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Switch Region Identity Plays an Important Role in Ig Class Switch Recombination

Palash Bhattacharya,¹ Robert Wuerffel,¹ and Amy L. Kenter

Ig class switch recombination (CSR) is regulated through long-range intrachromosomal interactions between germline transcript promoters and enhancers to initiate transcription and create chromatin accessible to activation-induced deaminase attack. CSR occurs between switch (S) regions that flank Cμ and downstream Cκ regions and functions via an intrachromosomal deletional event between the donor Sp region and a downstream S region. It is unclear to what extent S region primary sequence influences differential targeting of CSR to specific isotypes. We address this issue in this study by generating mutant mice in which the endogenous Sy3 region was replaced with size-matched Sy1 sequence. B cell activation conditions are established that support robust γ3 and γ1 germline transcript expression and stimulate IgG1 switching but suppress IgG3 CSR. We found that the Sy1 replacement allele engages in μ→γ3 CSR, whereas the intact allele is repressed. We conclude that S region identity makes a significant contribution to CSR. We propose that the Sy1 region is selectively targeted for CSR following the induction of an isotype-specific factor that targets the S region and recruits CSR machinery. The Journal of Immunology, 2010, 184: 6242–6248.

Class switch recombination (CSR) promotes diversification of IgH effector functions encoded in constant (Ck) regions while maintaining the original Ag-binding specificity arising from V(D)J recombination. The mouse IgH (Igh) locus contains eight Ck genes (μ, δ, γ3, γ1, γ2a, γ2b, ε, and α) that are located downstream of the V, D, and Jκ segments and each Ck region is paired with a complementary switch (S) region (with the exception of Cδ). CSR occurs between S region sequences leading to an intrachromosomal deletional rearrangement that results in the formation of composite Sμ-Sx junctions on the chromosome while the intervening genomic material is looped out and excised. Activation-induced cytidine deaminase (AID) is required for CSR (1) and initiates formation of S region-specific double-strand breaks (DSBs) that are processed through a cascade of events mediated by nonhomologous end joining (2, 3).

S region transcription is a defining feature of CSR. Ck genes are organized in transcription units consisting of a noncoding intronic (I) exon, the S region, and the Ck coding regions, and germline transcripts (GLTs) initiate from an intronic promoter located upstream of each I exon and terminate 3' of the Ck coding region (4). Downstream S regions are selectively targeted for recombination with Sp by directed activation of I exon promoters in response to combinations of Ag or mitogen, cytokines, and costimulatory signals (4). CSR occurs between highly repetitive S regions with overall length varying from 1–12 kb (5), and length influences CSR frequency both in vivo and in vitro (6, 7). Deletion of S regions or their replacement with non-S region sequences by gene-targeting methods reduces CSR, indicating that S regions are the specialized targets in this recombination event (6, 8–10). An unresolved issue is how S regions are recognized by AID and the CSR machinery.

The question of whether S region primary sequence contributes to CSR specificity has been highly controversial. The degeneracy of the S region repeats and the absence of an identifiable recombination motif (11) have led to models in which higher order structures originating from palindromic S sequences provide the recognition code for recruitment of CSR machinery (12, 13). Indeed, murine and human S regions are G-rich on the nontemplate strand, which contributes to G4 tetraplexes (14) and R-loop formation that contain ssDNA stretches as substrate for AID deamination (15). Targeted inversion of the Sy1 region led to R-loop loss and a significant reduction of CSR activity, indicating a physiological role for R-loops in CSR (16). Studies of stably integrated transcribed switch substrates analyzed in a single B cell line have been interpreted to suggest that primary S sequence does not play a role in targeting CSR (17). An alternative model is based on transient switch plasmid studies that indicate that S region isotypes are differentially targeted for participation in CSR (reviewed in Ref. 18). CSR on transient switch substrates is AID dependent and strictly correlated with the pattern of switching at endogenous loci (2, 19, 20). In switch substrates, a single consensus Sy3 or Sy1 repeat is sufficient for μ→γ3 or μ→γ1 CSR, respectively, and limited mutation of the Sy1 consensus repeat flipped its specificity to Sy3 (7, 19). These studies raise the intriguing possibility of a central role for S region primary sequence that may function to target AID to specific S substrates.

