Activation-Induced Cytidine Deaminase Splice Variants Are Defective Because of the Lack of Structural Support for the Catalytic Site

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Activation-Induced Cytidine Deaminase Splice Variants Are Defective Because of the Lack of Structural Support for the Catalytic Site

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Recently, conflicting results were reported on the hypermutation activity of activation-induced cytidine deaminase (AID) splice variants. With the generation of single point mutations, we studied the structure-function relationship of the amino acids that are commonly absent from all described splice variants. The results from this analysis pointed to several amino acids that are required for class switch recombination (CSR), without perturbing cellular localization or nucleocytoplasmic shuttling. A defect in deaminase activity was found to underlie this CSR deficiency. Interestingly, the most debilitating mutations concentrated on hydrophobic amino acids, suggesting a structural role for this part of the protein. Indeed, by generating homologous amino acid replacements, CSR activity could be restored. These results are in agreement with recent reports on the protein structure of the AID homolog APOBEC3G, suggesting a similar protein composition. In addition, the findings underscore that AID splice variants are unlikely to have preservation of catalytic activity.


By deaminating cytidines to uracils in the Ig variable (IgV) genes and switch regions, activation-induced cytidine deaminase (AID) generates the key lesions required for somatic hypermutation (SHM) and class switch recombination (CSR), two processes pivotal in the generation of high-affinity Abs (1). Although instrumental in adaptive immunity, these genome-modifying processes entail the risk of unwanted DNA damage, which is reflected in the various translocations involving the IgV genes and switch regions, characteristic for B cell non-Hodgkin’s lymphoma. In addition, aberrant targeting and deregulated expression of AID was described in human B cell lymphomas (2, 3). Indeed, several mouse models confirmed the oncogenic potential of AID (2). These findings have raised fundamental questions with respect to regulation of expression, targeting, posttranslational modifications, and the structure-function relationship of AID.

Studies related to AID expression have reported the existence of a number of AID splice variants (3–6). Considering a potential role of alternative splicing in the regulation of AID, functional studies of these isoforms were desired. Recently, we and others reported that three of these isoforms completely lack CSR activity (7, 8). Concordantly, we found that recombinant isoform proteins were incapable of deaminating a synthetic oligonucleotide substrate in vitro and that this deficiency was accompanied by a disturbed cellular localization. Alternative splicing takes place at the intersection of exons 3 and 4, reflecting the intrinsic weakness of the splice acceptor site of exon 4. Two of these splice variants, designated AID-ΔE4 and AID-ivs3, lack the entire C terminus encoded by exons 4 and 5, whereas a third isoform (named AID-ΔE4a) has a relatively small deletion of the first 10 aa encoded by exon 4. These 10 aa, absent from all three splice variants, encode the start of the previously designated “pseudocatalytic site”—in analogy to Escherichia coli cytidine deaminase—and constitute a part of the APOBEC-like C-terminal domain (CTD) of AID (9, 10). Although the C-terminal amino acids of AID, encoded by exon 5, were found to contain the nuclear export signal (NES) and are absolutely required for CSR but dispensable for SHM, very little is known about the function of the remaining part of the CTD of AID (11, 12). Studies on hyper-IgM patients have described three point mutations leading to single amino acid changes in this region (i.e., M139V, F151S, and R174S); all three lacking both CSR and SHM activity (13). More recently, T140 was found to be subjected to phosphorylation, and mutation of this amino acid also led to a reduced SHM and CSR in vivo (14). By functionally analyzing the effect of mutations introduced in the first 10 aa of exon 4, we have aimed to gain more insight in the structure-function relationship of this part of AID. Interestingly, among these are three putative phosphorylation substrates; two tyrosines (Y144 and Y146) and one threonine (T150).

Materials and Methods

AID mutants and vectors

AID wild-type (WT) and mutants were cloned into the following vectors: for the generation of retrovirus into LZRS-linker-IRES-YFP [a gift from H. Spits, Department of Cell Biology and Histology, Academic Medical Center, Amsterdam, The Netherlands (15)]; for the nucleocytoplasmic shuttling experiments into pcDNA3.1/CT-GFP-topo (Invitrogen, Carlsbad, CA); and for generation of GST-AID recombinant protein into pGEX5X-1 (GE Healthcare, Little Chalfont, U.K.).

