et al.* 2003; Pham *et al.* 2003; Sohail *et al.* 2003; Yu *et al.* 2004), as well as the severe CSR blockade and the SHM bias to transition mutations at dC:dG in the absence of UNG (Rada *et al.* 2002; Imai *et al.* 2003) appeared to favour the DNA-deamination model. Major evidence for the two models is summarized in table 1.

(d) Dissociation between *in vitro* DNA deamination activity and physiological functions of AID

According to the DNA-deamination model, one should expect that there should be a strong correlation between the relative ssDNA deamination activity and the relative CSR of AID, i.e. CSR should not occur in the absence of ssDNA deamination activity of AID. In order to study the correlation between AID deamination activity and the respective physiological activity, we generated a large series of mouse AID (mAID) mutants. ssDNA deamination activity of each mutant was estimated after *in vitro* translation using wheat-germ extracts. The ssDNA deamination reaction was performed with a ssDNA-oligo labelled with Alexa-fluor 680. The percentage of deamination for each mutant was estimated relative to the activity of the same amount of the wild-type mAID. As expected, mutants with variable levels of ssDNA deamination activity were identified. Among them, we focused on several single and composite mutants with alanine replacement just upstream of the catalytic domain (residues 45–55; figure 3a). We identified a cluster of mutations between F46 and N51 that affected the DNA deamination activity ranging from complete loss (N51A) to severe reduction (H48A, L49A and R50A; figure 3b,d). All mutants with double or triple substitutions, including N51A, had little or no DNA deamination activity.

We were also able to evaluate quantitatively the effect of each mutation on one of the biologic functions of AID, CSR. The measure for CSR activity of each mutant was the percentage of switching of AID^{-/-} B-cells to IgG1 after retroviral transfer of each mutant's cDNA and stimulation for 72 hours with IL-4 and LPS. Again the activity was estimated relative to the wtAID CSR activity. It became immediately obvious that the mutant with loss of ssDNA deamination

activity (N51A) showed half the wild-type level of CSR activity (figure 3c). That led us to perform a comprehensive analysis of the correlation between catalytic and biologic activities. We, therefore, plotted the relative ssDNA deamination activity of each mAID mutant versus its corresponding relative CSR activity (figure 3d). DNA deamination and CSR activities of most (7 out of 11) of the mutants were not proportional to each other, while the F46A, K52A, S53A and C55A mutants showed a somewhat weak correlation between the two activities. Extremely surprising was the finding that a cluster of mutants, H48A, L49A, R50A and N51A, showed much higher relative CSR activity than expected from their DNA deamination activity.

Because our findings were quite unexpected based on the assumption for CSR initiation through dC to dU deamination by AID, we had to exclude any experimental bias owing to the comparison of the activities of proteins from different expression systems (recombinant *in vitro* synthesized and from mammalian B-cells for the deamination activity and CSR assay, respectively) and to exclude the possibility that extremely low ssDNA deamination activity could promote significant levels of CSR activity. To address both issues, it appeared essential to titrate precisely the CSR activity of the wtAID and its corresponding deamination activity using proteins from the same expression system. In spite of the high CSR activity of native wtAID after retroviral expression in B cells, it shows a very low deamination activity. We therefore, assessed the CSR activity of wtAID and N51A fused to human ER by titration of 4-hydroxytamoxifen (4-OHT) as described previously (Doi *et al.* 2003). CSR activity of both wtAID-ER and N51A-ER showed a clear dose dependency on 4-OHT concentration (Shivarov *et al.* 2008). The maximum level of relative CSR activity we observed for N51A under these conditions was 20 per cent. This was probably because CSR activity was estimated only 48 hours after 4-OHT addition. Furthermore, we were able to demonstrate a significant level of dose-dependent DNA deamination activity for wtAID. No deamination activity was detected for the N51A-ER mutant. It is of note that if DNA deamination was the mechanism by which AID triggered CSR, no less than 50 per cent of the maximum DNA deamination activity would be sufficient to achieve the wild-type level of CSR activity. In regard to this observation, the R50A mutant clearly showed less than 50 per cent of the wild-type level of deamination activity (approx. 21%) but higher than the wild-type level of CSR activity. Thus, we confirmed that the dissociation between ssDNA deamination and CSR for mAID mutants is also true in B cells.

(e) Dissociation between DNA deamination activity and physiological function of APOBEC1

The AID/APOBEC family of cytidine deaminases is comprised four major groups of enzymes (AID, APOBEC1, APOBEC2 and APOBEC3; Conticello *et al.* 2005). To date, however, a strict correlation between catalytic activity and known physiological function has been demonstrated only for APOBEC1 that performs its biological function through cytidine deamination at position 6666 on ApoB mRNA to

Table 1. Evidence for the two models.

