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Mismatch Repair Proteins and AID Activity Are Required for the Dominant Negative Function of C-Terminally Deleted AID in Class Switching

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Activation-induced cytidine deaminase (AID) is essential for class-switch recombination (CSR) and somatic hypermutation (SHM) of Ig genes. The AID C terminus is required for CSR, but not for S-region DNA double-strand breaks (DSBs) during CSR, and it is not required for SHM. AID lacking the C terminus (∆AID) is a dominant negative (DN) mutant, because human patients heterozygous for this mutant fail to undergo CSR. In agreement, we show that ∆AID is a DN mutant when expressed in AID-sufficient mouse splenic B cells. To have DN function, ∆AID must have deaminase activity, suggesting that its ability to induce DSBs is important for the DN function. Supporting this hypothesis, Msh2-Msh6 have been shown to contribute to DSB formation in S regions, and we find in this study that Msh2 is required for the DN activity, because ∆AID is not a DN mutant in msh2−/− cells. Our results suggest that the DNA DSBs induced by ∆AID are unable to participate in CSR and might interfere with the ability of full-length AID to participate in CSR. We propose that ∆AID is impaired in its ability to recruit nonhomologous end joining repair factors, resulting in accumulation of DSBs that undergo aberrant resection. Supporting this hypothesis, we find that the S–S junctions induced by ∆AID have longer microhomologies than those induced by full-length AID. In addition, our data suggest that AID binds Sµ regions in vivo as a monomer.


Mouse and human AID have a deaminase domain between aa 56 and 94, a C-terminal domain reported to be required for CSR, but not for SHM, between aa 182 and 198 (2, 5–7), and a nuclear export signal located at the C terminus between aa 190 and 198 (13, 14). AID predominantly resides in the cytoplasm, and nuclear AID is a DN mutant, some data suggest that AID functions as a dimer or tetramer (5, 9–12) and that perhaps the full-length AID and ∆AID heterodimerize and fail to perform a function or fail to interact with a protein essential for CSR (5). However, dimerization of full-length AID and ∆AID would not explain the very low level of CSR in human HIGM patients because both proteins are likely to be induced simultaneously; therefore, some dimers of full-length AID should be present.

IgM (HIGM) human patients, who cannot undergo CSR, are heterozygous for mutant AID proteins lacking 8 or more amino acids at the C terminus of AID (∆AID), indicating that ∆AID is a dominant negative (DN) mutant (5, 8). Although it is not known why ∆AID is a DN mutant, some data suggest that AID functions as a dimer or tetramer (5, 9–12) and that perhaps the full-length AID and ∆AID heterodimerize and fail to perform a function or fail to interact with a protein essential for CSR (5). However, dimerization of full-length AID and ∆AID would not explain the very low level of CSR in human HIGM patients because both proteins are likely to be induced simultaneously; therefore, some dimers of full-length AID should be present.

A relevant difference between CSR and SHM is that the latter does not require DSBs or recombination. During CSR, the deoxyuridines (dUs) in S regions generated by AID are converted into DSBs in the donor (Sµ) and acceptor Sx regions (17). The dU bases are excised by uracil-N-glycosylase (UNG), and UNG deficiency causes a dramatic reduction in S-region DSBs and CSR (18–20). Apurinic/apyrimidinic endonuclease 1 and 2 (APE1/2) can nick the abasic sites generated by UNG and are important
for creating S-region DSBs during CSR (21). When the single-strand breaks (SSBs) created by APE activity on opposite DNA strands within Sµ are sufficiently near, they can form a DSB, which can recombine with a DSB in a downstream S region. When the SSBs created by AID-UNG-APE activities are too far apart to spontaneously form a DSB, mismatch repair (MMR) proteins are thought to convert these distal SSBs into DSBs during CSR, and S-region DSBs are decreased by 50–80% in MMR-deficient B cells (17, 22–24). The DSBs in two different S regions are recombined by nonhomologous end joining (NHEJ), resulting in CSR.

Retrovirally transduced AID tagged at the C terminus with the estrogen receptor (ER) binds to S regions, as assayed by chromatin immunoprecipitation (ChIP) in aid<sup>−/−</sup> splenic B cells (25). In that study (25), we reported that AID associated poorly with S-region DNA. However, our current ChIP results, now obtained many times, show that AID associates with Sµ, as well as full-length AID. We cannot repeat the previous result and do not understand the basis of this discrepancy; we published a correction (26). All other results reported previously are reproducible. The new finding that AID binds Sµ is in better agreement with the fact that AID is competent for introducing mutations and DSBs at the Sµ and Sy3 regions (7, 25, 27, 28).

AID-ER introduces as many mutations in unrearranged (germline) Sµ in B cells induced to undergo CSR as does Aid-ER (7); thus, AID can produce the DNA substrate for UNG and Msh2-Msh6 (MutSα). However, these repair proteins bind poorly to Sµ, in cells expressing AID, indicating the importance of the C terminus for their binding (25, 29). Consistent with the dependence on the C terminus for the binding of UNG, there is an increase in the proportion of transition mutations at G:C bp in the Sµ region in cells expressing AID, as expected if UNG does not readily access the mutations (7). CSR is reduced 2–3-fold in the proportion of transition mutations at G:C bp in the Sµ region in cells expressing AID, as expected if UNG does not readily access the mutations (7). CSR is reduced 2–3-fold in mice lacking deaminase activity does not have DN function and does not associate stably with Sµ in aid<sup>−/−</sup> cells. We also find that the DN function of AID depends upon the ability of AID to bind Sµ and to induce DSBs in S regions, as well as indicate that AID-induced DSBs are not recombined by NHEJ. It is possible that the inability of these DSBs to recombine by NHEJ results in inefficient S–S recombination and generation of aberrantly resected DSBs that interfere with the ability of full-length AID to induce normal DSBs that can be recombined properly during CSR.