In the wild-type (WT) control context, IL-4 reciprocally stimulates μ→γ1 and represses μ→γ3 CSR. To examine the role of S region sequence identity in CSR, we replaced the endogenous Sy3 region with a segment of Sy1 and compared μ→γ3 switching efficacy on the intact and targeted γ3 locus alleles upon activation with LPS in the presence or absence of IL-4. By using three independent measurements for CSR, including a newly devised method based on digestion-circularization (DC)-PCR to measure...
allele-specific CSR, we directly show that the γ3 allele containing the Sy1 replacement sequence is stimulated to switch μ→γ3 by the addition of IL-4, whereas the intact allele is repressed. Our findings demonstrate that in a physiological setting, S region sequence identity contributes to isotype selection and implies that μ→γ1 CSR is normally facilitated by an IL-4–induced factor specific for Sy1 DNA sequence.

Materials and Methods

Construction of targeting vectors and gene targeting

To create the S5 homology arm, a 5-kb region containing Sy3 was PCR amplified from an Iph containing bacterial artificial chromosome genome clone using Expand Long Template polymerase (Roche, Basel, Switzerland) and primers 5′-GTCCTAGAACGGGCGATCATTCGACCTG-3′ and 5′-GGCGCTTAGGAAGTCTATCTTCTTGCTT-3′ (nucleotide positions 1,475,648–1,480,625, accession number AJ851868; www.ncbi.nlm.nih.gov/nuccore/126349412). The amplified fragment was digested with XhoI to generate a 3.5-kb fragment that was cloned into the XhoI site of the pLNtk targeting vector. To generate the S5 homology arm, genomic configuration of D78343, Genbank, www.ncbi.nlm.nih.gov/nuccore/1663189 (nucleotide positions 1,484,682–1,489,641, accession number AJ851868) and cloned in the SalI site of the pLNtk vector. In both cases, PCR was carried out for 30 cycles (0.5 min at 94°C, 0.5 min at 55°C, and 3 min at 68°C with 0.2 min increments at 68°C every 20 cycles). The replacement 2-kb Sy1 region was designed from a bacterial artificial chromosome-derived BamHI fragment (nucleotide positions 1,511,993 and 1,514,053; accession number AJ851868) that was inserted between the neo cassette and the S5 homology arm into a NotI site that was introduced during the cloning of the S5 homology arm. This construct was transfected into the F1 embryonic stem (ES) cell clone. Appropriately targeted clones were identified by Southern blot analysis using a probe that hybridized upstream of the S5 homology arm. The genomic configuration of the targeted clones was confirmed by additional Southern analysis using probes that hybridized downstream of the S5 homology arm or to the neo regions. The 5′ probe (995 bp) was PCR generated with primers 5′-TGGAGAGATGGGTGGGCTTC-3′ and 5′-AGGTAATGATTACATCA-GTGAAC-3′ (nucleotide positions 1,476,126–1,477,120, accession number AJ851868) that was amplified from an EcoRI homology arm. This construct was used to program round 2. The primers for round 2 are Su.b 5′-GAAGGCTCTGACAGGTGTA-3′ (nucleotide positions 5,618,482–5,618,508) and C57g3.b 5′-GATCCACGGATGCTTGCATGCAC-3′ (nucleotide positions 5,544,363–5,544,380). PCR was carried out for 30 cycles at 94°C for 30 s, 72°C for 30 s, and 5 μl product is harvested at 26, 29, and 32 cycles. For the C57BL/6 (b) allele, the DC-PCR primers are Cs.7su.a 5′-GAAGGCTCTGACAGGTGTA-3′ (nucleotide positions 25,518,422–25,518,449) and Cs.7su.b 5′-CAAGGAGAAGTCTGAGTATAAC-3′ (nucleotide positions 25,544,482–25,544,463), and PCR was carried out for 15 cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s) in a 25-μl reaction and 2.5 μl used to program round 2. The primers for round 2 are Su.b 5′-GAAGGCTCTGACAGGTGTA-3′ (nucleotide positions 5,618,482–5,618,508) and Cs.7su.a 5′-GATCCACGGATGCTTGCATGCAC-3′ (nucleotide positions 5,544,363–5,544,380). PCR is carried out (94°C for 30 s, 63°C for 30 s; 72°C for 30 s) and 5 μl product is harvested at 26, 29, and 32 cycles. Nested μ→γ1 DC-PCR primers were designed to specifically analyze CSR on both the C57BL/6 and 129 alleles and are based on genomic sequence for C57BL/6 (accession number NT_166318) and 129 (accession number AJ851868) and using EcoRI digestion. PCR was carried out in a 25-μl reaction using 1.25 U Hot start Platinum Taq with 2.5 mM MgCl2 for both rounds 1 and 2. The primers for round 1 are Su.b 5′-GATCCACGGATGCTTGCATGCAC-3′ (nucleotide positions 5,618,482–5,618,508) and RWDCg1.a 5′-GCTATCTAGAGTGGGAGATGGCT-3′ (nucleotide positions 5,544,363–5,544,380). PCR is carried out for 30 cycles at 94°C for 30 s, 72°C for 30 s, and 5 μl product is harvested at 26, 29, and 32 cycles.