RNA-editing model	DNA-deamination model
de novo protein synthesis required for DNA cleavage in CSR; Doi <i>et al.</i> (2003) and Begum <i>et al.</i> (2004b)	recombinant AID deaminates C to U on ssDNA; Bransteitter <i>et al.</i> (2003), Chaudhuri <i>et al.</i> (2003, 2004), Dickerson <i>et al.</i> (2003), Pham <i>et al.</i> (2003), Sohail <i>et al.</i> (2003) and Yu <i>et al.</i> (2004)
nuclear-cytoplasm shuttling required for CSR; Brar <i>et al.</i> (2004), Ito <i>et al.</i> (2004) and McBride <i>et al.</i> (2004)	AID overexpression in <i>E. coli</i> and yeast induces predominantly C to T (G to A) mutations; Petersen-Mahrt <i>et al.</i> (2002) and Ramiro <i>et al.</i> (2003)
AID forms RNA-protein complex <i>in vivo</i> ; T. Nonaka, T. Doi, T. Honjo & K. Kinoshita 2008 (unpublished data)	UNG deficiency reduces CSR to 10% while no change in SHM frequency; Rada <i>et al.</i> (2002) and Imai <i>et al.</i> (2003)

Figure 3. (a) Structural and functional properties of AID. A schematic of the primary structure shows the motifs of AID. NLS, nuclear localization signal; NES, nuclear export signal. (b) *In vitro* DNA deamination activity of AID and its mutants. Representative polyacrylamide gel electrophoresis analysis of the deamination activity by AID and its mutants. The lower and upper bands indicate the product (deaminated and cleaved) ssDNA and substrate ssDNA, respectively. AID and its mutants were synthesized *in vitro* using wheat-germ extracts. An equal amount of each mutant protein was incubated with 0.1 pmol of labelled substrate (Alexa-5'-TTTTTTTTTTTAGCGTTTTTTTTTTT-3'), 0.4 unit of UDG and 1 µg of RNase for 60 min at 37°C in phosphate-buffered saline supplemented with 10 µM ZnCl₂ in a volume of 20 µl. The reaction was followed by incubation at 95°C for 8 min after addition of NaOH to 150 mM to cleave the alkali-labile abasic site. Samples were electrophoresed on 15% denaturing acrylamide gels and visualized using Odyssey (LI-COR). Cleavage ratio was determined by band quantitation using Odyssey. Deaminase activity of each mutant was shown as relative activity compared with that of wild-type AID. (c) CSR activity of each AID mutant was assessed by IgG1 surface expression of AID-deficient spleen B cells stimulated with LPS and IL-4 for 3 days after retroviral expression of AID and its mutants. The level of CSR was assessed by the percentage of IgG1⁺ cells among the infected AID^{-/-} spleen cells (GFP⁺). Bar represents mean ± s.d. from more than three independent experiments. Note that the N51A mutant of mAID showed a significant level of CSR. (d) Scattered plot of CSR versus ssDNA deamination activities shows clear dissociation between CSR and ssDNA deamination activity. Note the mutants (open diamonds) that show relatively higher CSR activity than expected from the ssDNA deamination activity. Data taken from Shivarov *et al.* (2008).

create a premature stop codon that in turn leads to the generation of a truncated ApoB peptide (Navaratnam *et al.* 1998). Furthermore, a specific APOBEC1 cofactor called APOBEC1 complementation factor (ACF) has been identified, and the RNA-editing activity of APOBEC1+ACF on the specific mRNA has been demonstrated *in vitro* (Mehta *et al.* 2000). Along with a considerable level of sequence homology,

APOBEC1 and AID appeared to show a significant level of functional similarities. We and others showed that both enzymes require nucleo-cytoplasmic shuttling for their proper functioning *in vivo* (Chester *et al.* 2003; Ito *et al.* 2004; McBride *et al.* 2004). Also, both enzymes have been shown to act *in vitro* on single-stranded DNA (Morgan *et al.* 2004), and to induce C to U mutations when expressed in *E. coli* (Harris *et al.*

Figure 4. Titration of AID, APOBEC1 and APOBEC1(N57A) proteins for *in vitro* ssDNA deamination activity. The experimental condition is the same as given in figure 3b except for the molar ratio of enzyme to substrate. Data taken from Shivarov et al. (2008).

2002a). However, APOBEC1 overexpression in mammalian cells cannot induce either CSR or SHM (Eto et al. 2003; Fugmann et al. 2004).