**Materials and Methods**

**Mice**

Mice were extensively (at least eight generations) backcrossed to C57BL/6 mice. AID-deficient mice were obtained from T. Honjo (Kyoto University, Kyoto, Japan) (1). Msh2-deficient mice were obtained from T. Mak (University of Toronto, Toronto, ON, Canada) (41). Msh6-deficient mice were obtained from R.M. Liskay (Oregon Health Sciences University, Portland, OR) (42). UNG-deficient mice were obtained from D. Barnes and T. Lindahl (London Research Institute, London, U.K.) (43). Msh6-deficient mice were obtained from W. Edelmann (Albert Einstein Medical College, New York, NY) (44). For each experiment, splenic B cells were isolated from littermates. Mice were housed in the Institutional Animal Care and Use Committee-approved specific pathogen-free facility at the University of Massachusetts Medical School; mice were used according to the guidelines from University of Massachusetts Medical School Institutional Animal Care and Use Committee.

**Abs**

Abs to ER (sc-8002X), GAPDH (sc-25778), Grb2 (sc-255), and Msh6 (sc-10798) were purchased from Santa Cruz, and Ab to lamin A/C was from Cell Signaling (#2032). Rabbit Abs to mouse AID (20) and UNG (23) were described previously. For ChIP experiments using untagged AID, we used rabbit AID Ab provided by J. Chaudhuri (The Rockefeller University, New York, NY) (10).

**Production of retroviruses in Phoenix-E cells**

pMX-PIE-AID-FLAG-ER-IRIS-GFP-puro and pMX-PIE-AID-FLAG-ER-FRS-GFP-puro (7) were received from Dr. V. Barreto and Dr. M. Nussenzeig (The Rockefeller University). The control retrovirus pMX-PIE-IRIS-GFP was constructed, and viruses were prepared as previously described (25). To create the AID<sup>DSDGEKESQ</sup> and AID<sup>DSDGEKESQ</sup> mutants, the AID-ER gene was subcloned into Bluescript (Stratagene), mutated using QuikChange (Stratagene), sequenced, and then reinserted into pMX-PIE. pMIG-AID and pMIG (45) were provided by J. Chaudhuri. pMIG-AID was created by converting amino acid position 189 to a nonsense codon.

**ChIP**

Live cells were isolated by flotation on Lymphocyte M (CEDARLANE, Burlington, ON, Canada) 24 h after RV transduction. After recovery and washing twice, ~2 10<sup>8</sup> live cells were resuspended in balanced salt solution and cross-linked with formaldehyde at a final concentration of 1% for 5 min at 37°C. Cross-linking was stopped by adjustment to 125 mM glycine and incubation for 5 min at room temperature. Cross-linked cells were intermediately sonicated at 4°C for a total of 22.5 min. Samples were filtered through glass wool. A total of 2 10<sup>8</sup> cell equivalents was incubated overnight with Ab at 4°C. On the following day, Protein G or Protein A Dynabeads (Invitrogen) were added to the samples and incubated for 2 h at 4°C. The beads were washed five times for 10 min, and reverse cross-linking was performed at 65°C in the presence of RNase A. The samples were treated with Proteinase K at 55°C, and DNA was recovered using phenol-chloroform extraction, followed by ethanol precipitation. ChIP results were assayed by real-time PCR using SYBR Green. Significance was calculated by a paired two-tailed t test. Primers for Sµ were forward primer DK99: 5'-AACCTAGGCTGCTAACAACGGAGATG-3' and reverse primer DK100: 5'-GTCACGTTAGCAGCATAGATTCA-3'. Primers for mb-1/CD79a were mb-1FW: 5'-CCACCGCATAGAGGAGAAGCTACA-3' and mb-1REV: 5'-CCCGGTACACTTCTCTGTTGACGG-3'. Primers for Cµ were forward primer Cµ-FG-P: 5'-TCTGACAGGAGGCAAGAAGACAGATTCTTA-3' and reverse primer Cµ-PG-R: 5'-GCCACGATTTCTATCACAGACAGGGG-3' (46), with the exception of the experiment shown in Fig. 5C, in which previously described Cµ primers were used (25).

**B cell purification and cultures**

Mouse splenic B cells were isolated from mouse cell depletion with Ab and complement (21). B cells were cultured at 10<sup>6</sup> cells/ml. LPS (25 µg/ml)
and anti-IgD dextran (0.3 ng/ml; Fina Biosolutions), with or without IL-4 (20 ng/ml), were used to induce CSR to IgG1 and IgG3, respectively. Human BlyS/BAFF (50 ng/ml; Human Genome Sciences) was included in all cultures. RV infection and assay of CSR by FACS was performed as previously described (25). Briefly, cells were activated for 2 d and then infected. One day later, they were harvested for all experiments. The one exception is noted in the Results. Statistical differences in CSR between different cultures were determined by a two-tailed t test.

