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C-terminal region of activation-induced cytidine deaminase (AID) is required for efficient class switch recombination and gene conversion

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Activation-induced cytidine deaminase (AID) introduces single-strand breaks (SSBs) to initiate class switch recombination (CSR), gene conversion (GC), and somatic hypermutation (SHM). CSR is mediated by double-strand breaks (DSBs) at donor and acceptor switch (S) regions, followed by pairing of DSB ends in two S regions and their joining. Because AID mutations at its C-terminal region drastically impair CSR but retain its DNA cleavage and SHM activity, the C-terminal region of AID likely is required for the recombination step after the DNA cleavage. To test this hypothesis, we analyzed the recombination junctions generated by AID C-terminal mutants and found that 0- to 3-bp microhomology junctions are relatively less abundant, possibly reflecting the defects of the classical nonhomologous end joining (C-NHEJ). Consistently, the accumulation of C-NHEJ factors such as Ku80 and XRCC4 was decreased at the cleaved S region. In contrast, an SSB-binding protein, poly (ADP)-ribose polymerase1, was recruited more abundantly, suggesting a defect in conversion from SSB to DSB. In addition, recruitment of critical DNA synapse factors such as 53BP1, DNA PKcs, and UNG at the S region was reduced during CSR. Furthermore, the chromosome conformation capture assay revealed that DNA synapse formation is impaired drastically in the AID C-terminal mutants. Interestingly, these mutants showed relative reduction in GC compared with SHM in chicken DT40 cells. Collectively, our data indicate that the C-terminal region of AID is required for efficient generation of DSB in CSR and GC and thus for the subsequent pairing of cleaved DNA ends during recombination in CSR.

Various studies on AID mutations in the N-terminal or C-terminal regions (4, 8, 9, 16–19) have shown that N-terminal AID mutants are compromised for CSR and are defective in SHM, indicating that the N-terminal region of AID is required for DNA cleavage (9, 16, 19). On the other hand, the C-terminal region of AID, which contains a nuclear-export signal and is responsible for AID’s shuttling activity between the nucleus and cytoplasm, is required for CSR-specific activity but not for DNA cleavage and SHM (8, 16). Among the series of AID C-terminal mutants examined, two mutants show characteristic features: P20, which has an insertion of 34 amino acids at residue 182 and normal nuclear-cytoplasmic shuttling activity, and JP8Bdel, which has a 16-aminoc acid truncation at residue 183, accumulates in the nucleus, and shows higher DNA break activity at the donor switch (S) region (16, 17). Although several reports suggest that the C-terminal region of AID is involved in protein stability (20, 21), C-terminal mutants of AID stabilized by fusing the hormone-binding domain of estrogen receptor (ER) also show similar CSR-defective phenotypes (8). Taken together, these data suggest that the DNA cleavage activity and CSR-specific activity depend on different regions of AID (8, 19). In addition, the C-terminal region of AID is essential for the interaction of AID with poly (A)\textsuperscript{+} RNA via a specific cofactor (22). Because CSR requires de novo protein synthesis, we proposed that after DNA cleavage the C-terminal region of AID may be involved in the regulation of the recombination step through generation of a new protein (8, 16, 22).

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The authors declare no conflict of interest.

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DSBs induced by AID during CSR ultimately are joined by the efficient DNA repair pathway that requires C-NHEJ factors such as Ku70/80 (12, 23). However, in the absence of C-NHEJ, the A-EJ pathway that relies on microhomology can join the broken DNA ends, although this pathway is associated with chromosomal translocations (11, 24). Previously, we reported that JP8Bdel enhances aberrant c-myc/IgH translocations and that it fails to carry out the efficient recombination between donor and acceptor S regions in the IgH locus (8). Therefore, it is important to examine whether the AID C-terminal mutants affect the S-S joining in CSR.

