AID from bony fish catalyzes class switch recombination

Vasco M. Barreto,1 Qiang Pan-Hammarstrom,3 Yaofeng Zhao,3 Lennart Hammarstrom,3 Ziva Misulovin,2,4 and Michel C. Nussenzweig1,2

Class switch recombination was the last of the lymphocyte-specific DNA modification reactions to appear in the evolution of the adaptive immune system. It is absent in cartilaginous and bony fish, and it is common to all tetrapods. Class switching is initiated by activation-induced cytidine deaminase (AID), an enzyme expressed in cartilaginous and bony fish that is also required for somatic hypermutation. Fish AID differs from orthologs found in tetrapods in several respects, including its catalytic domain and carboxy-terminal region, both of which are essential for the switching reaction. To determine whether evolution of class switch recombination required alterations in AID, we assayed AID from Japanese puffer and zebra fish for class-switching activity in mouse B cells. We find that fish AID catalyzes class switch recombination in mammalian B cells. Thus, AID had the potential to catalyze this reaction before the teleost and tetrapod lineages diverged, suggesting that the later appearance of a class-switching reaction was dependent on the evolution of switch regions and multiple constant regions in the IgH locus.
The emergence of class switch recombination after somatic hypermutation might depend on alterations in AID, the structure of the Ig heavy chain locus, or both (1, 6). We report that AID from species that do not undergo class switching can complement AID deficiency in mammalian cells. Therefore, all of the features of AID required for Ig switch region targeting and the resolution of switch lesions were present before the emergence of the switch reaction and were conserved throughout evolution.

RESULTS AND DISCUSSION
Teleost AID is active in *Escherichia coli* and *Saccharomyces cerevisiae*

The presence of antibody affinity maturation, AID, and Ig loci enriched for mutation hotspots suggest that somatic hypermutation occurs in bony fish (for review see reference 6). However, there is no precise information on the catalytic activity or targeting of fish AID in vivo. To examine the mutator activity of AID orthologs from mouse (*Mus musculus*; m-AID), zebra fish (*Danio rerio*; z-AID), and Japanese puffer fish (*Fugu rubripes*; f-AID), we expressed the respective cDNAs in *E. coli* or *S. cerevisiae*. We assayed AID in both systems because AID from cold-blooded animals might be thermolabile (18). The *E. coli* assay is performed at 37°C and measures mutations in an inactive kanamycin allele (KanL94P) that becomes active by mutation (CCA to TCA or CTA) (19). m-AID induces a higher frequency of mutations than z-AID in this assay, whereas f-AID is indistinguishable from the negative control (Fig. 1 A). The *S. cerevisiae* assay is performed at 30°C and measures inactivation mutations in *ura3*, resulting in resistance to 5-fluoroorotic acid (5-FOA). In contrast to the *E. coli* assay, numerous mutations in *ura3* result in loss of activity and resistance to 5-FOA (Tables S1 and S2, available at http://www.jem.org/cgi/content/full/jem.20051378/DC1). We found that z-AID was more active than m-AID in *S. cerevisiae*, whereas f-AID showed marginal levels of activity (Fig. 1 B). When the same experiment was performed in *ung* *S. cerevisiae*, we observed an increase in the frequency of mutations, and f-AID activity was clearly above the background level. Increased mutation or altered patterns of mutation in the absence of UNG have been reported in *E. coli*, *S. cerevisiae*, chicken, and mammalian cells, implicating UNG in the recognition and repair of AID-dependent mutations (10, 13, 20, 21). In the absence of UNG, fewer AID-induced lesions are repaired, thereby increasing

![AID from bony fish induces class switch recombination in mouse AID<sup>−/−</sup> splenocytes.](image-url)

(A) Representative flow cytometry profiles of IL-4 and LPS-stimulated B cells from AID<sup>−/−</sup> mice infected with retroviruses encoding m-, f-, or z-AID or an empty vector control. The y axis shows GFP expression, and the x axis shows cell surface IgG1. The percentage of cells in each indicated quadrant is shown. (B) Percentage of switching to IgG1 by GFP<sup>+</sup> cells. Points represent individual cultures. (C) Immunoblot analysis of protein extracts from AID<sup>−/−</sup> B cell cultures infected with retroviruses encoding FLAG-tagged AID. GFP expression was used as a loading control.
the sensitivity of the assay and underscoring the mutator activity of f-AID. We conclude that fish AID is catalytically active in *E. coli* and *S. cerevisiae*.

