A hallmark of active class switch recombination: Transcripts directed by I promoters on looped-out circular DNAs

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To specify when and where Ig class switch recombination (CSR) takes place, a good molecular marker closely associated with active CSR is required. CSR is accompanied by deletion of circular DNA from the Ig heavy chain locus. The circular DNA contains a DNA segment between Sμ and a target S region including its I promoter, which is driven by specific cytokine stimulation before CSR. We found that the specific I promoter is still active in looped-out circular DNA and directs production of I-Cμ transcripts termed "circle transcripts." Reverse transcription–PCR demonstrated transient induction of specific circle transcripts upon CSR in a murine lymphoma cell line, CH12F3-2A, as well as spleen B cells. Production of the circle transcripts appeared to depend on expression of activation-induced cytidine deaminase (AID), an essential factor for CSR. A comparison of kinetics between circle transcripts and circular DNA showed more rapid disappearance of circle transcripts. Thus, circle transcripts may serve as a hallmark for active CSR *in vitro* and *in vivo*.

Antigen stimulation of mature B lymphocytes often but not always leads to two types of genetic alterations: class switch recombination (CSR; ref. 1) and somatic hypermutation (SHM; ref. 2). It is well established that not all IgG or IgA has undergone SHM, and conversely, some IgM has SHM, indicating that neither CSR nor SHM is a prerequisite of the other (3, 4). Previous studies reported that SHM occurs in germinal centers (5), whereas CSR occurs not only within germinal centers (6) but also in the extrafollicular area or periarteriolar lymphoid sheath (PALS) in the T cell zone of lymphoid organs (7). Activated B cells often migrate from the site of stimulation, home to other tissues, and differentiate into plasma cells (8, 9). Some stimulated B cells and their progenies become memory B cells, which can respond to the secondary challenge by the same antigen with increased amplitude and affinity. Memory T cells are also shown to migrate to many nonlymphoid tissues (10). To understand the dynamic regulation of the immune response *in vivo* it is important to dissect the steps of B cell activation and differentiation not only temporally but also anatomically. However, it has been difficult to pinpoint when and where CSR actually takes place. This is because there is no good molecular marker that appears at the moment of CSR and disappears quickly after CSR.

There are several candidate markers that are associated specifically with CSR. CSR is preceded by the expression of germline transcripts (GLTs) initiated from I promoters, which are regulated specifically by various cytokines. Activation-induced cytidine deaminase (AID) has been shown recently to be induced specifically in activated B cells and essential to both CSR and SHM (11, 12). Because AID deficiency did not affect either GLT synthesis or nonhomologous end-joining repair, AID is most likely to be involved in a critical step of CSR. CSR is accompanied by looping-out deletion of a DNA segment containing Cμ and other C_H genes from the chromosome (13–15). A resultant circular DNA (CD) can be a good marker for active CSR if it decays rapidly. However, PCR amplification of CD generates products that are heterogeneous in size and thus appear smeary on gel electrophoresis unless single-cell PCR is undertaken. In addition, this method has limited sensitivity, because only a single copy of CD is generated in switched cells.

To overcome these problems we examined whether isotype-specific transcripts are generated from I promoters located on excised CDs and found that isotype-specific I-Cμ transcripts termed "circle transcripts" (CTs) were produced only in cells that express AID and undergo CSR. Kinetic analysis of CTs showed that they disappeared more quickly after removal of cytokine stimulation than GLT, CD, or AID. The results indicate that CTs are a hallmark to demonstrate active CSR *in vitro* and *in vivo*.

Materials and Methods

Cell Culture, Stimulation, and Flow Cytometry. CH12F3-2A, a subline of CH12F3 cells, was used for the time course analysis, cultured, and stimulated as reported previously (16). Cells were stained with phycoerythrin-conjugated anti-IgM antibodies and FITC-conjugated anti-IgA antibodies and analyzed as described previously (16). Red blood cell-depleted spleen cells (5×10^5/ml) from 5-week-old $AID^{+/+}$, $AID^{+/-}$, and $AID^{-/-}$ mice were cultured for 2 days in 9 ml of the culture medium (11) containing 50 μg/ml lipopolysaccharide (LPS, Sigma), 10 ng/ml mouse IL-4 (GIBCO), LPS + 10 units/ml mouse IFN-γ (Genzyme), or LPS + 1 ng/ml human transforming growth factor (TGF) β1 (R & D Systems). Freshly isolated spleen cells were used as nonstimulated controls.

