DNA-Dependent Protein Kinase Inhibits AID-Induced Antibody Gene Conversion

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Affinity maturation and class switching of antibodies requires activation-induced cytidine deaminase (AID)-dependent hypermutation of Ig V(D)J rearrangements and Ig S regions, respectively, in activated B cells. AID deaminates deoxycytidine bases in Ig genes, converting them into deoxyuridines. In V(D)J rearrangements, subsequent excision of the deaminated bases by uracil-DNA glycosylase, or by mismatch repair, leads to further point mutation or gene conversion, depending on the species. In Ig S regions, nicking at the abasic sites produced by AID and uracil-DNA glycosylases results in staggered double-strand breaks, whose repair by nonhomologous end joining mediates Ig class switching. We have tested whether nonhomologous end joining also plays a role in V(D)J hypermutation using chicken DT40 cells deficient for Ku70 or the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Inactivation of the Ku70 or DNA-PKcs genes in DT40 cells elevated the rate of AID-induced gene conversion as much as 5-fold. Furthermore, DNA-PKcs-deficiency appeared to reduce point mutation. The data provide strong evidence that double-strand DNA ends capable of recruiting the DNA-dependent protein kinase complex are important intermediates in Ig V gene conversion.

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Introduction

In humans and mice, primary antibody (Ig) diversity is produced by V(D)J recombination, which is dependent on the RAG-1 and –2 proteins [1]. Over a lifetime, primary repertoires are largely re-shaped by the processes of Ig somatic hypermutation (SHM) and class switching [2], independent processes which occur in B cells activated by infection or immunization. SHM and class switching absolutely depend on a mutator protein, activation-induced cytidine deaminase (AID or AICD), whose expression is restricted to activated B cells [3,4]. In humans and mice, Ig SHM predominantly involves point mutation of rearranged Variable (V) gene segments and the immediately downstream intron sequences, leaving the Constant region (C) gene segments largely unaffected [5,6]. In some species, including chickens, SHM of rearranged V genes also involves intrachromosomal gene conversion with related pseudo- (Ψ) V genes, in preference to point mutation [7]. A minority (5%-10%) of AID-induced mutations in Ig V(D)J genes in all species are small deletions and insertions, which might be due to nonhomologous DNA end joining (NHEJ) and template slippage during translesion synthesis [8–11]. Although class switching also involves AID-induced point mutation, now targeted to the Switch (S) regions located upstream of each C region gene in the IgH locus [6,12–14], its salient outcome is recombination between S regions via NHEJ and the concomitant deletion of kilobase regions of DNA [1].

There is now compelling evidence that AID represents a previously unrecognized class of DNA-editing enzymes vital for both antibody diversification and direct destruction of viral DNA [15]. AID deaminates deoxycytidine (dC) bases in targeted Ig gene regions, converting the targeted bases to deoxyuridine (dU), and thus directly causes transition mutations of dC/dG (deoxycytidine/deoxyguanosine) base pairs to dA/dT (deoxyadenosine/deoxythymidine) base pairs [10]. Excision of AID-deaminated bases by uracil-DNA glycosylase (UNG) or by mismatch repair leads to further mutation via translesion DNA repair [10,16–23]. In chicken Ig V genes, excision of AID-induced dU bases by UNG mostly leads to homology-directed gene conversion with ΨV genes by a process independent of translesion DNA repair, rather than to point mutation [21,24,25]. In yeast and vertebrate cell models, gene conversion is stimulated by the induction of a double-strand break (DSB), which produces the requisite free 3’-ends [26,27]. However, this does not imply that DSBs are obligatory for gene conversion because free 3’-ends are also generated during DNA replication. It is clear that the combined attack of Ig S regions by AID and UNG results in DSBs, which are required for class switching [28,29], but there

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Abbreviations: AID, activation-induced cytidine deaminase; dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand DNA break; dsDNA, double-stranded DNA; dT, deoxythymidine; dU, deoxyuridine; HDR, homology-directed repair; Ig, primary antibody; NHEJ, nonhomologous end joining; SHM, somatic hypermutation; UNG, uracil-DNA glycosylase