Cloning and DNA sequence analysis of S/S junctions

Su-S3y and Su-Su replacement Sy1(Sy1[3]) junctions were amplified by PCR with primers Su.UPA1F 5′-GGCTACGAGATGGGTTCCGCT-3′ (C57BL/6 accession number, NT_166318, coordinates 25,516,103–25,516,083; 129 accession number, AJ851868) and using EcoRI digestion. PCR was carried out in a 25-μl reaction using 1.25 U Hot start Platinum Taq with 2.5 mM MgCl2 for both rounds 1 and 2. The primers for round 1 are Su.b 5′-GATCCACGGATGCTTGCATGCAC-3′ (nucleotide positions 5,618,482–5,618,508) and RWDCg1.a 5′-GCTATCTAGAGTGGGAGATGGCT-3′ (nucleotide positions 5,544,363–5,544,380). PCR is carried out for 30 cycles at 94°C for 30 s, 72°C for 30 s, and 5 μl product is harvested at 26, 29, and 32 cycles.

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Results

Replacement of Sy3 with size-matched Sy1

To clarify the function of S region identity in CSR mechanisms, we sought to determine whether physiological μ→γ1 CSR is mediated by Sy1 sequence through an IL-4-inducible activity that is independent of GLT expression and AID. We used gene-targeted mutation techniques to exchange the endogenous Sy3 region and downstream flanking region with a 2-kb segment of the core Sy1 in the γ3b/γ3b F1 ES cell line (Fig. 1A). This Sy1 sequence was previously demonstrated to mediate CSR in transient switch plasmid studies (7, 19), is size matched with the endogenous Sy3, and contains a similar number of C residues. The γ3 GLT promoter, Sy3 exon and splice donor, and acceptors are unchanged. The F1 ES cell line derives from 129Sv-C57BL6 mice, and the two Igh alleles originate from the Ighγ (from 129Sv) and the Ighb (from C57BL/6) allotypes, respectively (16, 25). Our targeting construct was built using 129Sv DNA, and all targeting mutations were introduced into the Ighb allele while the Ighγ allele remained in the WT configuration. In the targeting vector, the inserted Sy1 is oriented in the physiological direction with respect to the germline Sy3 promoter and contained a neomycin resistance selectable marker gene, neo<sup>+</sup> (Fig. 1A). Neo<sup>+</sup> can interfere with germline transcription from I promoters (26) and was therefore flanked by 34-bp loxP sequences (27) and subsequently deleted by exposure to Cre recombines. The presence of sequence polymorphisms facilitates comparison of CSR levels on the modified Ighγ allele to that found on the unmodified Ighb allele. The size and integrity of the inserted Sy1 region was confirmed by Southern blot analyses with probes immediately flanking the 5′ and 3′ homology arms (Fig. 1B). On the targeted allele, the 6.3-kb and 12-kb EcoRI fragments are found when analyzed with the 5′ and 3′ probes, respectively. On the γ3b allele, an 18-kb fragment is found when analyzed with either probe. Cre deletion results in the removal of the neo gene, leaving a single loxP at the site. Following Cre-deletion, the 6.3-kb fragment is shortened to 4.5 kb, whereas the 12-kb fragment remains unchanged. To obtain mature B cells harboring the Sy1 knockin allele, referred to in this study as γ3<sup>3KI</sup>, the successfully targeted F1 ES cells were injected into RAG2-deficient blastocysts to generate chimeric mice.