In the current work we examined whether the C-terminal region of AID is involved in DNA synapse formation and recombination during CSR in CH12F3-2 and spleen B cells. We also examined the effect of AID C-terminal mutations on GC in chicken DT40 cells, which depends on HR between pseudo V genes and the downstream IgVδ region. Using these CSR- and GC-monitoring systems, we demonstrate that efficient CSR and GC require the C-terminal region of AID for the formation of DSB from SSB and subsequent end synapse. Considering these findings together, we conclude that, in addition to DNA cleavage, AID has a unique function in the generation of DSBs, which is required for S–S synapse formation and joining in CSR and recombination in GC.

Results and Discussion

AID C-Terminal Mutants Fail to Support NHEJ in CSR. Because the DNA cleavage in the Sμ region in AID C-terminal mutants remains unaltered during CSR (8), we examined whether the next step after DNA cleavage, namely recombination, is affected by these mutations. We analyzed junction microhomology of Sμ–Sγ1 recombination in AID-deficient splenic B cells retrovirally transfected with WT AID or P20 fused with GFP to monitor their expression (Fig. 1A and B). Before transfection, cells were prestimulated with LPS for 2 d, and their IgG1 switching was induced by incubation with LPS and IL-4 for another 3 d. Consistent with the previous report, IgG1 switching was impaired in cells expressing P20 (Fig. 1C) (8, 16). DNA was isolated from IgG1–switched cells and analyzed for the Sμ–Sy1 junction. Interestingly, we found that the Sμ–Sγ1 junctions in P20-expressing cells contain less C-NHEJ repair signature (0–3 bp) and more frequent insertions, which presumably were introduced during repair by A-EJ, than do cells expressing WT AID (Fig. 1D and E). The junctions with 0- to 3-bp microhomology occupied 59.2% and 28.9% in cells expressing WT AID and P20, respectively (P < 0.05). Our data showed longer average microhomology in the P20 transfectant: The average microhomology length was 5.65 bp for cells expressing P20 and 3.01 bp for cells expressing WT AID (P < 0.05).

We also examined the microhomology and insertions at the Sμ–Sα recombination in CH12F3-2 cells, which almost exclusively switch from IgM to IgA. CH12F3-2 cells expressing JP8Bdel-ER were activated by the addition of 4-hydroxytamoxifen (4-OHT) for 2 d, and IgA+ cells were sorted to analyze their DNA for Sμ–Sα junction sequences. The phenotypes of the Sμ–Sα recombination junctions generated by JP8Bdel were essentially identical to those described above for the P20 mutation, with less C-NHEJ and more frequent insertions than in WT AID (Fig. 1F and G). Our findings agree with the report of longer microhomology in the Sμ–Sα junctions in patients with the hyper-IgM syndrome type II (AID<sup>C-termina</sup> mutation R190X) than in healthy individuals (25). These results suggest that the C-terminal mutations of AID affect C-NHEJ drastically and affect A-EJ relatively less severely.

To confirm the NHEJ defect in AID C-terminal mutants, we examined the accumulation of bona fide repair proteins involved in C-NHEJ, such as Ku80 and XRCC4 in the Sμ region (23). SSBs were introduced by stimulating CH12F3-2 cells expressing WT AID-ER or JP8Bdel-ER with 1 μM 4-OHT for 3 h (8). Consistent with the microhomology analyses of the Sμ–Sγ1 and Sμ–Sα junction, our ChIP analysis showed a drastic reduction of Ku80 and XRCC4 in the Sμ region of CH12F3-2 cells expressing JP8Bdel-ER compared with those expressing WT AID-ER (Fig. 2). These results indicate that the abolishment of CSR by P20 and

![Fig. 1. Expression of AID C-terminal mutants suppresses C-NHEJ in CSR junctions.](image-url)
JP8Bdel mutants can be attributed to the impairment of end repairs and especially to the severe defect of the C-NHEJ pathway.