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is no a priori reason to expect a role for DSBs in AID/UNG-induced point mutation or gene conversion. On the contrary, nicking at AID/UNG-induced abasic sites could even prevent mutation, promoting faithful Ig V gene conversion with sister chromatids (in S-phase) or faithful base excision repair (in G1-phase) instead [11,30]. Attempts to directly demonstrate AID-dependent DSBs in mutating Ig V genes by ligation-mediated PCR have produced mixed results [31–35]. This is probably because DNA extracted from mutating cells carries a high background of breaks caused by, for instance, normal DNA replication, apoptosis, and even mechanical damage during DNA extraction. Although the frequency of staggered double-strand DNA (dsDNA) ends detected in the VDJ rearrangement of human CL-01 cells is increased by AID expression, there is no convincing evidence that physiological expression of AID causes the formation of double-strand DNA ends in antibody V genes, which appear to be prevented from participating in homologous recombination if they recruit DNA-dependent protein kinase.

Results/Discussion

Loss of DNA-Dependent Protein Kinase Subunits Increases sIg Gain and Gene Conversion in DT40 Cells

Ku70, Ku86, and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) form the heterotrimeric protein DNA-dependent protein kinase (DNA-PK), which is primarily responsible for processing dsDNA ends in G1-phase vertebrate cells, protecting them from inappropriate homologous recombination, and promoting rapid, usually faithful end re-joining by DNA ligase IV [1]. Deficiency for Ku70 or DNA-PKcs was previously reported to have no effect on sIg loss in DT40 cells [39], but the possibility that Ig V gene conversion might involve DSBs prompted us to re-assess whether sIg gain was affected by NHEJ. In DT40 cells, sIg fluctuation is complicated by the fact that the Ig V gene rearrangements mutate by both point mutation and gene conversion. The donor Vβ genes have varying homology to the acceptor V(D)J genes, but in the Igδ locus (and probably also the IgH locus) they usually code for nearly complete reading frames with only a few Vβ3 genes carrying premature stop codons [7]. Ig V gene conversion tracts frequently cover many codons [7]. Gene conversion is therefore a more efficient way to repair premature stop codons than is single base point mutation, because any gene conversions initiated near a deleterious mutation are biased toward repairing it. This is particularly true in the DT40-CL18 subline where sIg loss in the founder cell was due to a single base frame shift in the VJβ gene [40]. We can therefore infer that the rate of sIg gain in lines derived from CL18 cells is essentially an indirect measure of the Ig V gene conversion rate.

The DNA-PKcs- and Ku70-knockouts were originally generated in DT40 cells carrying the canonical CL18 VJβ frame shift [41], which we confirmed by DNA sequencing (unpublished data). We found that deficiency for either Ku70 or DNA-PKcs increased sIg gain relative to control CL18 cells at the 0.001 significance level (Figure 1). To our surprise, DNA-PKcs-deficiency had more of an effect on sIg gain than Ku70-deficiency: a repeat experiment where clones were cultured for 24 d, rather than 50 d, confirmed the reproducibility of these results (Figure 1B). This demonstrated that the power of sIg fluctuation analyses to detect small differences in Ig V mutation rates depends on the use of a large number of clones, rather than on the duration allowed for mutations to accumulate—a conclusion consistent with mathematical modeling of DT40 sIg fluctuation [42].

Similar phenotypes in two independent knockouts acting in the same DNA repair pathway (NHEJ) made it unlikely that the observed increases in sIg gain were artifacts due to unknown additional mutations. Nor were the increases due to preferential outgrowth of sIgδve cells in the NHEJ-deficient cultures, because the cloning efficiency of sIgδve and sIgδve NHEJ-deficient cells was the same (unpublished data). In our cultures, the doubling times for CL18, DNA-PKcs+/−, and Ku70−/−DT40 cells in log-phase growth were 11.2 h, 11.6 h, and 12.0 h, respectively. Using these doubling times in mathematical modeling [42] of our fluctuation data suggested that the mean rate of sIg gain in DNA-PKcs- and Ku70-deficient cultures was 5.0× and 2.9× that of control cells, respectively (Table 1).