Teleost AID catalyzes class switch recombination

Zebra fish and the Japanese puffer fish are teleosts, a group capable of mounting an adaptive immune response but lacking class switch recombination. A comparison of the sequences of bony fish AID orthologs with amphibians, birds, and mammals reveals that fish AID has a longer cytidine deaminase motif and extensive substitutions in the carboxy-terminal region that are required for class switch recombination (22). To determine whether AID expressed by species lacking class switch recombination can induce switching, we used retroviruses to express m-, z-, or f-AID in mouse AID*^/-^/H11002 lymphocytes. To facilitate switching and retroviral...
infection, AID-deficient mouse B cells were stimulated with LPS and IL-4. Virally infected cells were identified by enhanced GFP expression, and Ig class switching was measured by cell surface IgG1 expression. Virally expressed z-AID induced class switching in AID<sup>−/−</sup> B cells with a similar frequency as m-AID (Fig. 2, A and B). f-AID–expressing cells showed lower levels of switching than m- or z-AID (Fig. 2, A and B), as would be expected from the <i>E. coli</i> and <i>S. cerevisiae</i> assay. To monitor protein expression levels, we repeated these experiments using amino-terminal FLAG-tagged m-, f-, and z-AID (tm-, tf-, and tz-AID). The percentage of switching to IgG1 triggered by tm-, tf-, and tz-AID was lower than with the native counterparts but clearly above background levels for m- and z-AID (Fig. 2 B). In agreement with the observation that teleost AID may be thermolabile, immunoblotting revealed that AID from fish, notably tf-AID, was less stable than tm-AID in mouse B cells grown at 37°C (Fig. 2 C) (18). We conclude that teleost AIDs can induce switching to IgG1 in mouse B cells and that differences in the intrinsic deoxycytidine deamination activity and/or the stability of different AID proteins correlate with switching activity.

**Teleost AID produces normal switch junctions in mammalian cells**

<del>Sμ–Sy1 switch junctions frequently show regions of microhomology and the DNA region around the junction is enriched in somatic mutations (23). To determine whether switch junctions produced by teleost AID were similar to junctions produced by mammalian AID, we cloned Sμ–Sy1 switch junctions from m- or z-AID expressing AID<sup>−/−</sup> B cells. We found no substantial differences in the amount of donor/acceptor homology or mutations at the switch junctions produced by mammalian and teleost AID. The mean length of overlap for m- and z-AID–generated junctions was 1.2 (μ = 26) and 1.6 (μ = 26; Fig. 3, A and B), and the mutation frequency in the vicinity of the junctions (±50 bp) was 0.4 and 0.7 × 10<sup>−2</sup>, respectively. Furthermore, the breakpoint distribution in Sμ and Sy1 measured by scatter analysis was similar in m- and z-AID (Fig. 3 C). We conclude that z-AID generates normal Sμ–Sy1 junctions, suggesting that the proteins required for switching, including nonhomologous end joining, mismatch repair, excision repair, and base excision repair enzymes, were recruited by z-AID in mouse B cells (for review see reference 24).

Class switch recombination requires switch region transcription, AID targeting, cytidine deamination, mismatch detection, and repair (17). In addition to deamination, AID is thought to be involved in targeting and switch lesion repair. Three lines of evidence support the existence of a specific targeting mechanism. First, only a small number of genes outside of antibody genes are somatically mutated (17). Second, B cells stimulated to undergo class switching in vitro mutate Ig switch regions but not Ig V regions (25). Third, deoxycytidine deamination activity and transcription of the switch regions is not sufficient for switching because AID–related deaminases, such as APOBEC-1, and catalytically active carboxy terminus AID mutants fail to induce switching to IgG1 in IL-4/LPS-stimulated B cells (26–28). Transcription exposes single-stranded DNA, which is the substrate of AID, and it is also essential for Ig gene targeting. The dual role of transcription in class switch recombination and hypermutation has made it difficult to identify the function of individual cis elements or transcription factors in the reaction. The only exception is the observation that an E47 binding site appears to enhance mutation of a transgenic substrate independently of its effects on the rate of transcription, and it has been proposed that AID may be targeted to Ig DNA by interaction with transcription factors like E47 (29). Finding that zebra fish AID is completely functional for class switching in mouse B cells suggests that the interaction domains required for targeting AID to Ig switch regions evolved before the appearance of class switch recombination. This apparent paradox could be explained if the protein interaction domains evolved in the context of other DNA modification reactions and were later adapted for different switch recombination.

