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

Anna J. Ucher, Sanjay Ranjit, Tatenda Kadungure, Erin K. Linehan, Lyne Khair, Elaine Xie, Jennifer Limauro, Katherina S. Rauch, Carol E. Schrader and Janet Stavnezer

J Immunol 2014; 193:1440-1450; Prepublished online 27 June 2014;
doi: 10.4049/jimmunol.1400365
http://www.jimmunol.org/content/193/3/1440

References  This article cites 63 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/193/3/1440.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2014 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Mismatch Repair Proteins and AID Activity Are Required for the Dominant Negative Function of C-Terminally Deleted AID in Class Switching

Anna J. Ucher, Sanjay Ranjit, Tatenda Kadungure, Erin K. Linehan, Lyne Khair, Elaine Xie,1 Jennifer Limauro,2 Katherina S. Rauch,3 Carol E. Schrader, and Janet Stavnezer

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 (ΔAID) is a dominant negative (DN) mutant, because human patients heterozygous for this mutant fail to undergo CSR. In agreement, we show that ΔAID is a DN mutant when expressed in AID-sufficient mouse splenic B cells. To have DN function, ΔAID 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 ΔAID is not a DN mutant in msh2−/− cells. Our results suggest that the DNA DSBs induced by ΔAID are unable to participate in CSR and might interfere with the ability of full-length AID to participate in CSR. We propose that ΔAID 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 ΔAID 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. The Journal of Immunology, 2014, 193: 1440–1450.

A

citivation-induced cytidine deaminase (AID) initiates Ab gene diversification after immunization or infection by deamination of cytosines in Ig S regions, leading to double-strand breaks (DSBs) and class-switch recombination (CSR), as well as in recombined V(D)J gene segments, leading to somatic hypermutation (SHM) (1–4). It has been known for several years that the C terminus of AID is required for CSR, although it does not appear to have a role during SHM of Ab genes (5–7). Some hyper-

Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA 01655
1Current address: Queen’s University Medical School, Kingston, Ontario, Canada.
2Current address: Stemtech, Charlotte, NC.
3Current address: Center for Chronic Immunodeficiency, University Medical Center Freiburg, University of Freiburg, Freiburg, Germany.

Received for publication February 7, 2014. Accepted for publication June 3, 2014.

This work was supported by National Institute of Allergy and Infectious Diseases Grants R01 AI023283 and R21 AI099988 (to J.S.) and R03 AI092528 (to C.E.S.). J.L. and E.X. were supported by the Summer Undergraduate Research Experience fellowship program, funded by the University of Massachusetts Medical School.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Address correspondence and reprint requests to Dr. Janet Stavnezer, Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA 01655. E-mail address: janet.stavnezer@umassmed.edu

Abbreviations used in this article: AID, activation-induced cytidine deaminase; ΔAID, activation-induced cytidine deaminase lacking the C terminus; APE1/2, apurinic/apyrimidinic endonuclease 1 and 2; ChIP, chromatin immunoprecipitation; CSR, class-switch recombination; DN, dominant negative; DSI, double-strand break; dU, deoxuryridine; ER, estrogen receptor; HIGM, hyper-IgM; LM-PCR, ligation-mediated PCR; MFI, mean fluorescence intensity; MH, microhomology; MMR, mismatch repair; Msh2-Msh6; NHEJ, nonhomologous end joining; RV, retroviral; SHM, somatic hypermutation; SSB, single-strand break; T4 Pol, T4 DNA polymerase; UNG, uracil-N-glycosylase; WT, wild-type.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

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 (ΔAID), indicating that ΔAID is a dominant negative (DN) mutant (5, 8). Although it is not known why ΔAID 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 ΔAID 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 ΔAID 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.

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 also located at the C terminus between aa 190 and 198 (13, 14). AID predominantly resides in the cytoplasm, and nuclear AID undergoes rapid ubiquitin-mediated proteasomal degradation, causing the half-life of nuclear AID to be three times shorter than that of cytoplasmic AID (15). However, nuclear export is not required for CSR, because mouse AIDF198A is not exported from nuclei, and yet CSR and SHM are only modestly reduced in cells expressing this mutant (14). Also, some C terminus substitution mutants that retain a functional nuclear export signal cannot potentiate CSR (16).