In contrast, we found the more abundant recruitment of an SSB-recognizing protein, poly(A)-ribose polymerase1 (PARP1), in the $S_u$ region in CH12 cells expressing JP8Bdel-ER (Fig. 2A). This result is consistent with the previous reports that PARP1 facilitates the A-EJ pathway (26) and preferentially binds to SSB (27, 28). Similarly, our ChIP data exhibited more abundant accumulation of MSH2 (Fig. 2B), a critical mismatch protein, at the $S_u$ region. This observation also is in agreement with the report that MSH2 plays an important role in the absence of C-NHEJ factors during CSR reaction (29). These data demonstrate that the AID C-terminal mutants fail to generate DSBs from SSBs and consequently fail to recruit proteins required for C-NHEJ. Collectively, the data show that the C-terminal mutants P20 and JP8Bdel are inefficient at the recombination step, as characterized by more severe defect in the C-NHEJ pathway.

**AID C-Terminal Mutants Impair $S_u$–$S_o$ Synapse Formation.** Because the defect in CSR of the C-terminal mutants of AID appears to affect the recombination step, we examined whether the interaction between $S_u$ and downstream $S$ regions in the IgH locus also is affected. To this end, we examined the DNA synapse formation using the chromosome conformation capture (3C) assay in CH12F3-2 cells expressing WT AID-ER, G23S-ER (an N-terminal mutant), P20-ER, or JP8Bdel-ER. CSR was induced by 1 μM 4-OHT for 48 h. We first confirmed that CSR is compromised by the mutation at N-terminal region of AID and more severely by those mutations in C-terminal region, although their protein expression was not affected (Fig. 3 A and B). Subsequently, the stimulated cells were cross-linked in situ and subjected to a 3C assay (Fig. 3C), followed by restriction enzyme digestion and religation of the chromatin as described (SI Materials and Methods). The products were reverse cross-linked, and purified DNA was amplified by the specific primer pairs of two distant $S$ regions (Fig. 3D). Although the $S_u$–$S_o$ interaction in the cells expressing endogenous AID was similar among all AID transfectants when stimulated with the CD40 ligand, IL4, and TGF-β (CTT), cells expressing the AID C-terminal mutants P20 and JP8Bdel showed significant reduction in $S_u$–$S_o$ synapse formation upon 4-OHT addition (without CIT stimulation) (Fig. 3E). On the other hand, the G23S mutation, which has defect in DNA cleavage but not in CSR (8, 9), demonstrated a level of $S_u$–$S_o$ interaction similar to that of WT AID. We sequenced the $S$ synapse-associated and downstream $S$ regions in the IgH locus that can be disrupted by those mutations in C-terminal region. Impaired $S_u$ region was selected as a DNA break-negative control zone. ChIP analysis was performed following AID activation by 4-OHT for 3 h. Rabbit IgG was used as a ChIP control antibody, and the background value was subtracted. Values were normalized to the DNA input. In each dataset, the maximum value is considered to be 1.0. Similar results were obtained in repeated independent experiments.

**Fig. 2.** WT but not the C-terminally defective AID mutant supports DNA end repair and synapse factor recruitment in the $S_u$ region. (A) ChIP assay of known C-NHEJ and A-EJ repair proteins in CH12F3-2 cells expressing either AID-ER or JP8Bdel-ER. (B) 3C assay for 5–5 synapse-associated factors and the mismatch repair enzyme MSH2. Primers were specifically designed to examine the DNA break-prone $S_u$ region, and the $C_u$ region was selected as a DNA break-negative control zone. ChIP analysis was performed following AID activation by 4-OHT for 3 h. Rabbit IgG was used as a ChIP control antibody, and the background value was subtracted. Values were normalized to the DNA input. In each dataset, the maximum value is considered to be 1.0. Similar results were obtained in repeated independent experiments.
amplified bands to confirm that amplified DNA fragments represent the joining of the two appropriate S regions by the designed 3C-PCR primer pairs (Fig. S1).