PCR. Total RNA was extracted from cultured cells by using TRIzol (GIBCO) according to manufacturer instructions. Genomic DNA was purified from the same source after separation of RNA from TRIzol lysate. cDNA was synthesized with Superscript II (GIBCO) by using 2 μg of total RNA and 1 μg of poly $d(T)_{12-18}$ (Amersham Pharmacia) in a 20-μl reaction volume, one twentieth of which was used as a template for reverse transcription (RT)–PCR in a 25-μl reaction volume. Amplification of AID transcripts was done by an initial denaturing step of 94°C for 5 min followed by 22 cycles of PCR (94°C for 20 s, 58°C for 30 s, 72°C for 1 min) by using recombinant *Taq* polymerase (Takara) with a 119- and 118-primer pair (11). Amplification of αGLT was done by an initial denaturing step of 94°C for 5 min followed by 22 cycles of PCR (94°C for 5 min, 58°C for 30 s, 72°C for 1 min) by using recombinant *Taq* polymerase (Takara) with an IαF and CαR primer pair. CTs were amplified by an initial denaturing step

Abbreviations: CSR, class-switch recombination; SHM, somatic hypermutation; GLT, germline transcript; AID, activation-induced cytidine deaminase; CT, circle transcript; LPS, lipopolysaccharide; TGF, transforming growth factor; RT, reverse transcription; HPRT, hypoxanthine-guanine phosphoribosyl transferase; CD, circular DNA.

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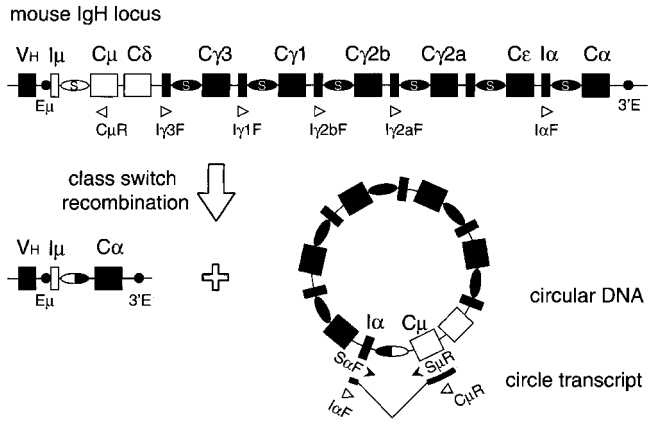

Fig. 1. CD and CTs. The mouse Ig heavy chain locus after VDJ rearrangement is shown schematically at the top. Ovals indicate S regions, and the rectangles before and after S regions are I exons and constant region exons (C), respectively. Variable exon (V_H) is shown by the left-most rectangle. Iμ, Sμ, Cμ, and Cδ are shown by open symbols. Locations of the Eμ and 3′ enhancers (3′E) are shown. The IgA CSR product is shown at the bottom. Thick lines below CD indicate exons of αCTs connected with a v-shaped line representing splicing. Open triangles indicate the position and direction of primers used in RT-PCR. Closed arrowheads below CD represent primers for PCR detecting CDs.