A relevant difference between CSR and SHM is that the latter does not require DSBs or recombination. During CSR, the deoxouridines (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−/− splenic B cells (25). In that study (25), we reported that ΔAID associated poorly with S-region DNA. However, our current ChIP results, now obtained many times, show that ΔAID 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 ΔAID binds Sµ is in better agreement with the fact that ΔAID is competent for introducing mutations and DSBs at the Sµ and Sγ3 regions (7, 25, 27, 28).

ΔAID-ER introduces as many mutations in unarranged (germline) Sµ in B cells induced to undergo CSR as does AID-ER (7); thus, ΔAID can produce the DNA substrate for UNG and Msh2-Msh6 (MutSox). However, these repair proteins bind poorly to Sµ in cells expressing ΔAID, 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 ΔAID, as expected if UNG does not readily access the mutations (7). CSR is reduced 2–3-fold in Msh2 or Msh6-deficient B cells relative to MutSα readily access the mutations (7). CSR is reduced 2–3-fold in Msh2 or Msh6-deficient B cells relative to MutSα.

ΔAID-ER introduces as many mutations in unarranged (germline) Sµ in B cells induced to undergo CSR as does AID-ER (7); thus, ΔAID can produce the DNA substrate for UNG and Msh2-Msh6 (MutSox). However, these repair proteins bind poorly to Sµ in cells expressing ΔAID, 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 ΔAID, as expected if UNG does not readily access the mutations (7). CSR is reduced 2–3-fold in Msh2 or Msh6-deficient B cells relative to MutSα (25). However, when ΔAID is expressed, the small amount of CSR induced (~10% of full-length AID) is not reduced further in MutSox-deficient cells. This indicates that MMR does not contribute to CSR efficiency in cells expressing ΔAID, consistent with the poor binding of Msh2 and Msh6 to Sµ in cells expressing ΔAID (25).

It is not understood why the AID C terminus is required for CSR, nor is it understood how its deletion creates a DN mutation. Aid−/− B cells transduced with ΔAID-ER have S-region DSBs (25, 27), indicating that the AID C terminus is important for events after DSB formation. However, it is possible that S-region DSBs are aberrantly processed and/or inefficiently introduced in cells expressing ΔAID and that they accumulate as a result of lack of repair and/or inefficient S–S recombination. Thus, the C terminus might be required for recruiting proteins involved in producing nonresected DSBs that are appropriate for NHEJ and/or for recruiting proteins involved in the end-joining process itself, as previously suggested (29, 34, 35). If AID functions as a dimer, it is possible that a heterodimer of AID and ΔAID cannot recruit these proteins. Numerous proteins were demonstrated to associate with AID (36), and several require the AID C terminus for their association (14, 37, 38). These proteins were shown to help export AID from the nucleus (14, 39), help maintain it in the cytoplasm (38), or help recruit AID to S regions (37, 40).

In this article, we show that the DN function of ΔAID observed in humans is also found in mouse B cells, and we also show that ΔAID lacking deaminase activity does not have DN function and does not associate stably with Sµ in aid−/− cells. We also find that the DN phenotype of ΔAID depends upon the MMR protein Msh2. Our results suggest that the DN function depends upon the ability of ΔAID to bind Sµ and to induce DSBs in S regions, as well as indicate that ΔAID-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, 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 Ab Ab provided by J. Chaudhuri (The Rockefeller University, New York, NY) (10).

Production of retroviruses in Phoenix-E cells

pMX-PID-FLAGER-ER-ires-GFP-puro and pMX-PID-AID-ER-ires-GFP-puro (7) were received from Dr. V. Barreto and Dr. M. Nussenzweig (The Rockefeller University). The control retrovirus pMX-PID-ER-IRES-GFP was constructed, and viruses were prepared as previously described (25). To create the ΔAID(326DE366) and AIDH56R/E58Q mutants, the AID-ER gene was subcloned into Bluescript (Stratagene), mutated using Quik-Change (Stratagene), sequenced, and then reinserted into pMX-PID. pMIG-AID and pMIG (45) were provided by J. Chaudhuri. pMIG-ΔAID was created by converting amino acid position 189 to a nonsense codon.