Western blotting

Preparation of extracts and Western blots was described previously (21).

RT-PCR

RNA was isolated using TRIzol reagent (Ambion) from splenic B cells after 2 d of activation under IgG3 switching conditions. The RNA was treated with DNase I twice (DNA-free Kit, Ambion), and cDNA synthesis was performed using oligo d(10) and SuperScript II reverse transcriptase (Invitrogen). Primers to amplify endogenous AID mRNA were located in exon 4 (forward primer: 5'-AACTTCGCGCCATCTTTTG-3') and in the 3' untranslated region (reverse primer: 5'-CGTTGACATCTGAGGT-3'). Primers to amplify RV-AID-ER included the same forward primer as for endogenous mRNA, and the reverse primer was located in the ER tag (5'-GGTTGGCAGCTCTCATGTCT-3').

Ligation-mediated PCR

After culture for 2 d, viable cells were isolated by flotation on Ficoll-Hypaque gradients (ρ = 1.09) or Lympholyte (CEDARLANE); cells were embedded in low-melt agarose plugs, and DNA was isolated as described (20). For linker ligation, 50 μl 1X ligase buffer was added to the plugs, which were heated to 68°C to melt the agarose. A total of 20 μl DNA (∼10,000 cell equivalents) was added to 2 μl T4 DNA Ligase (2 Weiss units; MBI Fermentas, Hanover, MD), 10 μl double-stranded annealed linker in 1X ligase buffer, 5 μl 10X ligase buffer, 5 μl 50% PEG 4000, and 25 μl dH2O and incubated overnight at 16°C. Linker was prepared by annealing 5 nmol each LMPCR.1 (5'-GCCGTAACCGGAGGATCTGAAATC-3') and LMPCR.2 (5'-GAATTCAACCTG-3') in 300 μl 1X ligase buffer, which results in a double-stranded oligo with a 14-nucleotide single-strand overhang that can only ligate unidirectionally. Ligated DNA samples were heat inactivated at 70°C for 10 min, diluted three times in dH2O, and heated to 70°C for an additional 20 min. This sample was assayed for mbl DNA (primer sequences same as used for ChiP) by PCR to adjust DNA input prior to ligation-mediated PCR (LM-PCR). The primer 5' SA1 (5'-GCAGAAAATTTAGATAAAATGGATACCTCAGTGG-3') was used in conjunction with linker primer (LMPCR.1) to amplify DNA breaks. Three-fold dilutions of input DNA (0.5, 1.5, and 4.5 μl) were amplified by HotStar Taq (QIAGEN) using a touchdown PCR program. PCR products were electrophoresed on 1.2% agarose gels and blotted at 50°C, and washed at 70°C with 2X SSC/0.1% SDS.

Amplification and sequencing of Sa–Sa junctions

Genomic DNA (100 ng) from RV-transduced cells induced to switch to IgA (47) is amplified (in 12 reactions/genotype) using two nested PCRs (Expand Long Template System; Roche), using the same program for each PCR. Primers for the first round were 5u1: 5’-AATGGATACTCCTAGTGGTTTATTAGTGTTGTTA-3’ and 5R3: 5’-CCCATCCCATCCATCCATCC-3’, and primers for the second round were 5S2: 5’-AACAAAGCTTGCTTACCCAGTGTCAT; and 5R2: 5’-CCAGCCCGCTGCAGGCAATTT-3’. The products were cloned using the Topo TA cloning kit (Invitrogen) and sequenced by Macrogen.

Results

ΔAID-ER is a DN mutant in aid+/− cells

To study the DN effect of ΔAID, we transduced RV constructs encoding full-length AID-ER, ΔAID-ER, or the ER tag alone into wild-type (WT) mouse splenic B cells 2 d after activation to induce CSR. The cultures were treated with tamoxifen at the time of RV transduction to induce nuclear localization of ER-tagged proteins, allowing AID to reach its target. We harvested the cells 1 d after transduction, because we found this was optimal for their viability (25). We detected no consistent difference in cell viability or recovery from cultures expressing full-length AID, ΔAID, or the ER tag alone at this time point; it averaged ~90%. Two days after transduction, cell viability was reduced to ~70% among all cultures. To compare expression of the AID constructs, we prepared extracts of transduced WT cells for Western blot analysis of AID and ΔAID expression. Full-length AID-ER and ΔAID-ER are expressed at similar levels in cytoplasmic and nuclear extracts from WT cells with similar GFP expression (Fig. 1A, three leftmost lanes). Note that the ER-tagged constructs have two forms of protein, which probably are monomers and dimers, although their apparent molecular masses do not have a clear 2-fold relationship on these gradient polycrylamide gels. To determine whether the transduced AID-ER or ΔAID-ER affects endogenous AID levels, we also assayed endogenous AID in total cell extracts and did not find a difference among cells transduced with the three retroviruses (Fig. 1B). This indicates that expression of ΔAID does not cause degradation of endogenous AID, ruling out one possible explanation for the mechanism of its DN effect. Because our AID Ab is specific for the C terminus of AID, which is where the ER tag is located, we cannot detect transduced ΔAID with this Ab. To compare the levels of transduced AID-ER and endogenous AID, we compared their relative mRNA levels by quantitative RT-PCR. As shown in

![Figure 1](http://www.jimmunol.org/)
Fig. 1C, the levels of mRNAs for endogenous and transduced AID are similar. Taken together, the data in Fig. 1 indicate that endogenous AID, AID-ER, and ΔAID-ER are expressed at similar levels in aid+/+ cells 1 d after transduction and that ΔAID-ER does not cause degradation of endogenous AID.