Human AID-WT was obtained from Ramos cells by RT-PCR. The generation of AID mutants was established using site-directed mutagenesis: first, two fragments were created by PCR (with Phusion polymerase; Finnzymes, Espoo, Finland), combining the AID-fw primer with a mutant reverse primer or a mutant forward primer with an AID-rev primer (Table I). These fragments were then used as input for a second PCR with the AID-fw and -rev primers, creating full-length mutant AID. AID-fw/rev1 primers were used for direct cloning into pcDNA3.1/CT-GFP-topo, resulting in

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Abbreviations used in this paper: AID, activation-induced cytidine deaminase; CSR, class switch recombination; CTD, C-terminal domain; LMB, leptomycin B; NES, nuclear export signal; SHM, somatic hypermutation; WT, wild-type.

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a fusion protein of AID and GFP connected through the linker sequence QGGQCRYQPQWRPLESR. The AID-fw and -rev2 primers were used for cloning into LZR5 and pGEX, of which the latter primer was equipped with a NotI site to enable easy transfer to the target vectors. The PCR products were cloned in Topo 2.1, verified using M13 primer-mediated sequencing, and used in subsequent digestion and ligation into the target vectors: BamHI/NotI for ligation into the LZR5s and EcoRI/NotI for pGEX5X-1. In the resulting GST-AID fusion proteins, GST and AID were linked by IEGRGIPFFALDFT.

**Virus production and titration**

LZR5 vectors containing the AID-encoding sequences were transfected with Fugene 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) into Phoenix Ecotropic packaging cells (American Type Culture Collection, Manassas, VA) to produce supernatant containing the retrovirus. The virus titer was measured by applying the supernatants to 3T3 cells in serial dilutions and in the presence of 10 μg/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands), with subsequent analysis of the percentage of infected cells by flow cytometry.

**CSR assay**

AID-knockout mouse splenocytes were depleted of erythrocytes and sorted for B cells with anti-B220 magnetic beads (Miltenyi Biotec, Utrecht, The Netherlands). Cells were cultured in IMDM with 10% FCS, 1% penicillin/streptomycin, 1% glutamine, BSA (100 μg/ml), 0.04% 2-ME, 25 μg/ml LPS (Sigma-Aldrich), and 10 ng/ml murine IL-4 (Pro-Tech, Rocky Hill, NJ). The next day, cells were transduced with equal titers of the AID-WT, AID mutants, or empty vector as negative control. On day 3 after transduction, cells were analyzed by flow cytometry, based on YFP expression and mRNA transduction experiments, but dominant negativity was not found in the presence of 10 μg/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands), with subsequent analysis of the percentage of infected cells by flow cytometry.

**In vitro deaminase assay**

pGEX5X-1 vectors were transformed into BL21 E. coli cells (GE Healthcare) and grown until OD 0.6–0.8, at which the bacteria were induced with 1 mM IPTG, for 16 h at 16˚C. Concentrated bacterial suspensions were lysed by sonication in the presence of 100 μg/ml lysozyme (Sigma-Aldrich) and 60 U/ml DNase I (Roche Diagnostics). GST-fusion proteins were purified from the lystate with GSTrap HP 1-ml columns (GE Healthcare) and dialyzed in 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.1 mM DTT, and 50% glycerol. Protein concentrations were measured on a NanoDrop ND-1000 Spectrophotometer (Isogen lifescience, Huelstein, The Netherlands) and confirmed by Coomassie staining of DNA-PAGE gels loaded with adjusted protein amounts.

**Results**

**AID mutants**

We generated AID point mutations (Table I) within the area of the deletion found in splice-variant AID-ΔE4a, ranging from D143 to V152 (i.e., the first 10 aa encoded by exon 4), as shown in Fig. 1A. The F151S mutation previously found in a hyper-IgM patient and mapping to this part of AID was included as well.

**Functional analysis of AID point mutants**

To measure CSR activity, retroviral reconstitution of AID-knockout mouse B cells and stimulation with LPS and IL-4 was used to specifically induce CSR to IgG1. As with AID-WT, some of the mutants were able to restore CSR, either completely (F145A) or partially (D143A, C147A, N149A, and T150A) (Fig. 1B, 1C). Five mutants appeared completely defective for CSR activity (i.e., Y144A, Y146A, W148A, F151A, and V152A). Several of the mutants were tested for inhibitory effects on AID-WT, using double transduction experiments, but dominant negativity was not found (data not shown).