ChIP

Live cells were isolated by flotation on Lympholyte M (CEDARLANE, Burlington, ON, Canada) 24 h after transduction. After recovery and washing twice, ~2 × 107 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 intermitently sonicated at 4°C for a total of 22.5 min. Samples were filtered through glass wool. A total of 2 × 107 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 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 intermittently sonicated at 4°C for a total of 22.5 min. Samples were filtered through glass wool. A total of 2 × 107 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 DK9: 5′-AACTAGGCTGCTAATACCGAGATG-3′ and reverse primer DK10: 5′-GGTCCAGTTAGCGAGGAGAGTAGTTA-3′. Primers for mb-1/C079a were mb-IW-FW: 5′-C10CCAGCTACTAGAGAGAGACTCA-3′ and mb-IREV: 5′-C10-CGCCTACCTTCTGTCGCCAGC-3′. Primers for Cµ were forward primer CuPG-F: 5′-TGTGACAGAGGACGCAAGACAGACAGTCTTA-3′ and reverse primer CuPG-R: 5′-G1CAGGACGACATCTTATACAGACAGGGG-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 by cell depletion with Ab and complement (21). B cells were cultured at 107 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(pTdT)10 and SuperScript II reverse transcriptase (Invitrogen). Primers to amplify endogenous AID mRNA were located in exon 4 (forward primer: 5'-AACTTTCCGAGATCCCTTTCGTTG-3') and in the 3' untranslated region (reverse primer: 5'-GCGTGACATTCCGAGGT-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'-GGTTGCAGCTCTCATGTCT-3').

Ligation-mediated PCR
After culture for 2 d, viable cells were isolated by filtration on Ficoll-Hypaque gradients (ρ = 1.09) or Lymphoprep (CEDARLANE); cells were embedded in low-melt agarose plugs, and DNA was isolated as described (20). For linker ligation, 50 μl 1× 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 1× ligase buffer, 3 μl 10× 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 LMP.CR.1 (5'-GGCGGTGCCAGCTCTCATGTCT-3') and LMP.CR.2 (5'-GAATTCTGAATTC-3') in 300 μl 1× ligase buffer, which results in a double-stranded oligo with a 14-nt 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 used for linker ligation.

Amplification and sequencing of Sμ–Sα 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 primer and PCR conditions for each PCR. Primers for the first round were 5′-CCAGCAGATGATGGCTT TATTAATGTGGGGTTTTA-3′ and SaR3: 5′-CCATCCCATCCTCCATCC ATC-3′, and primers for the second round were 5′-AAACACCTTG CCCTTACCCAGATGACGCTG-3′ and SaR2: 5′-CCAGGCAGCTCAGGG CCATT-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 polyacrylamide 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. Quantitation of transduced AID-ER and endogenous AID mRNA levels in splenic B cells induced to switch to IgG3.](http://www.jimmunol.org/DownloadedFrom/01442/DOMINANT_NEGATIVE_EFFERENT_AID_C_TERMINUS_DELETION/433/338.png)
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-
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 (\sim 70\%) than when harvested after 1 d (\sim 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\text{2/2} cells 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.
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 Sμ 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 Sμ in WT cells**

To further explore the mechanism of the DN effect, we asked whether ΔAID-ER binds Sμ 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 Sμ depend upon deaminase activity of ΔAID-ER. (A) ChIP of AID-ER proteins at Sμ and Cμ 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 Sμ and Cμ in aid^−/−^ cells relative to input DNA. Six ChIPs for AID, ΔAID, and ER and three ChIPs for AIDRQ and ΔAIDRQ 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) Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated RV-AID constructs in GFP^+^ WT splenic B cells. Results are normalized to AID-ER results in one of the cultures each for IgG1 and IgG3. Six cultures (three mice, two cultures each) were performed. CSR is plotted for GFP^+^ cells, which are ∼50% of the total GFP^+^ cells. Cells transduced with ER switch similarly to GFP^+^ cells in the same cultures (data not shown). (E) 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 Sμ region in AID-sufficient cells, similar to results in aid<−/−> cells (Fig. 5B). Binding of AID and ΔAID was not detected at the Cμ gene in WT or aid<−/−> cells.

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

We previously showed that AID lacking deaminase activity due to mutations in the catalytic domain, AID<sub>H56R/E58Q</sub> (AIDRQ) (10, 49), does not bind to Sμ and Sy3 in our ChIP assays in aid<−/−> cells (25) (Fig. 5B). This result differs from that reported by Vuong et al. (50), who found that AIDRQ expressed without a tag binds poorly to Sμ/Sy3. This difference was due to the ER tag. However, we found that untagged AIDRQ is also unable to bind Sμ 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 AIDRQ binds Sμ but not stably than WT AID.

