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.


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
a fusion protein of AID and GFP connected through the linker sequence GQGCRYFQWPRPLESR. The AID-fw and -rev2 primers were used for cloning into LZRS 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 LZRS and EcoRI/NotI for pGEX5X-1. In the resulting GST-AID fusion proteins, GST and AID were linked by IEGRGIFEEEDLLT.

**Virus production and titration**

LZRS 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 the presence of 10 ng/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 from erythrocytes and sorted for B cells with anti-B220 magnetic beads (Miltenyi Biotec, Uttaruch, The Netherlands). Cells were cultured in IMDM with 10% FCS, 1% penicillin/streptomycin, 1% glutamine, BSA (100 μg/ml), 0.6% 2-ME, 25 μg/ml LPS (Sigma-Aldrich), and 10 ng/ml murine IL-4 (ProTech, Rocky Hill, NJ). The next day, cells were transduced with equal titers of the AID-WT, AID mutants, or empty vector as negative control using Fugene 6 transfection reagent according to manufacturer’s instruction. After 2 d, half of the cells were incubated with 10 ng/ml Leptomycin B (Sigma-Aldrich), for 3–5 h. AID-GFP was visualized in live cells by confocal microscopy and in fixed cells by immunofluorescence of monoclonal anti-mouse CD45R/E-2 (BD Biosciences, San Jose, CA) and polyclonal anti-mouse IgG1, PE (Southern Biotechnology Associates, Birmingham, AL).

**Cytoplasmic localization**

CT-GFP-topo vectors were transfected into HEK293 cells with Fugene 6 transfection reagent according to manufacturer’s instruction. After 2 d, half of the cells were incubated with 10 ng/ml Leptomycin B (Sigma-Aldrich, St. Louis, MO), for 3–5 h. AID-GFP was visualized in live cells on a Leica DM500B microscope (Leica Microsystems, Deerfield, IL) at ×20 magnification, and recorded with a Leica DFC500 camera (Leica Microsystems).

**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 37˚C. Concentrated bacterial suspensions were lysed by sonication in the presence of 100 μg/ml lysozyme (Sigma-Aldrich) and 10 mM NaCl, 0.04% Triton X-100, and 0.1 mM IPTG, for 16 h at 16˚C. Concentrated bacteria suspensions were incubated overnight at 37˚C with 1–2 μg purified protein in 50 mM Tris (pH 7.5), 100 mM NaCl, and 2 μL MgcCl in a volume of 10 μL. Uracil excision by recombinant uracil DNA-glycosylase (New England Biolabs, Ipswich, MA) and breakage of the abasic site by NaOH heat treatment were performed as previously described by Larijani et al. (16, 17), with the modification that 1 μg of RNase A was added to the deamination reaction (18). Deamination products were separated by DNA-PAGE and visualized using the Typhoon Trio imager (GE Healthcare).

**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).

**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>CGTGAACCCACTATGGACAG</td>
<td>GAAATCCCAAGATGAGAAAGT</td>
</tr>
<tr>
<td>AID-rev1</td>
<td>TACATGCGCTCAGAAGATTTTTTAC</td>
<td>TGGAAATATTCTGAGAGGCGATG</td>
</tr>
<tr>
<td>AID-rev2</td>
<td>CACAGGTCTCAGAAGGTTTTTAC</td>
<td>GCAGATTTAATTACCTTGAG</td>
</tr>
<tr>
<td>T140A</td>
<td>CACATGCCCTCAAAGATTATTTTAC</td>
<td>GCCATTTAATTTACCTTGAG</td>
</tr>
<tr>
<td>D143A</td>
<td>CACTGACCTCAAGGGTTTTTAC</td>
<td>GCCATTTAATTTACCTTGAG</td>
</tr>
<tr>
<td>D143E</td>
<td>CACTGACCTCAAGGGTTTTTAC</td>
<td>GCCATTTAATTTACCTTGAG</td>
</tr>
<tr>
<td>Y144A</td>
<td>CCAAAGATGCTGCTGTTTTTAC</td>
<td>GCAGATTTAATTACCTTGAG</td>
</tr>
<tr>
<td>Y144P</td>
<td>CTTCAAGAGTTTTTGTCTGTTTAC</td>
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</tr>
<tr>
<td>F145A</td>
<td>CAAAGATATTAACTTCTGTTTAC</td>
<td>GCAGATTTAATTACCTTGAG</td>
</tr>
<tr>
<td>F145Y</td>
<td>CTTCAAGAGTTTTTTCTCTGTTTAC</td>
<td>GCCATTTAATTTACCTTGAG</td>
</tr>
<tr>
<td>Y146A</td>
<td>GTAATTTTCTCTGCTGTTTAC</td>
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</tr>
<tr>
<td>Y146F</td>
<td>GTAATTTTCTCTGCTGTTTAC</td>
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<tr>
<td>W148Y</td>
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<tr>
<td>N149A</td>
<td>TTATATTGCTGCTGTTTAC</td>
<td>AGTACCCGAGATTTAAATATAC</td>
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<tr>
<td>N149D</td>
<td>TTATTGCTGAGATTTTTGTTG</td>
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<td>T150A</td>
<td>CTGCTATGCTGCTGTTTAC</td>
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<tr>
<td>F151A</td>
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<tr>
<td>F151S</td>
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</tr>
<tr>
<td>V152A</td>
<td>GGAATATTTTCTGCTGTTTAC</td>
<td>GAGTTTCTTACAGAATTTTCTG</td>
</tr>
</tbody>
</table>

Catalytic competence was assessed for several of the mutants using an in vitro deaminase assay, in which the presence of a 30-nt digestion product is a reflection of AID-mediated DNA deamination. Similar to AID-WT, the mutants C147A, N149A, and T150A showed 60 U/ml DNase I (Roche Diagnostics). GST-fusion proteins were purified from the lysate 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, Jsselstein, The Netherlands) and confirmed by Coomassie staining of DNA-PAGE gels loaded with adjusted protein amounts.

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).

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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.
expressed in HEK293 cells. AID-WT as well as all mutants showed normal cytoplasmic localization in untreated cells and nuclear accumulation upon LMB treatment, demonstrating functional nucleocytoplasmic shuttling (Fig. 1E).

Interestingly, the mutants with the most severely defective phenotype corresponded to the most hydrophobic amino acids, suggesting a function in maintaining structural integrity. To test this hypothesis, several additional mutants were generated in which substitutions were made into amino acids with similar chemical characteristics (i.e., D143E, Y144F, F145Y, Y146F, W148Y, and N149D). Indeed, these homologous mutants were able to rescue partial or complete functionality, as demonstrated by restoration of CSR (Fig. 2).

**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 to 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-A4a, 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 sequence 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 shutting. 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).
Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References