**Catalytic activity**

AID catalytic activity was assessed using an in vitro deaminase assay, in which 100 nM of a FAM-labeled 64-nt substrate, containing one cytidine in a RGYW-hotspot motif (FAM, 5′-TAAAGGTGAAGAGGAGAGAGA-

**Table I. Primers used for the generation of AID mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Forward Primer 5′-3′</th>
<th>Reverse Primer 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>AID-fw</td>
<td>CGGACACCACATATGGACAG</td>
<td>GAAGTCCCAAGTACGAAATGC</td>
</tr>
<tr>
<td>AID-rev1</td>
<td>TGGACCGGCGGCTGAGTTTCTAATCAAAGTCA</td>
<td></td>
</tr>
<tr>
<td>AID-rev2</td>
<td>TGGACCGGCGGCTGAGTTTCTAATCAAAGTCA</td>
<td></td>
</tr>
<tr>
<td>T140A</td>
<td>CATATGTCCTTAAAGTATTATTTTAC</td>
<td>GTAAAATAATTCTTGGAGACCAGATG</td>
</tr>
<tr>
<td>D143A</td>
<td>CATGACCTTAAAGGTTTTTAC</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>D143E</td>
<td>CATGACCTTAAAGGGTTTTTAC</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>Y144A</td>
<td>CAAAGATGTCTTATGCG</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>Y144F</td>
<td>CTTCAAAGATTTTTTAC</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>F145A</td>
<td>CAAAGATGTCTTATGCG</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>F145Y</td>
<td>CTTCAAAGATTTTTTAC</td>
<td>GACAGTTAAAATAGCTTGGAGATG</td>
</tr>
<tr>
<td>Y146F</td>
<td>GATATTTCCTTTCCGTTGGAATAC</td>
<td>GTATTTCCAGCAGGCAAAATATC</td>
</tr>
<tr>
<td>Y146G</td>
<td>GATATTTCCTTTCCGTTGGAATAC</td>
<td>GTATTTCCAGCAGGCAAAATATC</td>
</tr>
<tr>
<td>C147A</td>
<td>TCTCAAGATTTTTTACCTTGG</td>
<td>TTTCAAGCCGTGAAATATTCTGG</td>
</tr>
<tr>
<td>W148A</td>
<td>TTTACTGCGGATCTCTTTTGTAG</td>
<td>AAAGTATGCGGGAAATATTCTGG</td>
</tr>
<tr>
<td>W148Y</td>
<td>TTTACTGCGGATCTCTTTTGTAG</td>
<td>AAAGTATGCGGGAAATATTCTGG</td>
</tr>
<tr>
<td>N149A</td>
<td>TTTACTGCGGATCTCTTTTGTAG</td>
<td>AAAGTATGCGGGAAATATTCTGG</td>
</tr>
<tr>
<td>N149D</td>
<td>TTTACTGCGGATCTCTTTTGTAG</td>
<td>AAAGTATGCGGGAAATATTCTGG</td>
</tr>
<tr>
<td>T150A</td>
<td>CTGTCGCTGAACCTTTTGTAG</td>
<td>CTATACCAAGTATTTTTTAC</td>
</tr>
<tr>
<td>F151A</td>
<td>CTGTCGCTGAACCTTTTGTAG</td>
<td>CTATACCAAGTATTTTTTAC</td>
</tr>
<tr>
<td>F151S</td>
<td>CTGTCGCTGAACCTTTTGTAG</td>
<td>CTATACCAAGTATTTTTTAC</td>
</tr>
<tr>
<td>V152A</td>
<td>GGAATATCTTTTCTGAGAAAAC</td>
<td>GGGTTTCTCCAGAAGTATTTCC</td>
</tr>
</tbody>
</table>
conversion of the substrate, whereas no activity was found for W148A, F151S, and V152A (Fig 1D). In the case of these three mutants, the lack of deaminating activity was in agreement with their null phenotype for CSR.

Similar to APOBEC1, one of its homologs, AID has been shown to shuttle between nucleus and cytoplasm, mediated by its C-terminal NES. This was demonstrated by incubation with the CRM1-exportin inhibitor leptomycin B (LMB), resulting in entrapment of AID in the cell nucleus. To see whether the catalytic defect of the mutants coincides with an aberrant cytoplasmic localization, as was previously found for the splice variants, GFP-tagged AID mutants were