Vβ3 rearrangements PCR-amplified from random Ku70- and DNA-PKcs-deficient DT40 clones carried more Ig Vδ gene conversions than those derived from control CL18 clones grown in parallel, while no gene conversions were detected in DNA amplified from control AID+ cells (Figure 2 and Table 2). The relative increases in Ig V gene conversion detected by sequencing were not large (Table 2), but this was probably due to sampling error. The effective sampling rate of the sIg fluctuation assay is much higher than that of DNA sequencing because the mutagenic gene conversion rate of DT40 cells is fairly low. We chose not to overexpress AID as a way of counteracting this problem because variation in AID
overexpression could have greatly increased data variance, and because mutation by overexpressed AID may not reflect physiological AID-induced mutation. The sequence data were consistent with the statistically highly significant increases in the rates of sIg gain calculated in Table 1. Furthermore, sequencing of 93 VJ rearrangements from CL18, DNA-PKcs+/−, and Ku70−/− clones, which started from sIg+/− cells (part of the dataset shown in Table 2), confirmed that sIg gain in these cells always involved a gene conversion that removed the canonical VJ frame shift (unpublished data). Overall, the sequence data confirmed that both DNA-PKcs- or Ku70-deficiency increased Ig V gene conversion.

DNA-PKcs-Deficiency Reduces sIg Loss and Ig V Point Mutation

In addition to the obvious increase in sIg gain, we were able to measure a small and reproducible decrease in sIg loss in DNA-PKcs-deficient cells, but not Ku70-deficient cells (Figure 1 and Table 1). Point mutation is far more likely to produce deleterious amino acid changes than it is to repair them. Thus, sIg loss should be more sensitive than sIg gain to changes in point mutation rates. This is illustrated by cells deficient for any of the Rad51-paralogs [39], such as XRCC3+/− cells, which were included in one of our sIg fluctuation assays as a control (Figure 1A). XRCC3-deficiency caused a little change in the rate of sIg gain in DT40 cells but had a dramatic effect on the rate of sIg loss (Figure 1A). Thus, the inverse changes in sIg gain and sIg loss in DNA-PKcs-deficient cells (Figure 1; Table 1) suggested that loss of the DNA-PKcs protein both promoted gene conversion and inhibited point mutation in DT40 cells. The data from XRCC3+/− cells also demonstrated the ability of our mathematical modeling [42] to estimate changes in mutation rate. The 21× increase in the rate of sIg loss estimated for XRCC3+/− cells (Table 1) corresponds well to their rate of point mutation determined by sequencing [43].

Reliable point mutation data were not collected in our initial sequencing of VJβ sequences because point mutations occurred in a control AID+/− dataset (unpublished data) and were therefore largely due to errors introduced by the “BioXact” polymerase mix used for PCR. However, sequencing of an additional 40–47 clones amplified with “Phusion” DNA polymerase (Finnzyme) yielded five, three, and zero point mutations in VJβ sequences amplified from CL18, Ku70−/−, and DNA-PKcs+/− cells, respectively (Table 3). Combined with the data summarized in Table 1, the sequence data indicate that increased gene conversion in DNA-PKcs-deficient DT40 cells is accompanied by reduced point mutation. Intriguingly, point mutation was either not reduced by Ku70-deficiency or the reduction was too small to be measured in our experiments.

Are AID-Induced DSBs Involved in Ig V Gene Mutation?

