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Reversible switching of immunoglobulin hypermutation machinery in a chicken B cell line

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Abstract

A chicken B lymphoma line, DT40, hypermutates immunoglobulin (Ig) genes spontaneously during culture. Thus, cultured DT 40 cells constitute a useful Ig library for screening antibodies (Abs) in vitro. To fix desirable Ig mutants by stopping hypermutation or to resume mutation for further improvement of Ab affinity, activation-induced cytidine deaminase (AID), a key enzyme responsible for the Ig mutation machinery, must be switched on or off. To this end, we generated a DT40 line whose one AID allele was disrupted, and the other allele was replaced by the loxP-flanked AID construct. In this engineered cell line designated as DT40-SW, AID expression could be switched reversibly by tamoxifen-regulated Cre recombinase. Devices were also introduced to discriminate between the "AID-ON" and the "AID-OFF" cells by GFP expression and puromycin resistance, respectively. Starting from a single DT40-SW cell, Ig gene repertoire was efficiently diversified during culture only when AID expression was on.

Keywords: DT40; Immunoglobulin gene; Hypermutation; Gene conversion; Antibody; Activation-induced cytidine deaminase

Several approaches have been exploited to select an antigen-specific antibody (Ab) in vitro. For instance, single-chain Abs can be selected from a phage-display library [1–3]. To improve the primarily selected clones, these may be diversified by secondary mutagenesis, followed by expression in appropriate host cells to assess their functions. Generally, these processes are laborious and time-consuming. This, however, is successfully accomplished in vivo in the immune system, where somatic hypermutation of immunoglobulin (Ig) genes coupled with selection of B cells expressing high-affinity Abs results in an increase in the affinity of secreted Abs, a process termed affinity maturation [4–6]. In this context, a chicken B lymphoma line, DT40, may be useful to mimic the affinity maturation process in in vitro culture [7], because DT40 cells produce IgM Abs, and undergo hypermutation in the variable region (V) of Ig genes spontaneously during culture [8–10]. Using DT40, mutagenesis in Ig genes and their expression can be sequentially performed in a single cell. Propagated DT40 cells have been shown to generate Ig diversity that was sufficient for selection of Abs against test antigens including protein A, streptavidin, and a rat IgG [11]. In addition, targeted integration of transfected genes occurs with exceptionally high frequency in DT40 [12], thereby making the cell line genetically tractable.

Chicken B cells including DT 40 cells have been shown to diversify Ig genes not only by point mutation, but also by another mechanism called gene conversion, which introduces partial sequences from upstream V pseudogenes into the expressed V gene [13,14]. Recently, activation-induced cytidine deaminase (AID) has been identified as an essential enzyme responsible for point mutation [15–18] and gene conversion [8,9]. DT40 cells have been shown to express AID constitutively

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[8,9,11]. To isolate a genetically stable clone producing a desirable monoclonal antibody, AID activity must be shut down for avoiding further mutation. On the other hand, AID expression should be switched on again to resume secondary mutation for improving the Ab affinity. To this end, we generated a DT40 line whose hypermutation machinery can be reversibly switched "ON" and "OFF" by controlling AID expression with an exogenous stimulus.

Materials and methods

Oligonucleotides and DNA polymerase. All primers used in this study are listed as follows: AID1, AID4, AID9 [8], ACT2 5′-CACC TCGAGGTGAGCCCCACGTTCTGCTT-3′, ACT4 5′-CCAGATC TTGTCGACATCATCCCAGTTGGTGACAAT-3′, AIDF1 5′-CAC CGTCTGAAACCCAGCAAGAGTAGATAG-3′, AIDR1 5′-CTCC AGGAGGTGAACCATGTGATGCGGTAG-3′ cmvE-R 5′-ACTCC ATATATGGGCTATGAACTAATGACC-3′, CITE1 5′-CGACAGA TCTAAGCTTGTAATACGACTCACTATAGG-3′, CITE2 5′-CAT AGGATCCGTCGACATGGTATTATCATCGTGTT-3′, cLL5 5′-C GGCGTGGGGATCCACAGCTGCTGGGATTC-3′, cCL3 5′-ACT CGGATCCCTTCAGGGTCTTCGTGATAG-3′, GFP1 5′-GCCCT GAGCAAAGACCCCAA-3′, and SV-1 5′-AACTCATCAATGTA TCTTATCATGTCTGG-3′. KOD plus DNA polymerase (TOYOBO) was used for PCR amplification as instructed by the manufacturer.

Plasmid construction. To express the Cre chimeric protein gene [19] under the control of the CMV promoter, a *Hin*dIII fragment from pANMerCreMer [19] was ligated into the *Nhe*I–*Not*I region of pEGFP-N1 that contains the CMV promoter and neomycin resistance gene, after filling up digested sites, to yield pCNMerCreMer.

Genomic DNA and cDNA of the AID gene were amplified by primers AID1 and AID4, cloned into the *Eco*RV site of pBluescript (Stratagene), and confirmed by sequencing. To construct the AID-disrupting vector pAID-KO, the *Bst*XI–*Bam*HI region of the AID genomic DNA, which contains the exon encoding the catalytic domain of AID, was replaced with a loxP-flanked blasticidin S resistance gene (Bsr) in a *Bam*HI fragment of pLoxBsr [20] after filling in the digested ends.