To confirm the defect in the long-range interaction in splenic B cells in the IgH locus, we expressed WT AID and P20 in AID-deficient spleen cells by retroviral infection and stimulated the infected cells with IL-4 and LPS for 3 d. WT AID or P20 was fused to GFP to monitor the level of protein expression in the infected cells. IgG1 switching by P20 was reduced drastically compared with WT AID in the GFP⁺ populations (Fig. 1C). The 3C assay in spleen B cells confirmed the reduction of the Sᵥ–Sᵥ₁ interaction in cells expressing P20 compared with cells expressing WT AID (Fig. 3F). The bands amplified by PCR were isolated and sequenced to exclude PCR artifacts (Fig. S1). These results clearly indicate that the long-range interactions in the IgH locus that form the S region synapse are severely impaired by the C-terminal mutants of AID. Taken together, these data demonstrate that the C-terminal region of AID is required for the formation of the S–S synapse during CSR. Wuerffel et al. (30) also reported the reduced levels of S–S synapsis in AID⁺/⁺ spleen B cells. The authors speculated that the reduction of the interactions could be caused by the C-terminal scaffolding activity of the AID protein to mediate the long-range interaction in the IgH locus, although they did not suggest any molecular mechanism.

Reduction of the Critical DNA Synapse Factors in the Sᵥ Region by JP8Bdel. To confirm further the defect of synapse formation by AID C-terminal mutants, we tested the occupancy of the critical DNA synapse factors such as DNA PKcs and 53BP1 at the Sᵥ region (31–34). Using CH12F3-2 cells expressing JP8Bdel-ER, we found a drastic reduction of DNA synapse-forming proteins at the break sites compared with cells expressing WT AID (Fig. 2B). The results are consistent with the report that long-range microhomology is favored in the absence of 53BP1 and that 53BP1 protects DNA ends from resection (35, 36). We also examined the binding of UNG at the Sᵥ region in CH12F3-2 cells expressing JP8Bdel (Fig. 2B). In agreement with Ranjit et al. (37), our UNG ChIP data demonstrated that the accumulation of this protein is clearly AID dependent and that its accumulation is obviously reduced in cells expressing JP8Bdel compared with cells expressing WT AID. In contrast, the accumulation of the other protein, an essential mismatch protein, was enhanced in the Sᵥ region. Taken together, these results indicate that the C-terminal mutants of AID are defective in recruiting critical synapse factors such as 53BP1, DNA PKcs, and UNG to the DSB terminal mutants of AID. Taken together, these data demonstrate that the C-terminal region of AID is required for the formation of the S–S synapse during CSR. Wuerffel et al. (30) also reported the reduced levels of S–S synapsis in AID⁺/⁺ spleen B cells. The authors speculated that the reduction of the interactions could be caused by the C-terminal scaffolding activity of the AID protein to mediate the long-range interaction in the IgH locus, although they did not suggest any molecular mechanism.

C-Terminal Mutants of AID Reduced GC Efficiency. Because GC and CSR share similar mechanistic features, namely recombination, we next examined whether mutations in the C-terminal region of AID also affect HR during GC events. WT AID, P20, and JP8Bdel fused to ER were retrovirally introduced into AID⁻/⁻ DT40 cells. The infected cells were selected with puromycin, and subcloning was performed. In this experiment, we used the cl18 DT40 chicken cell line, which carries a frameshift mutation in the IgH locus, although they did not suggest any molecular mechanism.

To verify the slgM reversion observed by flow cytometry, we cloned and sequenced the IgVᵥ region from slgM⁺ cells. To enrich the slgM⁺ population in P20-infected cells, the reverted populations from cells stimulated by 4-OHT for 4 wk were single or bulk sorted, and the IgVᵥ region was analyzed (Fig. S2 and Table S1). As expected, the majority of the mutations were accumulated within CDR1 region (Fig. S2). Interestingly, we observed that the GC percentage relative to PM is reduced in both P20-infected and JP8Bdel-infected cells compared with cells infected with WT AID, although this reduction is not as drastic as their abolishment of CSR (Fig. 4 C and D and Table S1). The ratios of PM combined with insertion/deletion (Ins/Del) events...
to the GC combined with ambiguous mutations (Amb) were augmented significantly in mutants as compared with WT AID (P < 0.01 for WT AID vs. P20 and P < 0.001 for WT AID vs. JP8Bdel) (Fig. 4E). This partial defect in GC by C-terminal mutants of AID may be caused by the defect in generating DBS from SSB, because GC is known to be initiated by either DBS or SSB but probably is initiated more efficiently by DBS (38–40). Collectively, these data indicate that the C-terminal region of AID is required for the efficient processing of cleaved DNA ends to enable efficient recombination during CSR and GC.