Increased Ig V gene conversion in DNA-PK-deficient DT40 cells implies competition between DNA-PK and homology-directed repair (HDR) factors for access to hypermutating Ig V genes in wild-type DT40 cells. How might DNA-PK be recruited to mutating Ig V genes? The generation of DSBs in Ig V genes is the most obvious mechanism, although we cannot rule out the possibility that DNA-PK or its subunits play roles in Ig V mutation independent of NHEJ. AID could generate staggered Ig V DSBs in any phase of the cell cycle if two AID-bearing complexes attacked both strands of an Ig V gene and thus recruited UNG and an abasic site-endonuclease or -lyase activity (Figure 3A). However, this scenario is unlikely to be a major cause of DSBs in DT40 cells because the rate of attack of the DT40 VJβ gene by AID (as revealed in UNG- and ΨV-deficient DT40 cells [25,38,44]) is not high enough for AID-induced cleavage of both strands of an Ig V gene to occur very frequently. Alternatively, dsDNA ends (“pseudo”-DSBs) could also be produced during S-phase if a replication fork encountered a single-stranded AID-induced nick (Figure 3B).
AID Induces dsDNA Ends in Ig V Genes

Ku70^-

DNA-PKcs^--

D4. 65

D28. 88

D28. 89

D28. 88,R

Dm25. 3

C18

Cp22. 1

Cp34. 4

Ku70^-

Kp22. 1

Ku21. 2

DNA-PKcs^--

D4. 65

D28. 88

D28. 89

D28. 88,R

Dm25. 3

Cp14. 1

Cp11. (p16)

Cp14. 2

Kp30. 5

Kp11. (p16)

Kp24. 1

Kp11. (p16)

Kp24. 1

Kp24. 1

Kp24. 1

Cp14. 1

Cp11. (p16)

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AID Induces dsDNA Ends in Ig V Genes

3B). In both Figure 3A and 3B, resection of dsDNA ends would produce 3’-extensions capable of initiating gene conversion with ΨΨV genes; dsDNA ends with both 5’- and 3’-extensions have been detected by LM-PCR in rearranged Ig V genes in human B cells expressing AID [35]. Our data provide indirect, less artifact-prone confirmation that physiological expression of AID does indeed generate dsDNA ends in Ig V genes.

Di Noia et al. [11] have recently argued that AID-induced dC/dG point mutation need not involve DNA breaks. Indeed, in Rad51 paralog-deficient cells, AID/UNG-induced abasic sites must be diverted from gene conversion to point mutation prior to excision of the abasic site, otherwise no lesion would be present to recruit translesion bypass and transversion point mutation [19,39,45]. This is illustrated in Figure 3A and 3B, where only intermediates 3 and 4 can divert to translesion bypass. We were able to envisage a scenario where NHEJ could inhibit Ig V conversion independently of nicking at abasic sites, but the scenario required nicks between Okazaki fragments to persist in template ΨΨV genes for some time after the replication fork had passed (Figure 3C). This requirement is more likely to be met in chicken B cells than in human or mouse B cells (which do not undergo AID-induced gene conversion) because the V genes are much closer together in chicken B cells, and furthermore, is consistent with the preferential use of closer ΨΨV genes as gene conversion donors [7]. However, we think scenario B in Figure 3 is more likely than scenario C, because it is clear that the combined activity of AID and UNG recruits DNA nicking to Ig S regions participating in switching [28]. Thus, it is reasonable to expect the same in Ig V regions. Nonetheless, there is no data available to rule out scenario C in Figure 3 yet.

We conclude that inhibition of mutagenic gene conversion by DNA-PK strongly implicates dsDNA ends as frequent, even obligatory precursors of Ig V gene conversion. The production of a dsDNA end by any of the scenarios shown in Figure 3 provides two 3’ DNA ends that can simultaneously prime strand invasion into an upstream ΨΨV gene. Trimming of mismatched 3’-ends (which frequently occurs when non-identical sequences participate in HDR [46]) after simultaneous strand-invasion provides a simple mechanism by which both strands of the ΨΨV gene are copied into the acceptor V(D)J gene (Figure 3). A good candidate for the nicking enzyme required for models A or B in Figure 3 is the abasic site-lyase activity of MRE11/RAD50 [47].