The targeting vector pAID-ctrl was prepared as follows. An IRES amplified from pCITE2a(+) (Novagen) by primers CITE1 and CITE2 was cloned into the *Bam*HI site of pEGFP-N1 (Clontech) after *Bgl*II/*Bam*HI-digestion. The IRES connected with the GFP gene was excised by *Nhe*I/*Hpa*I-digestion and inserted into the *Nhe*I–*Eco*47III region of pLoxPuro [20] to yield pLIG containing one loxP-flanked part. Next, a *Bam*H1–*Eco*RI fragment containing rabbit β-globin poly(A) from pCAGGS [21] was inserted into pPUR (Clontech) with modification of the *Eco*RI site to a *Not*I linker. The puromycin resistance gene (Puro^r) including SV40 poly(A) connected with the inverted β-globin poly(A) was excised by *Hind*III/*Not*I-digestion, and replaced with IRES and GFP genes in pLIG to create pLPsg containing another loxP-flanked part. Two *Spe*I/*Not*I-digested loxP-flanked parts from pLIG and pLPsg were ligated at the *Not*I sites and inserted into the blunted *Nhe*I site of pExpress [20] whose promoter had been replaced with the CAG promoter from pCAGGS [21] by *Spe*I/*Xba*I-digestion, to yield pCLPGIL. A *Pvu*II–*Sma*I fragment of the AID cDNA was inserted into the blunted *Eco*RI site of pCLPGIL to yield pCLPGIAL. Finally, the CAG-driven and loxP-flanked AID gene cassette was excised by *Spe*I/*Eco*RI-digestion and inserted into the *Bst*XI–*Bam*HI region of AID genomic DNA to complete pAID-ctr1 (Fig. 1A).

Cell culture and transfection. The DT40 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). DT40 cells were cultured in RPMI 1640 medium (ICN Biomedicals) supplemented with 10% fetal bovine serum (Life Technologies), 1% chicken serum (Sigma), 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mM pyruvic acid, 100 μg/ml penicillin G, and 50 μg/ml streptomycin at 37 °C in 5% CO_2 and 95% air. DT40 cells were suspended in 250 μl phosphate-buffered saline at 2×10^7 cells/ml and transfected with 15 μg of a linearized vector by electroporation using Gene Pulser Xcell (Bio-Rad) at 550 V and 25 μF in 4 mm cuvette. DT40 cells transfected with pCNMerCreMer were selected in a medium containing 2 mg/ml G-418 (Sigma). A DT40 clone bearing the 4-OHT-regulated Cre recombinase gene was stepwise transfected with pAID-KO or pAID-ctrl and selected in a medium containing 50 μg/ml blasticidin S (Invitrogen) or 0.5 μg/ml puromycin (Sigma), respectively. The targeted integration to AID loci was confirmed by PCR (40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 4 min at 68 °C) using primers AIDF1, AIDR1, SV-1, and cmvE-R for AID loci, or ACT2 and ACT4 for the β-actin gene. Transcription of the endogenous AID genes was assessed by RT-PCR (35 cycles of 15 s at 94 °C, 30 s at 65 °C, and 1 min at 68 °C) using primers AID1 and AID4, or cLL5 and cCL3 for the L chain gene.

Switching of AID expression by 4-OHT-treatment. DT40-SW cells were incubated with 50 nM 4-OHT for 48 h. After washing twice to remove free 4-OHT, the treated cells were cultured for 48 h. GFP expression was analyzed with FACSCalibur (BD Biosciences). GFP$^+$ (AID-ON) DT40-SW cells were sorted by using FACSAria equipped with Auto Cell Deposit Unit for single cell isolation (BD Biosciences). The purity of the sorted cells was more than 95%. To isolate AID-OFF DT40-SW cells, 4-OHT treated cells were cultured in a medium containing 0.5 μg/ml puromycin for at least 48 h. To confirm the inversion of the loxP-flanked AID gene, the GFP$^-$ or GFP$^+$ cells were genotyped by PCR (40 cycles of 15 s at 94 °C and 3 min at 68 °C). AID transcription was compared between wild type DT40 and AID-ON DT40-SW cells by RT-PCR using primers AID9 and AIDR1.

Analysis of gene conversion in V genes. cDNA was synthesized from total RNA of GFP$^-$ or GFP$^+$ DT40-SW cells as described previously [6]. The L chain genes were amplified by primers cLL5 and cCL3, cloned into pCR-Blunt (Invitrogen), and sequenced with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence changes were assigned to gene conversion or somatic point mutation by comparing mutated sequences with the published Vλ pseudogene sequences that could act as donors for gene conversion [13]. Maximal length of a Vλ1 sequence covering a mutation site that coincided with a donor pseudogene was indicated as a converted region illustrated in Fig. 2C. When a sequence spanning a mutation could not be assigned to any pseudogene, this was regarded as point mutation. Sequence data of the original Vλ1 (Lmn) and mutant (Lm1–Lm13) genes were deposited to DDBJ/EMBL/GenBank (Accession Nos.: AB193002, AB193003, AB193004, AB193005, AB193006, AB193007, AB193008, AB193009, AB193010, AB193011, AB193012, AB193013, AB193014, and AB193015).

Results and discussion

Integration of an AID-switching device into DT40

The strategy for switching AID expression is based on the replacement of the endogenous AID gene with the loxP-flanked counterpart whose orientation can be inverted by Cre recombinase [22,23]. First, DT40 cells were transfected with a modified pANMerCreMer vector that bears the gene encoding a chimeric protein between the Cre protein and a mutated hormone-binding domain of the murine estrogen receptor [19]. In this transfectant, Cre recombinase activity can be turned on by exposing the cells to an anti-estrogen drug, 4-hydroxytamoxifen (4-OHT) [19,20].