GLT γ3 is expressed upon treatment with LPS and low-dose IL-4

A requirement for analysis of CSR in the Sy1 replacement chimeras is activation conditions that include IL-4 and are supportive of γ3 GLT expression. Severinson and coworkers (28) have shown that LPS alone and in combination with reduced-dose IL-4 (1–10 ng/ml) synergizes to stimulate large amounts of γ3 GLT, whereas LPS and very high concentration IL-4 (20 ng/ml) suppress γ3 GLTs. We confirm in this study that γ3 GLTs are highly expressed in purified CD4<sup>+ </sup>splenic B cells that are unstimulated or activated with LPS alone or LPS plus IL-4<sup>4b</sup> (10 ng/ml), whereas the γ1 GLTs are LPS plus IL-4 dependent, and AID is induced by either LPS or LPS plus IL-4 (Fig. 2A). A similar GLT expression profile is found for splenocytes from C57BL/6 × 129 (WT F1) and a chimera (Chi 28) that are unstimulated or activated with LPS alone, LPS plus IL-4<sup>4b</sup> (1 ng/ml), or LPS plus IL-4<sup>4b</sup>, in which γ3 GLT is expressed under all three conditions, whereas the γ1 GLT is dependent on the combination of LPS plus IL4 (Fig. 2B), indicating that the culture conditions chosen for this analysis are appropriate.

Chimeric B cells produce appropriate levels of γ3 GLT in all activation conditions

WT F1 or chimeric knockin (Chi) mice were next stimulated with LPS and analyzed for γ3 GLT abundance. Allele-specific γ3 GLT RT-PCR products are distinguishable using a restriction site polymorphism that uniquely creates an additional PsI site on the γ3<sup>3</sup> allele. Intact and PsI-digested cDNA representing γ3 GLT derived from the a (129) or b (C57BL/6) or a and b (F1) alleles are clearly distinguishable (Fig. 2C, lanes 2–5). RT-PCR amplification of γ3 GLTs derived from WT F1 and three independent chimeras (Chi D, E, and F) indicates that Sy1 region replacement had no substantial deleterious effect on GLT expression from the Sy3 promoter, although GLT abundance from the γ3<sup>3KI</sup> allele appears somewhat underrepresented as compared with WT (Fig. 2C). To further examine this issue, allele-specific γ3 GLT abundance from WT F1 and Chi 1 cultures activated with LPS only, LPS plus IL-4<sup>4b</sup>, or LPS plus IL-4<sup>4b</sup> was assessed by RT-PCR followed by Southern blot analysis. In WT F1 samples, there is parity of γ3 GLT expression from both alleles, whereas in the Chi 1 γ3 GLT, quantities from the a allele are somewhat underrepresented as
compared with the b allele for all activation conditions (Fig. 2D).
These results confirm that γ3 GLTs from the intact and targeted alleles are expressed in the chimera cultures and that stimulation does not lead to overexpression of the γ3 GLT from the a allele.