We examined whether the introduction of a specific mutation at the N57 residue in APOBEC1 (homologous to N51 in AID) could affect its two known deamination activities on ssDNA and RNA. As it has already been shown in other studies, we observed a lower ssDNA deamination activity of rat APOBEC1 as compared to the wtAID activity (Morgan et al. 2004). N57A mutation, however, practically abolished APOBEC1 deamination activity on ssDNA even at the enzyme to substrate ratio of 7.5 (figure 4). When we assayed the N57A mutant for RNA-editing activity on the specific ApoB mRNA, we found that it retains approximately one-third of the wild-type APOBEC1 RNA-editing activity (table 2).

(f) Further confirmation of the non-canonical role of UNG in CSR

The severe effect of UNG deficiency on CSR has originally been considered as the strongest proof for the validity of the DNA-deamination model (Rada et al. 2002). Subsequently, we demonstrated that catalytically defective mutants of UNG (D145N) retained the wild-type level of switching (Begum et al. 2004a). Furthermore, we showed that mutations in the Vpr-interacting domain of UNG, WxxF, cannot rescue CSR (Begum et al. 2007). Neuberger's laboratory argued that UNG catalytic mutants could rescue switching because their very limited residual U removal activity would be sufficient (Di Noia et al. 2007). Currently, we have found that N-terminal deletion of UNG combined with catalytic site and WxxF mutations kills switching activity regardless of the residual U removal activity (N.A. Begum & T. Honjo 2008, unpublished data). Collectively, we could not observe a proportional correlation between U removal and CSR activities of those mutants (table 3).

2. CONCLUSION

We showed that several mutations in AID located outside the deaminase motif render CSR function of

Table 2. The APOBEC1 mutant has RNA-editing activity (Shivarov et al. 2008). (Apolipoprotein(apo)B RNA-editing activities of APOBEC1 and its mutant were determined by sequencing from two independent experiments. A 239 b RNA fragment containing C at nt 6666 of human apoB100 cDNA was synthesized *in vitro* and incubated at 30°C for 2 hours with the indicated proteins synthesized *in vitro*. RNA was extracted, reverse-transcribed and amplified by PCR for cloning. Sequences were determined to evaluate RNA editing at C₆₆₆₆.)

	no. of clones sequenced	C ₆₆₆₆ to U ₆₆₆₆ edited clones of ApoB mRNA (%)
APOBEC1 + ACF	91	18 (20)
N57A + ACF	116	8 (7)
APOBEC1 only	42	0 (0)

Table 3. Evidence for dispensability of U removal in CSR.

U-removal-negative (less than 1% wt) UNG mutants are fully active for CSR; Begum et al. (2004a) and Di Noia et al. (2007)
WXXF UNG mutants with U removal activity are inactive for CSR; Begum et al. (2007)
N-terminal portion is required for CSR activity not by wild-type UNG but by catalytic and WXXF mutants; N.A. Begum & T. Honjo (2008, unpublished data)
UNG ^{-/-} Msh2 ^{-/-} B cells can efficiently cleave S regions; Begum et al. (2007)

AID independent of the ssDNA deamination activity. It is of note that the deaminase activity *per se* is absolutely required for AID function in CSR, though the target remains disputable. Our findings suggest that AID initiates the double-strand DNA breaks required for CSR not through dC to dU deamination on ssDNA but most probably through C to U editing on RNA or through some other unprecedented mechanisms. Actually, the dissociation between known catalytic and physiological activities is not a phenomenon unique to AID. We demonstrated that N57A mutation in APOBEC1 (which is analogous to N51A mutation in AID) disproportionately affects the ssDNA-deamination and RNA-editing activities and hence the biological function. The same phenomenon has already been observed for the third well-studied member of the family, APOBEC3G, which helps protect cells from HIV infection by deaminating cDNAs of the HIV retrovirus genome (Harris et al. 2003). However, this DNA deamination activity is not required for the protection of host cells from HIV infection (Newman et al. 2005; Bishop et al. 2006) and the retrovirus restriction by APOBEC3G is not dependent on UNG (Langlois & Neuberger 2008). We also reconfirmed the dispensability of the U-removal activity of UNG for CSR and showed that residual U-removal activity is not sufficient for efficient CSR because, most probably, UNG plays a role in the repair step of CSR through recruitment of specific WxxF-interacting Vpr-like protein(s). Along with the disapproval of the DNA-deamination model for CSR, it is

clear that AID does interact with mRNA *in vivo* and thus we propose AID can exert C to U editing activity on RNA to initiate CSR.

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