To determine whether ΔAID-ER has a DN effect on CSR when expressed in WT mouse splenic B cells, we performed a dose-response assay to compare IgG1 and IgG3 CSR as a function of GFP mean fluorescence intensity (MFI), which is an indicator of AID and ΔAID expression levels. Increasing expression of full-length AID-ER results in increased IgG1 CSR (Fig. 2A, 2C), as previously reported (48), whereas increasing doses of ΔAID results in decreasing CSR to IgG1. Also, CSR to IgG3 increases with increasing expression of AID in WT B cells, but it decreases with increasing expression of ΔAID (Fig. 2B, 2D). These data demonstrate that ΔAID has a DN effect on CSR in WT mouse B cells. The weaker DN effect that we observed relative to the nearly complete inhibition of CSR found in most HIGM patients with the heterozygous C-terminal AID deletion (5, 8) is likely due to the fact that, in our experiments, endogenous full-length AID is expressed prior to RV transduction. Endogenous AID is already highly expressed in mouse splenic B cells 2 d after treatment with switch inducers (20), which is the day of RV transduction and 1 d prior to harvesting.

Untagged ΔAID has DN activity

To better compare with endogenous ΔAID in HIGM patients, we tested whether an untagged ΔAID expressed in the pMIG retrovirus would show DN function in RV-transduction experiments. Fig. 3A shows that CSR to IgG3 and to IgG1 is significantly lower in WT cells expressing ΔAID than in cells expressing the empty pMIG, demonstrating that the DN effect is not dependent upon the ER tag. In these experiments, we did not add tamoxifen to the cultures, and we infected the cells 1 d after activation and harvested them 2 d later. The increased duration of infection used in experiments with untagged AID (2 d instead of 1 d) was required to obtain the DN effect with these constructs, probably because, unlike ER-tagged ΔAID, untagged ΔAID appears to be poorly expressed, as reported previously for human ΔAID (29, 39). This is consistent with the facts that ΔAID lacks a nuclear export signal, and AID is degraded more rapidly in nuclei than in cytoplasm (15). We could not examine the expression of ΔAID di-

FIGURE 2. Dose-response assays of IgG1 (A and C) and IgG3 (B and D) switching in aid+/+ mouse splenic B cells expressing increasing levels of AID-ER or ΔAID-ER, as assayed by GFP expression (MFI). Each data point in (A) and (B) represents the average percentage of IgG1 or IgG3 (± SEM) at the indicated MFI [binned as shown in (C) and (D)] of three cultures from one mouse transduced with the indicated retrovirus relative to the percentage of CSR in cells transduced with the empty ER retrovirus. Data points on the y-axis (MFI = 2.5) in (A) and (B) represent CSR in GFP− cells expressing only endogenous AID. CSR in the GFP− cells are shown in the leftmost boxes of the FACS dot plots in (C) and (D). The procedure used for gating is illustrated in Fig. 7A.
directly, because our AID Ab is directed against the C terminus. We tested the two commercially available Abs that were reported to be against different epitopes in mouse AID; however, unlike our AID Ab, they did not detect mouse AID in our Western blots. Again, we did not detect consistent differences in cell viability or recovery postinfection with the different viruses, although the cells were less viable when harvested 2 d postinfection (∼70%) than when harvested after 1 d (∼90%), independent of the particular AID expression construct. Although it was speculated that untagged D\(_{\text{AID}}\) poorly induces CSR because of its low expression, the finding that both D\(_{\text{AID}}\)-ER and untagged D\(_{\text{AID}}\) poorly induce CSR, and both have DN effects on CSR in WT cells, indicate that the phenotype of D\(_{\text{AID}}\) is not solely due to its low expression.

Because untagged D\(_{\text{AID}}\) might be less stable than D\(_{\text{AID}}\)-ER, we asked whether untagged D\(_{\text{AID}}\) might destabilize endogenous AID. As shown in Fig. 3D, the levels of endogenous AID in WT cells expressing D\(_{\text{AID}}\) are not reduced relative to untransduced WT cells or cells transduced with pMIG, similar to the results obtained with D\(_{\text{AID}}\)-ER and confirming that D\(_{\text{AID}}\) does not exert a DN effect by causing degradation of endogenous AID.

We used LM-PCR to determine whether untagged D\(_{\text{AID}}\) induces DSBs in the S\(_{\mu}\) region in aid\(^{-/-}\) cells and found that, although it does, there are many fewer than in cells expressing full-length AID (Fig. 3B). This result differs from what we observed in transduced with untagged AID and D\(_{\text{AID}}\) expressed in the retrovirus pMIG and then probed with Ab to AID and to Grb2 for loading controls. Cells were cultured and transduced as in (A), but similar results were observed if cells were cultured for 2 d prior to transduction and harvested 1 d later.