In contrast to inactivation of DNA-PK, the inactivation of Rad51 paralogs causes a dramatic increase in point mutation in DT40 cells [39]. Thus, the ability of NHEJ to compete with Ig V gene conversion does not, at first glance, appear to be comparable to the ability of HDR to inhibit translesion bypass. However, this is probably because the majority of Ig V gene conversions induced by AID are non-mutagenic: using ΨΨV genes, which have regions of identity to the 3’ acceptor VJ gene or the sister chromatid, as repair templates. Thus, any increase in Ig V gene conversion increases the rate of faithful gene conversion as much as it increases mutagenic gene conversion. In fact, it is only when gene conversion is inhibited that the rate of attack of Ig V genes in DT40 cells by AID is “unmasked” as being much higher than the mutation rate of wild-type DT40 cells would suggest [25,39,44,45]. A 2- to 5-fold increase in mutagenic gene conversion in the absence of DNA-PK therefore implies that DNA-PK in fact blocks Ig V gene conversion most of the time.

A Ku-Independent Role for DNA-PKcs in Ig V Gene Mutation?

Gene conversion-mediated repair of I-Sce I-induced DSBs is elevated much more by Ku70-deficiency than it is by DNA-PKcs-deficiency [41], probably because Ku70 directly competes with the gene conversion machinery for access to dsDNA ends, while DNA-PKcs does not. This contrasts with AID-induced Ig V gene conversion, where DNA-PKcs appears to be more inhibitory than Ku70 (Tables 1 and 2). Perhaps some of the inhibitory activity of DNA-PKcs is independent of Ku70. Wu et al. showed that DNA-PKcs, and not Ku, associates with AID in a DNA-dependent manner [48]. It is unclear whether the reported association between AID and DNA-PKcs was physiological because it was enhanced by addition of

Table 2. Gene Conversions Detected by Sequencing Random Clones from Experiment A

| DT40 Line | Number of Clones Analyzed | Number of Sequences per Clone | Total Number of Sequences | Number of Gene Conversions | Gene Conversion Rate |
|-----------|---------------------------|-------------------------------|---------------------------|---------------------------|---------------------|
| CL18      | 22                        | 3–7                           | 88                        | 3                         | 1×                  |
| DNA-PKcs−/− | 21                        | 2–6                           | 86                        | 7                         | 2.4×                |
| Ku70−/−   | 22                        | 3–6                           | 90                        | 5                         | 1.6×                |
| AID−/−    | 8                         | 4–6                           | 34                        | 0                         | —                   |

*Shown in Figure 2.

Relative to control CL18 cells.

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Figure 3. Models for Ig V Gene Conversion Based on Attack of Both DNA Strands by AID and Attack of One DNA Strand by AID

(A) Attack of both DNA strands by AID. In step 1, two complexes containing AID attack opposed strands at the same time. If both complexes directly recruited DNA-PKcs molecules, DNA-PKcs could dimerize and perhaps inhibit base excision by UNG. In step 2, excision by UNG and a lyase or exonuclease creates a staggered DSB. Step 3 shows that gene conversion requires the production of 3'–protruding ends, which may involve a 5'–3' exonuclease, depending on the relative placement of the nicks. The 3'–protruding ends initiate gene conversion by invading a homologous \( V \) gene. This step could be inhibited by the binding of dsDNA ends to the DNA-PK complex. In steps 4–6, nonhomologous ('mutated') sequences are copied from the \( V \) gene by mismatched end trimming, primer extension, template switch, further end trimming, and ligation.

(B) Attack of one DNA strand by AID. In step 1, AID deamination in G1-phase may be ignored until S-phase, based on the model in [11]. In S-phase, shown in step 2, the dU base produced by AID is encountered by a replication fork, enabling access by UNG, which then creates an abasic site. Step 3 shows the abasic site is excised to create a dsDNA end, which can recruit the DNA-PK complex. In step 4, if DNA-PK is not recruited, the dsDNA end promotes gene conversion with an upstream \( V \) gene.