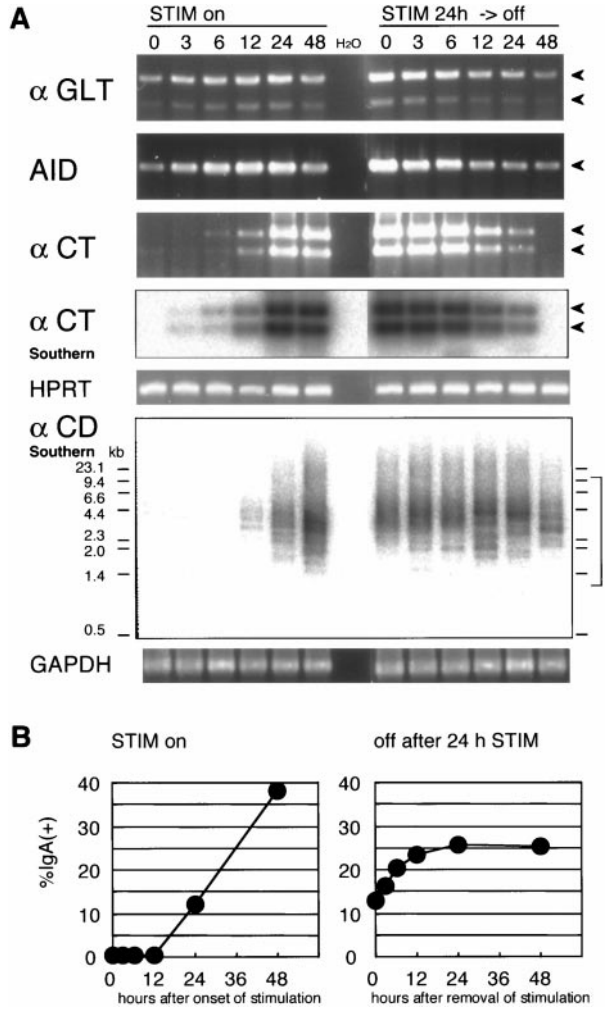

Fig. 2. Time course of appearance of CD and CTs. (*A*) αCDs, αGLT, αCTs, and AID transcripts were detected by PCR in CH12F3-2A cells at 0, 3, 6, 12, 24, and 48 h after stimulation. Similarly, the same set of molecules is detected in the cells prestimulated for 24 h at 0, 3, 6, 12, 24, and 48 h after removal of stimulation. For αCTs and αCDs, PCR products were transferred to nylon membrane and probed with internal oligonucleotides. The right bracket indicates range of αCD PCR products (1–10 kb) expected from recombination between Sμ (5 kb) and Sα (4 kb). The glyceraldehyde-3-phosphate dehydrogenase sequence and HPRT transcripts are amplified as an internal control for genomic PCR and RT-PCR, respectively. Lanes denoted with H_2O indicate blank controls for cDNA. (*B*) Time courses of the increase in IgA^+ cells after onset (right) or removal (left) of stimulation are shown.

of 95°C for 9 min followed by 35 cycles of PCR (94°C for 30 s, 58°C for 1 min) by using AmpliTaq Gold (Perkin–Elmer) in the presence of 2.0 mM Mg^{2+} with pairs of CμR and one of the isotype-specific I region primers Iγ3F, Iγ1F, Iγ2bF, Iγ2aF, and IαF. Expected sizes of PCR products are 332 bp (Iγ3F), 408 bp (Iγ1F), 311 bp (Iγ2bF), 538 and 420 bp (Iγ2aF), and 458 and 318 bp (IαF). CTs were detected after transfer to Hybond N+ membrane (Amersham Pharmacia) by a ^{32}P-labeled CμP oligonucleotide probe located 41 bp upstream of the CμR primer. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) transcripts were amplified by 25 cycles of PCR (94°C for 30 s, 50°C for 30 s, 72°C for 1 min) by using recombinant *Taq* polymerase (Takara) with an HPRT-7 and HPRT-9 primer pair (17). αCD were PCR-amplified from 20 and 80 ng of genomic DNA from CH12F3-2A cells and splenocytes, respectively, by a denaturing step of 95°C for 1 min followed by 33 cycles at 98°C for 20 s and 68°C for 6 min by using LA *Taq* (Takara) with SαF and SμR primers in the presence of 2.5 mM Mg^{2+}. The PCR products were separated with 1% agarose gel electrophoresis, transferred to Hybond N+ membrane, and probed with ^{32}P-labeled SμP oligonucleotide located 35 bp upstream of the SμR primer. Control PCR for the input of genomic DNA was done by amplification of the glyceraldehyde-3-phosphate dehydrogenase gene by using GF and GR primers (18) by a denaturing step of 94°C for 5 min followed by 25 cycles of PCR (94°C for 20 s, 60°C for 30 s, 72°C for 1 min). DNAs electrophoresed in agarose gel were stained with SYBR Green I (Molecular Probe) and recorded by LAS-1000 Plus (Fuji Film). The following primers and oligonucleotide probes were used: Iγ3F, 5′-TGG GCA AGT GGA TCT GAA CA-3′; Iγ1F, 5′-GGC CCT TCC AGA TCT TTG AG-3′; Iγ2bF, 5′-CAC TGG GCC TTT CCA GAA CTA-3′; Iγ2aF, 5′-GGC TGT TAG AAG CAC AGT GAC AAA G-3′; IαF, 5′-CCA GGC ATG GTT GAG ATA GAG ATA G-3′; CμR, 5′-AAT GGT GCT GGG CAG GAA GT-3′; CμP, 5′-CAG CCC ATG GCC ACC AGA TTC TTA TCA GAC-3′; CαR, 5′-GAG CTG GTG GGA GTG TCA GTG-3′; SαF, 5′-ATA TCG ATG CTT CCT GGA AAG CAG CAA CAG GAG ACT-3′; SμR, 5′-ATG GTC GAC AAA GAG AAA TGG AGG GGG TAA GAA TCT GTC T-3′; and SμP, 5′-TCT TGC CTC CTG TCA GAC AGG AGA TTC CTC TAC AC-3′.