FIGURE 3. Untagged D\(_{\text{AID}}\) has DN activity, induces very few S\(_{\mu}\) DSBs, and does not cause degradation of endogenous AID. (A) Compilation of IgG1 and IgG3 CSR results (+SEM) for the untagged D\(_{\text{AID}}\) in WT cells relative to CSR in cells expressing pMIG. The control retrovirus pMIG does not affect CSR significantly, because GFP^+ cells switched similarly to cells transduced with pMIG (data not shown). In these experiments, cells were activated to switch for 24 h, transduced, and harvested 2 d later. The procedure for gating is illustrated in Fig. 7A. In addition, we gated on the brightest GFP^+ cells (∼50% of the cells). Results from two independent experiments (two mice) with two cultures each are shown. (B) LM-PCR assay of S\(_{\mu}\) DSBs in aid\(^{-/-}\) cells induced to switch to IgG3 transduced with untagged AID, D\(_{\text{AID}}\), or the empty retrovirus pMIG. Transduction was performed on day 2 after activation, and cells were harvested on day 3. The vertical line indicates where the image was cut to remove irrelevant lanes. Three-fold titrations of input DNA were used, and the mb-1 gene was amplified as an internal control for template input. The mb-1 PCR bands shown were obtained by electrophoretic analysis on a QIAxcel Advanced instrument, which subjects each sample to electrophoresis in a capillary and provides an image and quantitation of each lane. (C) LM-PCR assay of S\(_{\mu}\) DSBs in aid\(^{-/-}\) cells induced to switch to IgG3 transduced with AID-ER, D\(_{\text{AID-ER}}\), or the control retrovirus ER. Methods used were similar to those described for (B). (D) Whole cell extracts of WT and KO aid\(^{-/-}\) cells expressing untagged AID and D\(_{\text{AID}}\) constructs (left) or pMXpie expressing ER-tagged AID constructs (right). Methods used were similar as described in Fig. 3.

FIGURE 4. LM-PCR assays of S\(_{\mu}\) DSBs in WT (aid\(^{+/+}\)) or aid\(^{-/-}\) cells induced to switch to IgG3 transduced with pMIG expressing untagged AID constructs (A) or pMXpie expressing ER-tagged AID constructs (B). Methods are the same as described in Fig. 3.
cells transduced with ΔAID-ER, in which the DSB frequency is similar to that observed in cells transduced with AID-ER (Fig. 3C) (25). This difference is consistent with the likely reduced expression of untagged ΔAID relative to that of ΔAID-ER. Note that LM-PCR only detects DSBs that are blunt; if T4 DNA polymerase (T4 Pol) is added prior to ligation, staggered DSBs can be detected. However, addition of T4 Pol does not change the relative frequency of DSBs induced by AID-ER or ΔAID-ER (25). If ΔAID induces extensively end-resected DSBs, as suggested by the results of Zahn et al. (29), these DSBs would not be detected by this assay.

To determine whether ΔAID might affect Sm DSBs detected in WT cells, we performed LM-PCR experiments in WT cells transduced with untagged and tagged AID viruses, using T4 Pol to fill in staggered ends. In WT cells, we detected similar numbers of DSBs in cells expressing ΔAID as in cells expressing full-length AID, whether ΔAID was untagged (Fig. 4A) or tagged (Fig. 4B). Because the intensity of the signals is slightly greater in cells expressing full-length AID, it is possible that these DSBs are slightly more numerous or more efficiently amplified. Similar results were obtained in the absence of T4 Pol (data not shown). Again, extensively end-resected DSBs would not be detected. As pointed out previously (25, 27), the role of the C terminus of AID appears to manifest itself subsequent to DSB formation.

**ChIP assays show that ΔAID binds Sm in WT cells**

To further explore the mechanism of the DN effect, we asked whether ΔAID-ER binds Sm in WT cells expressing endogenous AID or whether endogenous AID might compete and, thereby, inhibit its binding. WT B cells transduced with ER were used as

**FIGURE 5.** Both the DN effect and association of ΔAID-ER with Sm depend upon deaminase activity of ΔAID-ER. (A) ChIP of AID-ER proteins at Sm and Cm in WT cells relative to input DNA using anti-ER Ab. Error bars indicate SEM. Five ChIPs (two independent experiments, two mice) were performed, with the exception of the no Ab control for FL-AID, for which four were performed. ChIP was analyzed by quantitative PCR; percentage input was calculated, and percentage input in the absence of Ab was subtracted. (B) ChIP of the AID-ER proteins at Sm and Cm in aid−/− cells relative to input DNA. Six ChIPs for AID, ΔAID, and ER and three ChIPs for AID-RQ and ΔAID-RQ were performed (two mice). Analysis was done as in (A). (C) ChIP experiments performed with aid−/− cells transduced with untagged AID pMIG constructs, immunoprecipitated with anti-AID Ab (10). Error bars indicate SEM. Five or six ChIPs (two independent experiments, two mice) were performed. ChIPs were analyzed by quantitative PCR; percentage input was calculated, and percentage input in the absence of Ab was subtracted. The p values were determined by the two-tailed t test. (D) Western blots of AID-ER in nuclear, cytoplasmic, and whole-cell extracts from transduced WT splenic B cells. A total of 20 μg protein was loaded in each lane. *Unknown irrelevant protein.
negative controls. As shown in Fig. 5A, ΔAID-ER is recruited at least as well as AID-ER to the Sm region in AID-sufficient cells, similar to results in aid<sup>−/−</sup> cells (Fig. 5B). Binding of AID and ΔAID was not detected at the Cµ<sup>-</sup> gene in WT or aid<sup>−/−</sup> cells.