**Chimeric B cells express γ3 PSTs upon activation with LPS and IL-4**

To further assess CSR in WT and chimeric cultures, μ→γ3 and Iμ-Cγ3 and Iμ-Cγ1 PST expression patterns, indicators of successful CSR, were analyzed by RT-PCR. In WT cultures from purified B cells or splenocytes that were either unstimulated (U) or activated with LPS (L) alone or LPS plus IL-4 (1 ng/ml) or LPS plus IL-4 (10 ng/ml) for 48 h, RT-PCR analyses of the Gαpd or Hprt transcripts were used as loading controls. A, Purified B cells were unstimulated or stimulated and analyzed as indicated, and then analyzed for γ3 or γ1 GLT expression. B, LPS-activated WT F1 and chimeric (Chi D, E, F) splenocyte cultures, activated as indicated, and then analyzed for γ3 or γ1 GLT expression. C, LPS-activated WT F1 and chimeric (Chi D, E, F) splenocyte cultures are analyzed for γ3 GLTs. Allele-specific products were distinguished by PstI digestion, which generates 180- and 125-bp restriction fragments derived from the C57Bl/6 (b) and 129 (a) alleles, respectively. Samples in lanes 3–7 were digested with PstI. Samples are m.w. marker, M (lane 1), intact γ3 GLT (lane 2), and γ3 GLT from the 129 a allele (lane 3), the C57Bl/6 b allele (lane 4), the 129 × C57Bl/6 (F1) a and b alleles (lane 5), and Chi mice (lanes 6–8). D, WT F1 and chimeric (Chi 1) splenocyte cultures activated as indicated were assessed for allele-specific γ3 GLT by RT-PCR followed by Southern blot analysis.

Strikingly, in chimera cultures (Chi D, E, and F), γ3 PSTs were detected following induction by LPS or LPS plus IL-4, indicating that μ→γ3 CSR was active in the presence of IL4lo or IL4hi, whereas γ1 PSTs were induced by LPS plus IL-4 but not by LPS alone, which verifies appropriate IL-4 activity (Fig. 3B). The PST analyses indicate that μ→γ3 CSR occurs in the chimeric cultures activated with LPS plus IL-4, conditions that normally suppress switching in the WT. These studies do not distinguish whether in the chimeras one or both γ3 alleles are involved in CSR.

**LPS and IL-4 induce μ→γ3 CSR on the Sγ1 replacement allele**

In WT B cells, CSR tends to occur on both Igh alleles and to the same isotype (29–31). Newly devised allele-specific semi-quantitative DC-PCR assays were performed to determine which γ3 allele is responsible for high levels of Iγ3 expression in chimeric B cells following LPS plus IL-4 treatment. The DC-PCR strategy is diagrammed, and a portion of the Iγ3 locus before and after μ→γ3 recombination is shown (Fig. 4A). CSR for μ→γ3 generates a new S/S hybrid configuration, 5'-Sα3/Sγ3-3', in which EcoRI sites that flank the 5' and 3' ends of the Sα and Sγ3 regions, respectively, are preserved. Postdigestion with EcoRI, the DNA is ligated under low concentration conditions that favor intramolecular ligation. Nested primer sets specific for sites at the 5' end of Sα and the 3' end of Sγ3 amplify the region spanning the circle joint and yield a specific Sα3/Sγ3 DC-PCR product. Newly designed allele-specific DC-PCR primers amplify μ→γ3 events on the γ3a or γ3b alleles as demonstrated using DNA from 129 or C57Bl/6 B cells, respectively (Fig. 4B). The nAChR gene DC-PCR product functions as a control for the assay (Fig. 4B). Accumulation of DC-PCR products from both the γ3a and γ3b alleles is dependent on increasing cycles of amplification and is in the linear range of detection (Supplemental Fig. 1). In LPS-activated control WT F1 cells, μ→γ3 CSR is almost equivalently stimulated on both the γ3a and γ3b alleles (Fig. 4C). Stimulation with LPS in the presence of increasing concentrations of IL-4 yields diminished μ→γ3 CSR on both alleles and increased μ→γ1 switching as compared with LPS alone (Fig. 4C, 4D) in agreement with the pattern of γ3 PST expression (Fig. 3). In LPS-induced chimeric splenocyte cultures, CSR on the γ3a(SK1) allele is ~3- to 4-fold higher than for the intact γ3b allele (Fig. 4C). Upon LPS plus...
IL-4 activation, there is robust switching $\mu^a \rightarrow \gamma^3a(KI)$, whereas $\mu^a \rightarrow \gamma^3b$ is undetectable. When Chi D templates are concentrated 5-fold, then $\mu^a \rightarrow \gamma^3b$ DC-PCR products, denoted $\mu^a \rightarrow \gamma^3b*$, are detectable from cells induced with LPS plus IL-4lo but not with LPS plus IL-4hi (Fig. 4C). These findings demonstrate that switching on the intact b allele is repressed at least 10-fold by IL-4hi and to an even greater extent by IL-4hi treatment, whereas CSR on the Sy1 replacement allele is active. We also find that the level of $\gamma^3$ GLT produced from the $\gamma^3a(KI)$ allele is somewhat reduced as compared with the intact $\gamma^3b$ allele under identical culture conditions (Fig. 2D). Hence, the enhanced $\mu^a \rightarrow \gamma^3a(KI)$ CSR detected when chimera cultures are activated in the presence of LPS and IL-4 may be an underestimate of CSR potential for this allele.