Results and Discussion

The best marker of active CSR should be expressed specifically during CSR and disappear quickly after CSR. In addition, it is desirable that an easy and sensitive assay method is available for that marker. Although looped-out CDs, which are always produced on CSR, may be a good marker of CSR, their assay is not so easy and sensitive. We therefore examined whether an I promoter, the activation of which by cytokine stimulation precedes CSR, remains active even on CDs looped-out on CSR. We first designed primers to detect transcripts produced from CSR-derived CD in a mouse lymphoma cell line, CH12F3-2A, which undergoes CSR efficiently from IgM to IgA (Fig. 1). The primer pair of IαF and CμR is specific to transcripts from circle

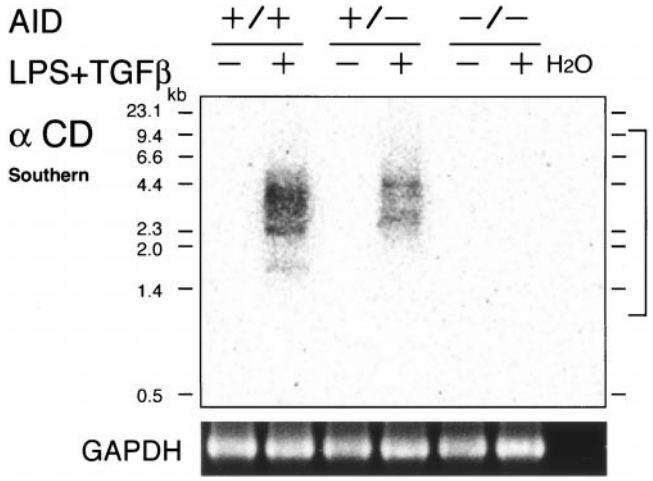

Fig. 3. Lack of CD formation in AID-deficient B cells. PCR analysis of αCDs is shown. Template DNAs were prepared from spleen cells of $AID^{+/+}$, $AID^{+/-}$, and $AID^{-/-}$ mice with or without stimulation with LPS and TGFβ for 2 days. PCR products were transferred to nylon membrane and probed with an internal oligonucleotide. The glyceraldehyde-3-phosphate dehydrogenase sequence was amplified as an internal control. The right bracket indicates a range of αCD PCR products (1–10 kb) expected from recombination between Sμ (5 kb) and Sα (4 kb). A lane denoted with H_2O indicates blank controls for cDNA.

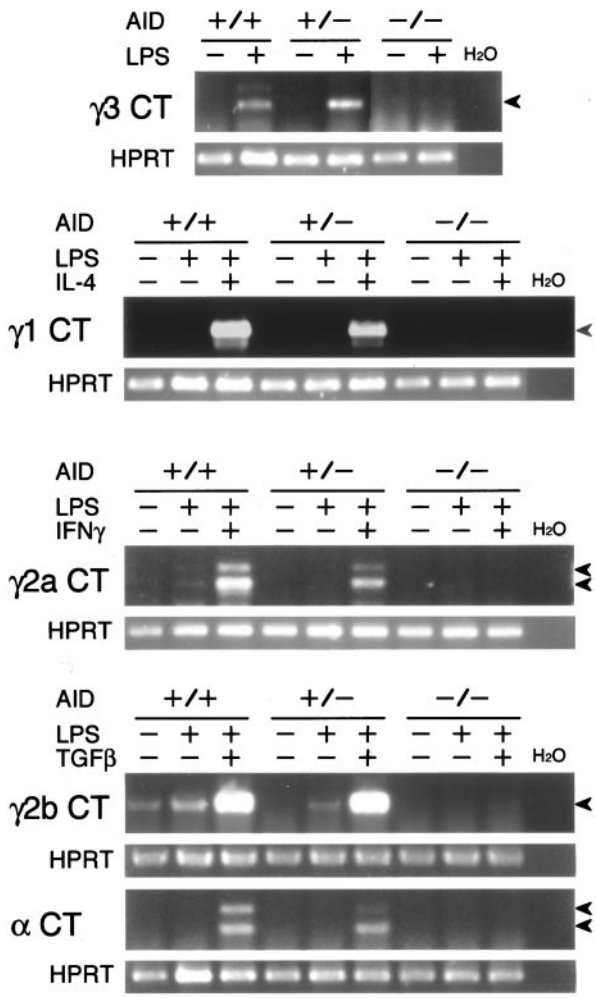

Fig. 4. Dependence of CTs on AID. CTs from Iγ3, Iγ1, Iγ2b, Iγ2a, and Iα promoters on CD detected by RT-PCR. Template cDNAs were prepared from spleen cells of $AID^{+/+}$, $AID^{+/-}$, or $AID^{-/-}$ mice with or without the indicated stimuli for 2 days. Two bands of γ2a and αCTs represent alternatively spliced forms. HPRT transcripts were amplified as an internal control. Lanes denoted with H_2O indicate blank controls for cDNA.