**ΔAID lacking deaminase activity is not a DN mutant and does not bind Sm in WT cells**

We previously showed that AID lacking deaminase activity due to mutations in the catalytic domain, AID<sub>H56R/E58Q</sub> (AID<sup>RQ</sup>) (10, 49), does not bind to Sm and Sy3 in our ChIP assays in aid<sup>−/−</sup> cells (25) (Fig. 5B). This result differs from that reported by Vuong et al. (50), who found that AID<sup>RQ</sup> expressed without a tag binds similarly as WT AID to Sm; therefore, we asked whether the difference was due to the ER tag. However, we found that un-tagged AID<sup>RQ</sup> is also unable to bind Sm under our ChIP conditions (Fig. 5C). There are several differences between the ChIP conditions in our laboratory and those of Vuong et al. (50), and we do not know which ones are important. Taken together, the data from our laboratory and the Chaudhuri laboratory (50) suggest that AID<sup>RQ</sup> binds Sm but less stably than WT AID.

We next asked whether ΔAID<sup>RQ-ER</sup> would have DN function by analyzing CSR in WT cells transduced with ΔAID<sup>RQ</sup> in comparison with ER and full-length AID<sup>RQ</sup> and ΔAID. Fig. 5D shows the combined CSR data, normalized to CSR in cells expressing ER alone. Again, CSR in cells expressing ΔAID is significantly lower than in cells expressing ER. However, there is no DN effect observed in cells expressing ΔAID<sup>RQ</sup> or AID<sup>RQ</sup>. This is consistent with our finding that ΔAID<sup>RQ</sup> or AID<sup>RQ</sup> do not stably associate with Sm in WT cells (Fig. 5A). As shown in the Western blot in Fig. 5E, the AID<sup>RQ-ER</sup> proteins are expressed at similar levels to ΔAID-ER in nuclei, cytoplasm, and whole-cell extracts, ruling out the possibility that ΔAID<sup>RQ</sup> does not have a DN effect due to lack of expression. These data suggest that the DN effect depends upon the ability of ΔAID to bind Sm and/or the ability of ΔAID to deaminate cytosines and to induce Sm DSBs.

If AID<sup>RQ</sup> and ΔAID<sup>RQ</sup> heterodimerize with endogenous AID, they might be expected to bind Sm in WT cells. Although AID and ΔAID are detected at Sm in WT cells, neither AID<sup>RQ</sup> nor ΔAID<sup>RQ</sup> binds (Fig. 5A), suggesting that a functional deaminase domain must be present in both partners of the heterodimer for detectable binding to Sm, that endogenous WT AID does not heterodimerize with catalytically inactive AID, or that AID functions as a monomer.

**Sm–Sα junctions show increased microhomology in cells expressing ΔAID**

Because the deaminase activity of AID is essential for S-region DNA breaks, we hypothesized that the DN function of ΔAID depends upon its ability to induce S-region breaks. Perhaps the breaks are aberrantly processed and cannot participate in S–S recombination by NHEJ. They might interfere with normal S–S recombination induced by full-length AID. To begin to address this possibility, we examined the S–S junctions in aid<sup>−/−</sup> cells expressing ΔAID to determine whether they showed increased amounts of microhomology (MH) as would be expected if they were not recombined by NHEJ, which is the predominant mechanism for recombining S–S junctions. We examined Sm–Sα junctions because Sα has the greatest amount of homology to Sm of any S region, thus increasing the sensitivity of the assay (51). Indeed, we found that Sm–Sα junctions showed an average of 2.3-fold greater lengths of MH in aid<sup>−/−</sup> cells expressing ΔAID-ER compared with cells expressing AID-ER (Fig. 6C), similar to observations in human HIGM patients expressing both C-terminally deleted AID and full-length AID (35), as well as recent reports studying a human AID with a 17-aa C-terminal deletion in mouse aid<sup>−/−</sup> B cells (29, 52). We also examined junctions in cells expressing untagged ΔAID and found a 2.8-fold increase in MH lengths in comparison with cells expressing full-length untagged AID (Fig. 6C). There also were significant differences in the distribution of MH lengths between cells expressing AID and ΔAID, whether it was tagged or untagged (Fig. 6A, 6B, respectively). Note that in cells expressing ΔAID, we observed junctions with up to 15 and 27 bp of MH. These data suggest that the AID C terminus is involved in directing S-region DSBs toward recombination by NHEJ, as suggested previously (29, 35, 52).

**The DN effect of ΔAID depends on the presence of Msh2**

Msh2 and Msh6 are required for optimal CSR in B cells expressing full-length AID-ER but do not contribute to CSR in B cells expressing ΔAID-ER, as indicated by our laboratory and the Chaudhuri laboratory (50). They might interfere with normal S–S recombination by NHEJ. They might interfere with normal S–S recombination by NHEJ. They might interfere with normal S–S recombination by NHEJ. They might interfere with normal S–S recombination by NHEJ.