In cultures activated with LPS alone, the incidence of switching on the $\gamma^3a(KI)$ allele was unexpectedly high (Fig. 4C) if CSR is strictly dependent on IL-4. Switching $\mu \rightarrow \gamma 1$ in response to LPS alone was higher in chimera as compared with WT cultures, suggesting the presence of some endogenous IL-4 (Fig. 4C). To explore the possibility that endogenous IL-4 accounts for $\mu \rightarrow \gamma 3$ switching on the $\gamma^3a(KI)$ allele in the LPS-activated cultures, we analyzed a fourth chimera, Chi 1, in which $\mu \rightarrow \gamma 1$ CSR was undetectable by DC-PCR when activated with LPS alone, demonstrating minimal levels of endogenous IL-4 (Fig. 4D). In Chi 1, activation by LPS alone led to relatively more $\mu \rightarrow \gamma 3$ switching on the intact $\gamma^3b$ allele than the Sy1 replacement allele, whereas this pattern is reversed following induction with LPS plus IL-4lo. Thus, CSR on the $\gamma^3a(KI)$ allele is ~4- to 5-fold higher compared with the intact $\gamma^3b$ allele poststimulation with LPS plus IL-4lo and suggests dependency of the Sy1 replacement allele on IL4 for $\mu \rightarrow \gamma 3$ switching. Irrespective of the level of IL-4 present, it is clear that the chimera cultures permit some endogenous $\mu \rightarrow \gamma 1$ CSR when activated with LPS alone. We favor the view that the chimera environment is permissive for the participation of Sy1ki allele in CSR within the context of the $\gamma 3$ locus because of higher IL-4 levels. However, we cannot formally rule out the possibility that Sy1 sequence is intrinsically a more robust substrate than Sy3 when compared side by side for CSR within the same locus.