Because we previously have shown that the CSR depends on de novo protein synthesis, we proposed that AID edits an mRNA to produce an unknown protein. We have shown that AID interacts with poly (A)-containing RNA at the C-terminal region of the AID protein (22). Considering our previous findings together with present results, we propose that mRNA edited by AID may encode an unknown factor that is essential for processing SSBs to DSBS that subsequently recruits synapse-forming proteins such as S3BP1, DNA PKcs, and UNG.

The Biochemical Step That Requires the Product of the C-Terminal Region of AID. It is tempting to speculate about the mechanism for generation of DSBS from AID-induced SSBs. We have provided a series of evidence that topoisomerase 1 (Top1) is responsible for generating SSBs immediately after AID activation (41, 42). Top1 generates SSBs only when it irreversibly forms a complex with the 3′ end of DNA. Top1 is known to associate covalently with non-B structure–forming DNA, which is abundant at repetitive sequences in the S region. For DNA repair, DNA-bound Top1 must be removed by proteasome, followed by TDP1/TPP digestion to expose the 3′ end of SSB (43–45). Because the S region is very rich in repetitive sequences, it can be assumed that SSB is generated on both strands of DNA within a reasonable distance that allows conversion of SSB to DSBS by digestion by either exonuclease or endonuclease, extension by DNA polymerase, or unwinding by DNA helicase. There are several exonucleases and endonucleases that may be involved in CSR, including the MRN complex, Apel, FEN1, and XPF-ERCC1. Because SHM is not affected by the C-terminal mutation of AID (7, 8, 16), it is unlikely to regulate the specific recruitment of any one of the enzymes that can convert SSB to DSBS. Collectively, the AID C-terminal region product may affect all the enzymes involved in DSBS processing.

The AID C-terminal product also may be involved in synapse formation after DSBS formation. Consistently, DSBSs generated by I-SceI (46) were not as efficient as those generated by AID, suggesting that AID may have another function in facilitating CSR. It is likely that the AID C-terminal product may share some functions with UNG, because both are required for efficient CSR and GC. However, UNG deficiency differs from the AID C-terminal mutation, having more severe phenotypes on GC (6), indicating that the two proteins have different roles after an AID-induced DNA break. Elucidation of the precise molecular function of the AID C-terminal production is critical for understanding the regulation of CSR.

Materials and Methods

Constructs, Transfection, and Retrovirus Infection. All AID proteins fused to GFP (used in spleen cell experiments) (17) or to the ER-purumycin-resistant gene (puro) (used in the DT40 experiment) have been described previously (8).

Cell Culture. Spleen B cells were obtained from 8- to 12-wk-old AID−/− mice with a C57BL6 background. B cells pretreated with LPS were retrovirally infected with WT AID and P20-GFP and were stimulated with LPS and IL-4 for 3 d. The DT40 −/− cell line (C18) (47) was retrovirally infected by AID or various AID C-terminal mutants and was cultured with RPMI medium 1640 containing l-glutamine (Invitrogen), Penicillin-Streptomycin Mixed Solution (Nacalai Tesque) supplemented with 10% (vol/vol) FCS (Gibco) and 1% chicken serum (Gibco) at 39 °C. Twenty-four hours after infection, cells were treated with puromycin (0.3 μg/mL) for 3 d and then were applied in limited dilution in 96-well plates for 1 wk. The established single clones were stimulated with 50 nM 4-OHT and incubated for 4 wk. Cell medium was refreshed every 2 d.