**FIGURE 6.** MH at Sm–Sα junctions in aid<sup>−/−</sup> cells transduced with AID-ER, ΔAID-ER, and untagged AID and ΔAID. (A) Percentage of junctions with the indicated lengths of MH cloned from aid<sup>−/−</sup> B cells (29, 52). We also examined junctions in cells expressing untagged ΔAID and found a 2.8-fold increase in MH lengths in comparison with cells expressing full-length untagged AID (Fig. 6C). There also were significant differences in the distribution of MH lengths between cells expressing AID and ΔAID, whether it was tagged or untagged (Fig. 6A, 6B, respectively). Note that in cells expressing ΔAID, we observed junctions with up to 15 and 27 bp of MH. These data suggest that the AID C terminus is involved in directing S-region DSBs toward recombination by NHEJ, as suggested previously (29, 35, 52).

The DN effect of ΔAID depends on the presence of Msh2.
expressing ∆AID-ER (25). It is clear that at least one of the roles of MMR in CSR is to increase S-region DSBs, most likely by converting distal SSBs to DSBs (17, 22–24). Furthermore, in the absence of the MMR proteins Mlh1 or Pms2, S–S junctions show increased MH (53–55), similar to the junctions in cells expressing AID. To determine whether the DN effect depends on MMR, we expressed AID-ER and ∆AID-ER in AID-sufficient msh2<sup>−/−</sup> or mlh1<sup>−/−</sup> B cells and analyzed CSR by flow cytometry. Fig 7A

FIGURE 7. FACS analyses of CSR in WT, msh2<sup>−/−</sup>, and mlh1<sup>−/−</sup> cells demonstrate that the DN effect of ∆AID-ER requires the presence of Msh2. (A) Gating strategy used for all FACS experiments in this article, proceeding from left to right. Example is from (D): WT cells transduced with AID-ER transduced and induced to switch to IgG1. Retrovirally infected cultured cells were stained with 7-aminoactinomycin D (7AAD) to detect dying/dead cells and with Ab F(ab’)<sub>2</sub> to IgG1 or IgG3 conjugated to PE. The percentage of viable (7AAD<sup>−</sup>) retrovirally infected (GFP+) cells that were IgG1+ or IgG3+ was determined as shown. Compensation and gating were performed using FlowJo software (TreeStar). (B) FACS results for one representative CSR experiment comparing the DN effect of ∆AID-ER in WT and msh2<sup>−/−</sup> B cells (expressing endogenous AID). The FACS plots show only viable and GFP<sup>+</sup> cells; the gates represent PE-IgG1/PE-IgG3+ cells within the GFP<sup>+</sup> populations, as indicated. The entire GFP<sup>+</sup> population was analyzed in these experiments. (C) Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated constructs in WT and msh2<sup>−/−</sup> cells relative to CSR in WT cells expressing AID-ER. Two independent experiments (two mice, three cultures each) were performed. IgG1 and IgG3 CSR are significantly increased in WT cells expressing AID-ER relative to ER (p = 0.004 and p = 0.011, respectively). IgG1 and IgG3 CSR in msh2<sup>−/−</sup> cells expressing AID-ER relative to ER were not significantly increased (p = 0.065 and p = 0.284, respectively). (D) FACS results for one experiment comparing the DN effect of ∆AID-ER in WT and mlh1<sup>−/−</sup> B cells. (E) Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated constructs in WT and mlh1<sup>−/−</sup> cells relative to CSR in WT cells expressing AID-ER. Three independent experiments (three mice, three cultures each) were performed. IgG1 and IgG3 CSR were significantly increased in WT cells expressing AID-ER relative to ER (p = 0.002 and p = 0.027, respectively). IgG1 CSR in mlh1<sup>−/−</sup> cells expressing AID-ER relative to ER was significantly increased (p = 0.004), but IgG3 CSR was not (p = 0.256). SSC, side scatter.
shows the gating strategy used in these experiments. Fig. 7B and 7D provide examples of the switching results in the GFP + transduced cells, and Fig. 7C and 7E present the compiled results. Demonstrating the DN effect, CSR is significantly lower in WT cells expressing ΔAID than in cells expressing ER (Fig. 7C, 7E). However, ΔAID does not have a DN effect in msh2 mutant cells, because CSR is not significantly lower in ΔAID-expressing msh2 mutant cells than in ER-expressing cells (Fig. 7B, 7C).

The levels of RV-AID proteins or endogenous AID in transduced cells are not affected by lack of the MMR protein Msh2 (Fig. 1A, 1B). In msh2 mutant cells, the DN effect borders on significance (Fig. 7E), perhaps due to the presence of Msh2-Msh6 in these cells. MMR is partially functional in the absence of Mlh1-Pms2 (17, 56). These results suggest that the DN effect of ΔAID in AID-sufficient cells depends upon a function that Msh2 provides. At first glance, this seems surprising because we showed previously that MMR does not contribute to CSR induced by ΔAID and because the AID C terminus is important for recruiting MMR proteins to S regions (25). However, endogenous AID can recruit MMR proteins in cells expressing ΔAID; thus, it is possible that the ability of MMR to increase S-region DSBs is important for the DN effect.

**Discussion**

ΔAID functions as a DN mutant in HIGM patients that retain one allele encoding WT AID (5), and we addressed the mechanism of this DN effect in this study. We show that the DN effect of ΔAID requires Msh2 and that ΔAID must have deaminase activity. The deaminase activity is required for binding of ΔAID-ER, Msh2-Msh6, and UNG to Sp, in our ChIP assays (25) (and data herein). However, deaminase activity is also essential for AID to induce DNA breaks, suggesting that the DN effect might require the induction of DNA breaks. Consistent with this hypothesis, MMR proteins are important for creating DSBs during CSR (23).