**Preferential usage of the $\gamma^3a(KI)$ allele in S-S junctions is induced by IL-4**

To independently examine the influence of IL-4 on isotype usage during CSR, we analyzed allelic frequencies of S/S junctions that were directly amplified by PCR from control WT F1 and five independent chimeric splenocyte cultures. Splenocytes were stimulated with LPS or LPS plus IL-4lo for 5 d, and genomic DNA was harvested. The PCR primers used to amplify these S/S junctions were identical in all cases (Fig. 5). DNA sequence polymorphisms distinguish S3/Sy3 junctions derived from the a and b allele (see Materials and Methods). Most normal CSR breakpoints occur within and just beyond the S tandem repeats (11). In WT F1 and chimeric DNAs, S3 recombination breakpoints occurred at various positions and were fused to complementary sites within intact Sy3 or Sy3ki alleles (Fig. 5A). S/S junctions from chimeric cultures were generally focused to the 5′ region of S3 and the 3′ region of Sy3 and Sy3ki, sequences similar to the pattern found for WT S3 and Sy3 fusion sites (Fig. 5B). S3/Sy3 recombination junctions carried insertions, deletions, and mutations typical of S/S junctions, and only unique junctions were considered (Supplemental Figs. 2, 3). In WT F1 controls, similar numbers of S3/Sy3 and S3ki/Sy3ki junctions were isolated following activation with LPS or LPS plus IL-4lo, indicating the equivalent usage of the a and b alleles in CSR (Table I). Similarly, in LPS-activated splenocyte cultures from Sy1 replacement chimeras, the distribution of S/S junctions was well balanced between the intact S3/Sy3 and replacement S3ki/Sy3ki alleles. Strikingly, when these cultures are stimulated with LPS plus IL-4lo, the frequency of S3ki/Sy3ki junctions is significantly overrepresented ($p > 0.011$), demonstrating that upon LPS activation, IL-4 signaling facilitates CSR targeted to Sy1 sequences even when in the context of the $\gamma 3$ locus (Table I).
and are analyzed in Table I. In the SPCR products and are indicated by the symbols beneath the locus dia-
breakpoints were determined by DNA sequence analysis of the cloned
the arrows under each locus schematic. The locations of the recombination
WT F1 (with LPS in the presence or absence of IL-4lo for 5 d. S-S junctions from

targeting of the S region sequence identity plays
in which
GLT expression, we established conditions
a role in focusing CSR to specific isotypes. Because CSR is

demonstrates a role for primary S region sequence in CSR.

The S1 replacement allele was capable of supporting some CSR in response to LPS alone. There are several possible ex-
planations for this observation. Our studies show that low levels of
endogenous IL-4 were present in the splenocyte cultures, which
could account for participation of the γ3a(KI) allele in μ→γ3 CSR. There may be cryptic sequences within the residual Sy3-Cγ3 in-
tronic region that confer the ability to recombine in response to
LPS alone. Transcribed S regions contain R loops that are de-
pendent on the presence and orientation of the S region (8, 15, 32).
Deletion of endogenous Sy1 essentially abolished μ→γ1 CSR, but
knockin of a random synthetic G-rich sequence that was also
capable of transcription-dependent R-loop formation provided
nearly 10% of CSR as compared with WT CSR activity (8). Therefore, any transcribed DNA sequence situated in the γ3a-Cγ3 intron that forms R-loops, such as the S1 replacement sequence, may function as a low-efficiency substrate for CSR in response to
LPS stimulation.

CSR is mediated by AID attack, resulting in DSB formation in
Sμ and a downstream S region, long-range synthesis between
targeted S regions and joining of DSBs by constituents of the
nonhomologous end joining pathway (2, 3, 21). How might S region sequence identity influence CSR targeting in vivo? One potential explanation is that Sy1 is simply a better target for CSR. If this were so when holding GLT expression constant, then the
differential CSR frequency between Sγ3 and Sy1 will remain
equivalent under all activation conditions. We find the converse,
that preferential usage of the Sy1 replacement allele is IL-4 de-
pendent, demonstrating that Sy1 is not simply a better substrate
for switching but rather differentially targeted for CSR under
those conditions. Our data are not inconsistent with the notion that
Sy1 might be a better substrate for CSR than Sy3 and also be
facilitated in CSR by an IL-4–inducible process. Additional studies using a germline Sy1KI allele in mice will be required to
resolve this issue. Another question posed by our studies is
whether the influence of Sy1 sequence identity on CSR can be
generalized to all isotypes. It is notable that the overall mechanism
of CSR is the same for Sy1 as for other isotypes. However, in
B cells harboring a deletion of hs3b,4, a critical component of 3’
Exo, some μ→γ1 switching is detected, whereas all other isotypes
are inert (33). Chromatin conformation capture studies demonstrate that in the hs3b,4 deleted B cells activated with LPS plus
IL-4, the γ1 locus is uniquely capable of some interaction with
Sμ, thereby creating Sμ/Sy1 proximity (21). Thus, it is possible