We next asked whether ΔAID<sup>RQ</sup>-ER 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 AIDRQ. This is consistent with our finding that ΔAID<sup>RQ</sup> or AID<sup>RQ</sup> do not stably associate with Sμ in WT cells (Fig. 5A). As shown in the Western blot in Fig. 5E, the AID<sup>RQ</sup>-ER 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 Sμ and/or the ability of ΔAID to deaminate cytosines and to induce Sμ DSBs.

If AID<sup>RQ</sup> and ΔAID<sup>RQ</sup> heterodimerize with endogenous AID, they might be expected to bind Sμ in WT cells. Although AID and ΔAID are detected at Sμ 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 Sμ. That endogenous WT AID does not heterodimerize with catalytically inactive AID, or that AID functions as a monomer.

Sμ–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<−/−> 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 Sμ–Sα junctions because Sα has the greatest amount of homology to Sμ of any S region, thus increasing the sensitivity of the assay (51). Indeed, we found that Sμ–Sα junctions showed an average of 2.3-fold greater lengths of MH in aid<−/−> 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<−/−> 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

FIGURE 6. MH at Sμ–Sα junctions in aid<−/−> 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<−/−> 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

FIGURE 6. MH at Sμ–Sα junctions in aid<−/−> 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<−/−> 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

FIGURE 6. MH at Sμ–Sα junctions in aid<−/−> 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<−/−> 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

FIGURE 6. MH at Sμ–Sα junctions in aid<−/−> 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<−/−> 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

FIGURE 6. MH at Sμ–Sα junctions in aid<−/−> 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<−/−> 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 (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 ΔAID. To determine whether the DN effect depends on MMR, we expressed AID-ER and ΔAID-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 ΔAID-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 ΔAID-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<sup>+</sup> 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 ΔAID-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−/− cells, because CSR is not significantly lower in ΔAID-expressing msh2−/− cells than in ER-expressing cells (Fig. 7B, 7C). The levels of RV-AID proteins or endogenous AID in the transduced cells are not affected by lack of the MMR protein Msh2 (Fig. 1A, 1B). In mlh1−/− 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 Sµ 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 Sµ, 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 Sµ. 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 Sµ 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 only 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 aid−/− 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 AID, DSBs might undergo extensive end resection. We hypothesize that the reason why the numbers of DSBs in S regions in both aid−/− 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 and UNG to Sµ in our ChIP experiments (25) suggests that UNG and MutSα require their DNA substrates, dU and U:G mismatches, respectively, for binding to DNA. It is pos-

![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.](http://www.jimmunol.org/)

- AID deaminates several Dcs in S region
  - U U U U U
  - U
  - U
  - MMR recruited to help convert SSBs to DSBs; Excision and displacement DNA synthesis
  - Msh2, Msh6, Mlh1-Pms2
  - DNA synthesis to create blunt DSBs
  - U
  - NHEJ proteins are recruited, perhaps as part of a complex; they bind DSBs and perform S–S recombination.
  - Sµ, Sx

- ΔAID deaminates several Dcs in S region
  - U U U U U
  - U
  - U
  - MMR is poorly recruited, therefore few SSBs
  - U
  - MMR is poorly recruited, so fewer DSBs form
  - U
  - NHEJ proteins are not recruited, so S–S recombination is inefficient.
  - Unrepaired DSBs accumulate and are aberrantly processed/resected.
  - Accumulated DSBs with long ss tails attempt to use alternative end-joining for S–S recombination.
sible that NHEJ proteins are also part of a complex that recruits AID-induced dU 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 S4. If heterodimers of AIDΔDQ 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.

Acknowledgments

We thank J. Chaudhuri for pMIG, pMIG-AID, and anti-AID Ab, as well as for very helpful discussions; V. Barreto and M. Nussenzweig for the pMX-PIE-AID-FLAG-ER-IRES-GFP-puro and pMX-PIE-AID-FLAG-ER-IRES-GFP-puro plasmids; and the University of Massachusetts Medical School Flow Cytometry Core Facility for excellent technical assistance. We also thank Drs. T. Honjo, T. Mak, R.M. Liskay, and W. Edelmann for knock-out mouse lines.

Disclosures

The authors have no financial conflicts of interest.

References