We showed that Msh2 and Msh6 are recruited to Sp, dependent upon both the C terminus of AID and deaminase activity (25). Thus, it seemed surprising that the DN effect of ΔAID depends upon Msh2, because ΔAID poorly recruits Msh2 to Sp. However, full-length endogenous AID would recruit Msh2-Msh6 in cells expressing ΔAID, which could increase S-region DSBs. Thus, the dependence of the DN effect upon Msh2 supports the hypothesis that DSBs are required for the DN effect. MMR is normally involved in postreplicative repair during S phase, whereas CSR occurs during G1 phase (23, 57), so it is possible that MMR must be specifically recruited to increase DSBs during CSR. Because ΔAID does not recruit UNG to Sp as well as does full-length AID (25, 29, 52), it is also likely that ΔAID inefficiently induces SSBs. Thus, it is possible that, in cells expressing haploid amounts of both AID and ΔAID, DSBs might be even more dependent upon MMR compared with cells expressing full-length AID. MMR appears to help generate DSBs that can be recombined by NHEJ and perhaps contributes to recruitment of NHEJ proteins, because S–S junctions in cells lacking Mlh1 or Pms2 show increased MH, similar to those in cells lacking NHEJ proteins (53, 54, 58, 59).

It is clear that the DSBs induced in ΔAID cells expressing ΔAID cannot undergo efficient S–S recombination by NHEJ, because CSR is 90% reduced compared with cells expressing full-length AID, and the S–S junctional MH is increased. It is possible that AID, but not ΔAID, recruits NHEJ proteins, which normally perform S–S recombination. Thus, in cells expressing ΔAID, DSBs might undergo extensive end resection. We hypothesize that the reason why the numbers of DSBs in S regions in both ΔAID and WT cells expressing ΔAID-ER appear similar in LM-PCR experiments to those in cells expressing AID-ER is because, although DSBs are created less efficiently as a result of poor recruitment of UNG and MMR, those that are made are also repaired less efficiently as a result of poor recruitment of NHEJ. Also, ΔAID was reported to have higher deaminase activity than full-length AID (28, 29). To explain how the putative inability of ΔAID to recruit NHEJ proteins could have a DN effect, it is possible that aberrantly resected DSBs predominate over breaks induced by endogenous full-length AID, because of their inability to recombine efficiently, and these DSBs interfere with recombination by NHEJ. Fig. 8 presents an outline model of this hypothesis.

UNG and Msh2 do not appear to bind AID directly, and we only detected their interaction with AID by ChIP, dependent upon the C terminus of AID (25). The fact that deaminase activity is required for binding of Msh2 to Sp, in our ChIP experiments (25) and data herein), suggests that the DN effect might require the induction of DNA breaks. Consistent with this hypothesis, MMR proteins are important for creating DSBs during CSR (23).

**FIGURE 8.** Model for role of the AID C terminus in the introduction of S-region DSBs and S–S recombination. (A) Cells expressing full-length AID. (B) Cells expressing ΔAID.

**TABLE 1.** Summary of the results from the experiments shown in Fig. 7.
sible that NHEJ proteins are also part of a complex that is recruited by AID, dependent upon the C terminal and deaminase activity. DNA-PKcs, a DNA-dependent kinase, is very important for NHEJ (60). It was reported to bind to the AID C terminal, and only in the presence of DNA (34). Consistent with this, DNA-PKcs was shown to associate in ChIP assays with Sg in CH12 B lymphoma cells expressing full-length AID but not human ΔAID (52). Also, KU70 was recently shown to bind by ChIP at Sg in mouse splenic B cells expressing human AID but not ΔAID (29). Recent evidence indicates that APE1 and AID interact directly, dependent upon phosphorylation of AID at S38 (50). Thus, NHEJ proteins could be recruited as part of a complex that converts AID-induced dsDs to DSBs and then forms S–S junctions.

If AID functions as a dimer, then AID lacking deaminase activity should be able to heterodimerize with endogenous WT AID, because the catalytic site is not located in regions thought to be involved in AID dimerization (11, 12) or in DNA binding (61). However, because we did not detect binding of AID lacking deaminase activity (AIDΔDQ) to S regions in WT cells, one possible explanation for our results is that both subunits of the putative AID dimer must have deaminase activity for AID to bind detectably to Sg. If heterodimers of ΔAIDΔDQ and AID cannot bind DNA, they should not interfere with the function of WT AID, because transduced AID was not expressed in excess over endogenous AID in our experiments. Alternatively, our results could be interpreted to indicate that AID functions as a monomer. Although several reports suggested that AID functions as a dimer or tetramer (5, 9, 62), another report indicated that purified monomer. Although several reports suggested that AID functions as a monomer. However, our results is that both subunits of the putative AID dimer must have deaminase activity for AID to bind detectably to Sg. If heterodimers of ΔAIDΔDQ and AID cannot bind DNA, they should not interfere with the function of WT AID, because transduced AID was not expressed in excess over endogenous AID in our experiments. Alternatively, our results could be interpreted to indicate that AID functions as a monomer. Although several reports suggested that AID functions as a dimer or tetramer (5, 9, 62), another report indicated that purified monomer.

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Disclosures

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