Discussion

We replaced the endogenous Sy3 region with Sy1 DNA by gene

targeting to determine whether S region sequence identity plays
a role in focusing CSR to specific isotypes. Because CSR is
critically dependent on GLT expression, we established conditions
in which γ3 GLTs from both the intact and Sy1 replacement loci
are well expressed even in the presence of IL-4, in agreement with
previous studies (28). Successful expression of γ3 GLTs on both
alleles allowed us to determine whether the Sy1 replacement allele
is preferentially targeted for μ→γ3 switching in the presence of
IL-4. In LPS-activated WT splenocyte cultures, μ→γ3 events
diminish on both alleles as a function of increasing IL-4 con-
centration as detected in Pst assays, in allele-specific DC-PCR,
and in S/S junction frequency studies, whereas, in chimeric
splenocytes, IL-4 stimulation induces γ3 Pst expression coupled
with preferential usage of the γ3a(KI) allele in μ→γ3 events as
directly visualized in DC-PCR analyses and reflected in S/S
junction frequency assays. These findings suggest that γ3a(KI)
effectively competes with the γ1 allele for CSR machinery as
a consequence of IL-4 stimulation. In summary, when chimeric B
cells are activated with LPS and IL-4, the Sy1 replacement se-
quence becomes a high-efficiency substrate in μ→γ3 switching in
contrast to the intact allele, which ceases to engage in CSR. This
finding is even more striking given the relatively lower level of γ3
GLT from the γ3a(KI) allele as compared with the intact allele and
demonstrates a role for primary S region sequence in CSR.

Table I. Switch junction analyses

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<th>Chimeras</th>
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<td>LPS</td>
<td>LPS plus IL-4lo</td>
<td>Sμa-KI</td>
<td>Sμb-KI</td>
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<tr>
<td>p Value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>n</td>
<td>20</td>
<td>21</td>
<td>24</td>
<td>10</td>
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*p values were calculated using a χ2 analysis.

n indicates the number of unique junctions analyzed.
that when triggered by IL-4, the Sy1 replacement allele is inherently better at synapsis with Sμ, leading to enhanced CSR frequencies. S/S junctions are created by joining DSBs in Sμ with those of a distal S region by means of DSB repair proteins, including the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). Although CSR is profoundly reduced in DNA-PKcs-deficient B cells, some μ→γ1 switching survives (34), perhaps indicating that Sy1 is more facile than other S regions in the joining reaction. Alternatively, detection of residual μ→γ1 CSR in hs354 deleted- or DNA-PKcs-deficient B cells might be a byproduct of assay sensitivity because μ→γ1 CSR normally occurs at a higher frequency relative to other isotypes. Our studies do not directly distinguish whether the Sγ1 replacement allele is preferentially targeted by AID or preferentially involved in synapsis with Sμ or is a better substrate for end joining as compared with Sγ3. Nevertheless, our findings demonstrate that S region sequence identity plays a role in CSR, and the effect is dependent on IL-4.

An intriguing explanation for our results is suggested by the findings that isotype-specific CSR occurs on switch-specific substrates in transient assays (7), and S region-specific DNA binding proteins have been detected (reviewed in Ref. 18). Together, these studies imply that recognition motifs in S regions interact with DNA binding proteins to recruit the CSR machinery. In this context, it is possible that a factor specific for the Sγ1 replacement allele is induced (or a repressor is repressed) by IL-4 in LPS-activated B cells and promotes μ→γ1 CSR. An independent gene-targeting study showed that a Sy3 replacement allele within the endogenous γ1 locus supported CSR following B cell activation with LPS plus IL-4 (35). This observation was predicted by switch substrate studies in which μ→γ3 CSR was stimulated by LPS but not repressed by IL-4 (7). Alternatively, the Sy3 replacement study could mean that any S region is capable of supporting CSR with the exception of Sy1, which is a special case. Additional work is required to investigate the step at which S region sequence identity functions in the CSR reaction, whether Sy1 is unique among S regions, and whether isotype-specific factors are involved in this process.

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Disclosures

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References