(C) Attack of one DNA strand by AID. As in (B), AID deamination is ignored until S-phase. In step 2, the dU base produced by AID is encountered by a replication fork, enabling access by UNG, which then creates an abasic site. Step 3 shows the abasic site is excised to create a dsDNA end, which can recruit the DNA-PK complex. In step 4, if DNA-PK is not recruited, the dsDNA end promotes gene conversion with an upstream \( V \) gene.

Table 3. Point Mutations Detected by Sequencing Random Clones from Experiment A

| DT40 Line | Number of Clones Analyzed | Number of Sequences per Clone | Total Number of Sequences | Number of Point Mutations | Point Mutation Rate\(^a\) |
|-----------|---------------------------|-------------------------------|--------------------------|--------------------------|--------------------------|
| CL18      | 12                        | 3–7                           | 46                       | 5                        | 1\(\times\)                |
| DNA-PKcs \(-/\-\) | 11                        | 2–6                           | 40                       | 0                        | —                        |
| Ku70 \(-/-\) | 11                        | 3–6                           | 47                       | 3                        | 0.6\(\times\)             |

\(^a\)Relative to control CL18 cells.

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exogenous DNA [48]. Nonetheless, one can speculate that in wild-type cells simultaneous recruitment of AID to both DNA strands of a V gene could directly promote DNA-PKcs dimerization, partially inhibiting access by UNG or HDR factors to the deaminated site, and thus inhibiting gene conversion whilst promoting point mutation (Figure 3A).

Relevance to Mammalian B Cells

The scid mutation, which is generally considered to essentially inactivate DNA-PKcs, has no detectable effect on Ig V hypermutation in mouse Peyer’s patch B cells [49]. However, this finding needs to be interpreted cautiously. Ig class switching is only reduced 50%–60% by the mouse scid mutation [13], in contrast to the almost complete abrogation of switching in mouse DNA-PKcs

transcription. Experiments were designed to ensure that differences in Ig fluctuation rates between groups did not arise because of fluorescent antibody detachment over time, variations in machine parameters over the course of data collection, or because of human bias in data analysis.

Mathematical modeling. The frequencies of sIg-gain and sIg-loss were estimated using the formula shown in Appendix 2 of [42]. Let \( f \) be the median proportion of cells that gained sIgM in sIgM

clones. Let \( b \) be the median proportion of cells that lost sIgM in sIgM

clones. Let \( g \) be the number of generations in the experiment, calculated from the cell line’s doubling time. Let \( s = b/g \). Then the estimates, \( \Phi \) and \( \beta \) of the rates of sIg-gain and sIg-loss, respectively, are \( \Phi = s/f + 1 \) and \( \beta = s - f \).

DNA sequencing. Genomic DNA was extracted from multiple random clones cultured for 50 d (i.e., experiment B). The VJ

and 3’-flanking sequences were PCR-amplified with published primers [39] using “BioXact Short” DNA polymerase mix (Bioline, http://www.bioline.com) or “Phusion” DNA polymerase (Finnzymes Oy, http://www.finnzymes.fi) and cloned into plasmids. To minimize the acquisition of redundant sequences, only a few plasmids derived from each clone were sequenced, as indicated in Tables 1–3.

Supporting Information

Accession Numbers

The Ensembl (http://www.ensembl.org) accession numbers for the genes discussed in this paper are AID (ENSBTAG00000018849), DNA-PKcs (ENSGALG00000011932), Ku70 (ENSGALG00000011932), and XRCC3 (ENSGALG00000011553).

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Author contributions. CJJ conceived and designed the experiments, AJLC, JMR, KKE, A.J., and CJJ performed the experiments. AJLC and CJJ analyzed the data. RSH and ST contributed reagents/materials/analysis tools. AJLC and CJJ wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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The Ensembl (http://www.ensembl.org) accession numbers for the genes discussed in this paper are AID (ENSBTAG00000018849), DNA-PKcs (ENSGALG00000011932), Ku70 (ENSGALG00000011932), and XRCC3 (ENSGALG00000011553).

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