We conclude that the molecular attributes required for AID-induced class switching did not coevolve with switch recombination and that the critical event for emergence of class switching in the last 400 million years was the evolution of switch regions and multiple constant region genes in the Ig heavy chain locus.

**Materials and Methods**

Fugu (<i>F. rubripes</i>) and zebra fish (<i>D. rerio</i>) full-length AID cDNA. Fugu spleen total RNA (provided by M. Toshiaki, Fukui Prefectural University, Obama, Japan) was reverse transcribed into first-strand cDNA (GE Healthcare) and used as a template for amplification of the full-length AID transcript. The primers were Fugu-AID (5′-ATCCCCGCGCGGAGTGTCAAA-3′) and Fugu-AIDas (5′-GAGAGAGACAGATGACGAAGATG-3′). All cDNA clones were derived from aberrantly spliced RNA, as a short intron was retained. To obtain a fugu AID cDNA with a correct translation frame, we used Fugu-AID-EcoRs (5′-TTTTGGAATTACCCTACCATGATCCCAAGCTA-3′) and Fugu-AIDas (5′-GACGAAGACGAGATGACGAAGATG-3′). Finding that zebra fish AID is completely functional was derived directly from a cDNA as previously described (22) and differs from the sequence deduced by genomic sequencing in which exon boundaries were incorrectly deduced (30).

**Mutation in <i>E. coli</i> and <i>S. cerevisiae</i>.** We measured the deoxycytidine deaminase activity of AID in <i>E. coli</i> as described previously (19), with the exception that the codon usage in the AID sequences was not optimized for expression in prokaryotes. For experiments in <i>S. cerevisiae</i>, mouse, zebra fish, and fugu, AID cDNAs with a Kozak consensus sequence for the initiation of translation were cloned into a galactose-inducible expression vector (pESC-His; Stratagene) and transformed into <i>E. coli</i> YPH500 (m<sup>1</sup>/m<sup>2</sup> ade2<sup>1</sup> his1<sup>1</sup> A1001) or <i>S. cerevisiae</i> YPH100, engineered to contain a constitutively expressed ung<sup>1</sup> gene in the HO locus (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051378/DC1). Targeted deletion of the ung gene was performed with a Trp-selectable cassette amplified from pUC18-TRP1 with the oligos ung<sup>1</sup> (5′-
GTGGTGCGATGAGAGATTTGGCAAAATCTATTAGCGGTGGAATAAACACATAGGCACCTGATAGCATGC-3') and ungRHA (5'-R-HATCAAGGTCCTCTGTCTCTGGGCTAGCTGCGCTGACGTTGACGGACTGCTGACGAGTC-3'). Targeted integration was confirmed by PCR.

Colones selected in his-ura (his-ura-trp for the urg strain) medium were expanded in 2% glucose liquid medium (then washed in water), and 2 OD were then grown in 2% galactose/1% raffinose his for 48 h. During this period, the cultures were diluted periodically to keep the cells dividing. Cells were plated in 5-ura 500 ng of DNA per 50-

We thank Dr. Miyadai Toshiaki for providing fugu RNA and Drs. Thiago Carvalho, Revat Maslaman, Kevin McBride, Nina Papavasiliou, and Almudena Ramiro for critical readings of the manuscript.

This work was supported by the Swedish Research Council and grants from the National Institutes of Health (1000879-01-CD300067-1-07552-113620) and the Leukemia Society (both to M.C. Nussenzweig). M.C. Nussenzweig is an investigator with the Howard Hughes Medical Institute.

The authors have no conflicting financial interests.

Submitted: 11 July 2005
Accepted: 12 August 2005

REFERENCES