DNA of IgA switching. After stimulation of CH12F3-2A cells with CD40L, IL-4, and TGFβ, RT-PCR products of Iα-Cμ transcripts (αCTs) began to be detected at 6 h by SYBR Green I staining and at as early as 3 h by Southern blotting (Fig. 2*A*). They reached a plateau 24–48 h after stimulation. Specificity of PCR amplification was verified by direct sequencing of PCR products (data not shown). The appearance of two bands corresponds to the presence of alternative splicing donors in the Iα exon. The estimated numbers of surface IgA-positive cells used for the PCR template are 6 at 0 h, 5 at 3 h, 7 at 6 h, 18 at 12 h, 498 at 24 h, and 2,063 at 48 h. Because the time lag between DNA recombination and surface IgA expression is estimated to be less than 12 h, the number of cells that finished recombination at 12 h may be ≈500. Thus, the PCR condition applied here can easily detect 500 recently switched cells without enhancing the signal by Southern hybridization. When stimulation was removed 24 h after the onset of stimulation, the amount of αCTs decreased quickly and fell below a detection limit 48 h after stimulation removal even by RT-PCR coupled with Southern blotting.

Similarly, AID transcripts and αGLTs (Iα-Cα transcripts) were measured by RT-PCR, and αCDs (Sα-Sμ) were detected by genomic PCR using the appropriate primers (SαF and SμR; Fig. 1). Induction of AID transcripts and αGLTs was augmented quickly after CSR stimulation (Fig. 2*A*), and both continued to be detected 48 h after removal of stimulation. αCDs appeared as faint smeary bands 12 h after stimulation. It is important to note that αCTs disappeared completely 48 h after removal of stimulation even though αCDs, AID, and αGLT were still detectable.

The frequency of IgA^+ cells measured by flow cytometry continued to increase until 24 h after removal of stimulation and remained constant between 24 and 48 h, suggesting that no additional CSR events were induced in this interval. Because the cell number increased only 4-fold during this period (data not shown), it is reasonable that CDs were still detectable at 48 h by PCR amplification. Quicker disappearance of αCTs than CDs suggests that Iα promoters on CD may be responsive to cytokines similar to those on the chromosome, although the former have lost link with both the Eμ and 3′ enhancers (Fig. 1). Proper cytokine regulation of Iγ1 and Iγ2a promoters independent from the 3′ enhancer was reported previously (19, 20).

To test whether generation of CTs detected by RT-PCR depends on CSR, AID-deficient mice were used. We first confirmed that αCDs could be amplified from $AID^{+/+}$ and $AID^{+/-}$ but not $AID^{-/-}$ B cells stimulated with LPS plus TGFβ (Fig. 3). Then, freshly isolated splenocytes from AID-deficient mice were stimulated *in vitro* with LPS in the presence or absence of IL-4, IFN-γ, or TGFβ for 2 days. RT-PCR analyses of their cDNAs showed that γ3 (Iγ3F-CμR), γ1 (Iγ1F-CμR), γ2b (Iγ2bF-CμR), γ2a (Iγ2aF-CμR), and α (IαF-CμR) CTs were detected in $AID^{+/+}$ and $AID^{+/-}$ but not $AID^{-/-}$ spleen cells (Fig. 4). The specificity of PCR amplification was confirmed by Southern blot hybridization and direct sequencing (data not shown). The CTs thus detected cannot be explained by simple PCR artifacts such as template switching or *trans*-splicing between two GLTs, because GLTs are normally induced in AID-deficient B cells by stimulation *in vitro* (11). Because it is known that CSR is blocked in AID-deficient B cells (11), AID dependence of CTs provided direct evidence for their association with CSR.

Taken together, CTs appear to serve as a better molecular marker for active CSR than GLT, AID, or CD. Detection of CTs specifically reflects CSR events and seems to be easier and more sensitive than that of CDs. This method can be applicable to detect B cells undergoing CSR *in situ* when coupled with single-cell PCR.

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