**FIGURE 1.** Functional analysis of AID mutants. A, The first 10 aa of exon 4 were each mutated to alanine for functional analysis. The hyper-IgM–mutant F151S was included as well (indicated with an asterisk). B, A representative experiment of CSR by reconstitution of AID-knockout B cells with AID-WT, empty vector, and AID mutants. Depicted is the percentage of IgG1⁺ cells among transduced cells. C, Relative CSR activity compared with AID-WT (set to 100%). Depicted is the mean of five measurements, with the SE of means illustrated in error bars. CSR activity was completely absent for the mutants of hydrophobic amino acids Y144A, Y146A, W148A, F151A, and V152A. D, In vitro deaminase activity measured for GST-coupled AID-WT and AID-mutants and GST-only as a control. Deamination at the cytidine in the RGYW hotspot is visible as the 30-nt product after treatment with recombinant UDG and subsequent alkaline cleavage. AID-WT, C147A, N149A, and T150A have preserved deaminase activity, whereas for GST, W148A, and F151S, no deaminase-product is evident. E, Cellular localization of GFP and AID-GFP fusion products, with and without treatment the CRM1 inhibitor of nuclear export LMB. All AID variants are by default localized in the cytoplasm but accumulate into the nucleus upon LMB treatment, demonstrating functional nucleocytoplasmic shuttling.
Discussion

Recently, we showed that three of the reported AID splice variants encode truncated proteins that lack catalytic activity and have abnormal cellular localization. Alternative splicing takes place downstream of exon 3, a sequence normally encoding a part of the APOBEC-like C terminus. Although conserved throughout many of the APOBEC family members, the function of this domain is still largely unknown. The most C-terminal 10 aa encode a functional NES, and mutation of this sequence resulted in a defect in CSR, whereas SHM remained intact (11, 12). In contrast, deletion of 10 aa from the upstream side of this domain, as with splice variant AID-AE4a, was sufficient to give a complete defect in catalytic activity in our experiments. By generating artificial point mutants, we studied the function of this area in more detail. Our results showed that the significance of this area is confined to the most hydrophobic amino acids, which, when mutated to the neutral alanine, are defective for CSR and deaminase activity. By introducing point mutations, we have generated relatively subtle defects. This has revealed that the CSR deficiency of these mutants is not due to impairment in cellular localization or nucleocytoplasmic shuttling. When converting the hydrophobic amino acids to homologous hydrophobic residues, the functionality was restored, indicating that these amino acids are important in maintaining structural integrity. These results rule out a role for phosphorylation at Y144 and Y146. In contrast, T150A showed only a partial defect in CSR, which was not directly reflected in the cytidine deaminase assay. Hence, phosphorylation on this residue cannot be excluded, moreover, as similar results were recently reported for the phosphorylation site at T140 (14).

The 10-aa stretch is highly conserved in AID: 100% in other vertebrates, such as mouse, rat, and the African clawed frog and to a great extent in other cytidine deaminases, as is shown in the alignment with APOBEC2 and APOBEC3G in Fig. 3. On the basis of the crystal structures of APOBEC2 and APOBEC3G, it can be deduced that they constitute an α-helix (α5), positioned parallel to the β-sheet, antiparallel to the α1-helix and opposite to the side containing the DNA-binding and Zn-coordinating motifs (21–25). Chen, et al. (22) confirmed their APOBEC3G structure model by extensive functional mutant analysis. Among the numerous APOBEC3G mutants assayed for mutation frequency in bacteria, five were analogous to the AID mutants presented in this study: F343A, C346A, W347A, F350A, and V351A in APOBEC3G, corresponded to Y144A, C147A, W148A, F151A, and V152A in AID, respectively. Only one of these five mutants displayed mutation activity detectable above background (i.e., C346A), whereas the other four lacked mutation activity, which matches with the outcome of our experiments. The APOBEC3G α5-helix makes extensive stabilizing hydrophobic contacts with the β-strand platform and the α1-helix, which together form a strong hydrophobic core, supporting the catalytic site (21–23). Similarly, we can conclude that the hydrophobic amino acids of the α5-helix in the APOBEC-like C terminus of AID is of essential structural importance in enabling deaminase activity. The pivotal significance of the hydrophobic core, as indicated by the single amino acid mutants, infers that the complete lack of this domain in the splice variants must have severe consequences for AID functionality (7).

**FIGURE 2.** Rescue of CSR activity by homologous mutation. CSR activity can be rescued when replacements are made with amino acids of similar biochemical properties.

**FIGURE 3.** Clustal-W sequence alignment of human AID, the CTD of hAPOBEC3G and hAPOBEC2 (19, 20). Asterisks indicate identical residues; colons and periods indicate conservation of strong and weak groups, respectively. The 10 aa subjected to mutation in this study, are shaded gray. The predicted secondary structure of APOBEC3G-CTD is illustrated above the sequence (23).
Acknowledgments
We thank N. Blom for his expertise on protein mutagenesis and protein modeling, D. Speijer for help with Typhoon imaging, A. Martin and M. Larjani for their help on the deaminase assay, H. Spits for the LZRS vector, and T. Honjo for the AID-knockout mice.

Disclosures
The authors have no financial conflicts of interest.

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