AID from bony fish catalyzes class switch recombination

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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.

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Antigen receptors found in amphibians, reptiles, birds, and mammals are products of lymphocyte-specific DNA modification reactions including V(D)J recombination, somatic hypermutation, and class switch recombination. Whereas V(D)J recombination is a site-specific recombination reaction that assembles Ig gene segments into a functional transcription unit, somatic hypermutation introduces point mutations in the assembled antibody gene. Both reactions alter the specificity of antibody combining sites and are found in all living jawed vertebrates (1). However, they are mechanistically distinct and catalyzed by different enzymes. V(D)J recombination is catalyzed by the recombination-activating genes 1 and 2, whereas somatic hypermutation is initiated by activation-induced cytidine deaminase (AID) (2–4).

Class switch recombination was the last of the lymphocyte-specific DNA modification reactions to emerge in evolution. It is a region-specific recombination reaction that does not alter the specificity of the antibody but replaces the constant region of the Ig heavy chain and, consequently, its effector function (5). Class switch recombination appears at the time of the divergence of the amphibians. It is absent in bony fish, including Japanese puffer and zebra fish, but is conserved in all tetrapods (6).

Despite the differences between somatic hypermutation and class switch recombination, the two reactions are catalyzed by AID (3, 4, 7, 8). AID initiates these reactions by deaminating cytosine residues in target DNA producing U:G mismatches that are recognized by uracil DNA glycosylase (UNG) or mismatch repair enzymes (9–13). The molecular mechanisms that mediate lesion formation, mismatch recognition, and initial processing appear to be common to both class switch and somatic hypermutation, but the requirements for completion of the two reactions are distinct. For example, efficient class switch recombination requires H2AX and ataxia telangiectasia mutated protein, whereas somatic hypermutation does not (14–16). In addition, the carboxy-terminal domain of AID is required for class switch recombination but not for somatic hypermutation, whereas the amino terminus is required for efficient somatic hypermutation and dispensable for switch recombination (for review see reference 17).
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

![Figure 1. m- and z-AID are catalytically active in *E. coli* and *S. cerevisiae*.](image1)

![Figure 2. AID from bony fish induces class switch recombination in mouse AID−/− splenocytes.](image2)
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−/− lymphocytes. To facilitate switching and retroviral
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 zebrafish 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 class 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 (F. rubripes) and zebra fish (D. rerio) 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-N (5′-ATCCCCCGCCGAGGTGTCAAA-3′) and Fugu-AID-as (5′-GACGAAGAGCGATGACGAAGATG-3′). All cDNA clones were derived from aberrantly spliced RNA, as a short intron was retained. To obtain a full AID cDNA with a correct translation frame, we used Fugu-AID-EcoRs (5′-TTTTGATTCCACCATGATCACCAGCTGATA-GACAGTA-3′), Fugu-fusion1-as (5′-CCACAAATTTCCGCAGAATT-GAAATGTCCTTTTGAGCTCACCAGT-3′), Fugu-fusion1-as (5′-GCGTAGGAGGTCTCTGTAGTGAGCTACAAAGACTATTTCTAT-GACAGTA-3′), and Fugu-AID-Not Ias (5′-TGTCCGAGGAGGTCTCTGTAGTGAGCTACAAAGACTATTTCTAT-GACAGTA-3′). The Zebrafish fish sequence used in this study 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 E. coli and S. cerevisiae.** We measured the deoxycytidine deaminase activity of AID in E. coli 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 S. cerevisiae, 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 by electroporation into YPH500 (ura3, ade2, leu2, his3, trp1, met15, lys2, can1, his3, leu2, ura3, ade2, trp1, his3, lys2, can1) cells and grown on raffinose plates (22). Cells were induced for AID expression by growing in glucose containing medium and selecting for uracil auxotrophy. The cells were then induced with 1% galactose and grown for 48–72 h before harvesting.
GTGGTGCAATGAGAGATGCTCAAAATTTCAATGTGACTCGGT
GTGAAATATAAGCTAAGGCACACTTGGAGTCTGG-3′) and ungRHA
(5′-RHIATCAAGGTCCTTTGACCTCTAAGGAGCATT-TGCACAGGTACGTTCTGACG-3′). Targeted integration was con-
figured by PCR.

Colonies selected in his−ura− (his− ura− trp− for the ung′ strain) me-
dium were expanded in 2% glucose liquid medium (then washed in water),
and 2 OD were then grown in 2% galactose/1% raffinose his− ura− trp− for
the ung′ strain) liquid medium for 48 h. During this period, the cultures were
diluted periodically to keep the cells dividing. Cells were plated in ura− his− ura− trp− for the ung′ strain) 2% glucose medium with or without 750
μg/ml 5-FOA to count viable cells and cells with null mutations in
ura3, respectively. All S. cerevisiae experiments were performed at 30°C.

Mouse B cell cultures and transduction. Mouse, zebra fish, and fugu
AID with a Kozak consensus sequence for the initiation of translation were
cloned into the pMX-PIE. Amino-terminal FLAG and hemagglutinin-
tagged AIDs were derived by PCR by adding the following sequence 5′ of
the second codon of AID by PCR: 5′-ATGGACTACAAGGAC-
CGATGCAAGGAGGAATATGACTGGTCTCTTCTGACG-3′. Targeted integration was con-
figured by PCR.

Amplification and cloning of Spt–Syl junctions. For the cloning of
Spt–Syl from AID−− splenocytes transduced with retroviruses, genomic
DNA was prepared according to standard procedures from samples that had
been transduced 48 h earlier. Switch junctions were amplified from 200–
500 ng of DNA per 50–μl reaction with oligos Spt3 (5′-AATGGATAC-
CCTACGTGGTTTTTAAAGTGGTCTTTA-3′) and y1-R (5′-CTAAT
AGCTCCTGCTCTTCTTG-3′) and cloned, as described previously (14). Colonies were then screened by PCR with oligos Spt-nested (5′-
CTAATTTAGATACGTTAAGGGAC-3′) and y1-R and sequenced
with an M13rev oligo.

Flow cytometry. Mouse B cells were stained with biotin-labeled anti-
mouse IgG1 antibodies and visualized with streptavidin APC (all from Bec-
ton Dickinson). Dead cells were excluded on the basis of forward side scat-
tering. Data were acquired on a FACSCalibur and analyzed with CellQuest
software (both from Becton Dickinson).

Online supplemental material. Fig. S1 describes the AID–expressing
plasmids and the ura3 reporter for AID activity in S. cerevisiae. Table S1
shows oligos used to generate the ura3 mutation reporter construct. Table
S2 shows the mutations found in the ura3 gene from 25 independent S-FOA
resistant S. cerevisiae clones recovered after induction of m-AID. Online
supplemental material is available at http://www.jem.org/cgi/content/full/
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