Analysis of Sμ–Sγ1 and Sμ–Sα4 Junctions. The Sμ–Sγ1 region was amplified using high-fidelity PrimeSTAR DNA polymerase (TaKaRa) as previously described (48). After sorting of the switched cells and DNA extraction, nested PCR was performed. The primers used for the amplification of the Sμ–Sγ1 region are listed in Table S2. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System and were cloned into pGEM-T Easy Vector (all from Promega). Sequencing was performed using magnetic beads (Anti-Mouse IgG1 Magnetic Particles-DM; BD IMag; BD Biosciences). All FACS analyses were carried out using FACSCalibur (Becton Dickinson). For analysis of the Sμ–Sα4 junction, Igα−/− cells were sorted by FACSaria (BD Biosciences). Chicken IgM antibody (Bethyl) was labeled with the Alexa Fluor 647 kit (A20173) according to the provided protocol. After puromycin selection and limited dilution all DT40 transclones from all DT40 transfectants were amplified by 4-OHT for 4 wk and were stained using chicken anti-IgM labeled with Alexa Fluor 647. To enrich the IgM+ population in P20 cells for sequencing, bulk or single sorting was performed by FACSaria (BD Biosciences).

IgV, GC, and PM Analysis. After 4-OHT treatment for 4 wk, the P20 transfectant was sorted. The bulk-sorted IgM+ cells were cultured for 5 d before genomic DNA (gDNA) extraction, whereas gDNA from the single-sorted cells was extracted immediately and used subsequently for nested PCR. The IgV, GC region was amplified and cloned according to the sorted method. For bulk-sorted cells, gDNA was extracted using phenol-chloroform protocol. The IgV, GC region was amplified (47), cloned, and analyzed as described above. For single-sorted cells, DNA was extracted using Tween20 and proteinase K. The rearranged IgV, fragments were amplified by nested PCR using 5′-GGTAT AACAG GGCAT GCCAC-GC CC-3′ (forward) and 5′-TACCC TCTC CATGG CGCA-3′ (reverse) as outer primer (35 cycles). The inner primer was the primer used for IgV, amplification in bulk-sorted cells (47). The PCR product was purified by the Exo-SAP-IT kit (78000; Affymetrix) and applied directly for sequencing as described above. All modifications within entire rearranged IgV, fragments were categorized into four groups: GC, Amb mutation, PM, or InDel. This classification is based on the accessibility of the pseudo V donor genes, searched in the database http://blast.ddbj.nig.ac.jp/blast/blastn?lang=en. The pseudo V donor can be considered as a template mutation tract harboring a >9-bp string homologous to the pseudo V donor was considered a GC. If there was only one hit in the sequence, and such a mutation was found in database, it was categorized as an Amb mutation. A mutation that was not found in donor pseudo genes was identified as a PM. In this categorization, any additional or deleted base along the IgV, region was considered an In or Del, respectively.

ChIP. We used the Active Motif ChIP-IT Express Kit according to the manufacturer’s instructions with slight modifications as reported elsewhere (49). Briefly, 5 × 106 CH12 AID/IJP8del ER cells were fixed for 5 min in the presence of 1% formaldehyde at room temperature. To quench the reaction, glycine to a final concentration of 0.125 M was added with rotation for 5 min. After the cross-linking reaction was washed, cell lysis and sonication were performed. The shearable samples were subjected to reverse cross-linking and were treated with proteinase K and RNaseA. Following electrophoresis, the sheared chromatin that yielded a smear between 200 and 1,000 bp was used for immunoprecipitation. Antibody (2–3 μg) was added to the lysate with rotation at 4 °C overnight. After the beads were washed, the immunoprecipitated DNA was used for real-time PCR using SYBR Green Master Mix (Applied Biosystems). The quantitative PCR signal was normalized by the input. The dataset was recalculated and the maximum value was considered as 1.0. Primers and antibodies used are listed in Tables S2 and S3, respectively.

3C Assay. The 3C assay was performed as described previously (30), with minor modifications in CH12 cells. Additional details of the 3C assay are given in SI Materials and Methods.
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