

Accessibility control and machinery of immunoglobulin class switch recombination

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Abstract: Immunoglobulin (Ig) class switching is a process by which B lymphocytes shift from production of IgM to other Ig classes and subclasses via Ig class switch recombination (CSR). Multiple cellular and molecular processes are involved in CSR. Induction of a given IgH germline transcription initiates CSR processes. Ig germline transcription is selectively activated and induced by specific cytokine(s) via cytokine-specific signal pathways, synergized by CD40 signaling, and optimized by the 3' Ig α enhancers through locus control region function. Following Ig germline transcription, the switch-region DNA undergoes conformational changes so that it can serve as an appropriate substrate for nicking and cleavage by switch recombination machinery. Finally, the double-strand breaks in donor and acceptor switch DNAs are processed, repaired, and ligated through a general nonhomologous end join pathway. CSR generates a new transcriptional unit for production of a class-switched Ig isotype. *J. Leukoc. Biol.* 73: 323–332; 2003.

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INTRODUCTION

Immunoglobulin (Ig) class switching, or isotype switching, is a process by which B lymphocytes shift from production of IgM to one of the IgG3, IgG1, IgG2b, IgG2a, IgE, or IgA classes and subclasses in mouse or to IgG3, IgG1, IgA1, IgG2, IgG4, IgE, and IgA2 in humans [1–3]. This process is mainly mediated by the deletional DNA recombination between the switch (S) region of the Ig heavy chain (IgH) constant region μ gene (S_{μ}) and one of the downstream S regions located 5' to each IgH except for the δ gene [4]. This process is known as Ig class switch recombination (CSR). Ig CSR creates a new, transcriptional unit encompassing the original variable/diversity/joining (VDJ) fragment, plus the IgH chain to be expressed for production of a class-switched Ig isotype therefore generates a new type of Ig molecule with original antigen-binding specificity and novel effector functions associated with the IgH chain [1, 2]. This process provides the basis for the versatile, humoral, immune functions of Ig molecules.

CSR requires the participation of multiple cellular and molecular processes, which are schematically diagrammed in

Figure 1. CSR process can be divided into three major steps. Transcription of a given germline IgH gene, termed Ig germline transcription (GT), is the initial step for CSR. This process, which selectively determines the accessibility of a given IgH locus for CSR, is activated and directed by cytokine(s) and synergized by the costimulation of CD40. Ig GT appears to be optimized by the 3' Ig α enhancer via its locus control region (LCR) function providing for efficient GT and CSR. Following Ig GT, S region DNA undergoes a conformational change so that it can be served as an appropriate substrate for S region nicking and cleavage through an AID-dependent mechanism. Finally, the induced DSB in the S regions are appropriately processed, repaired, and ligated to join the broken ends through a general nonhomologous end-joining (NHEJ) pathway. This final step of CSR generates a recombined chimeric S region in the chromosome, accompanying with the loop-out and deletion of the intervening DNA between the two CSR partners. Understanding CSR has significantly advanced in the past several years with progress especially occurring in the characterization of Ig germline promoters and the role of AID (reviewed in refs. [5, 6]). This review will focus on the recent progress in CSR, emphasizing Ig accessibility control and the CSR machinery.

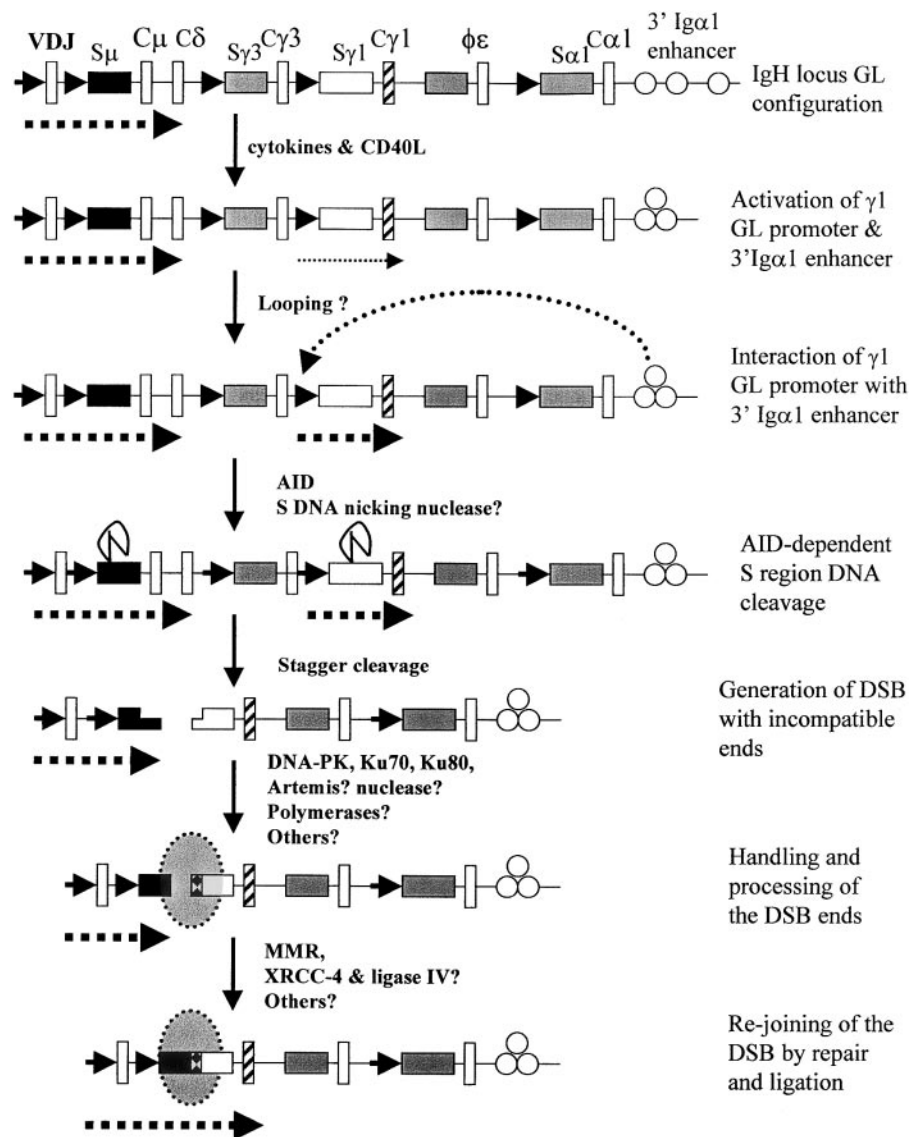
ACCESSIBILITY CONTROL FOR CSR

Accessibility model of CSR

It has been well established that cytokine(s) direct appropriately activated B cells, such as lipopolysaccharide (LPS) and CD40 ligand (CD40L), B lymphocyte-stimulator protein, and a proliferation-inducing ligand, toward switching to specific isotype(s) [4–7]. In mouse, interleukin-4 (IL-4) preferentially directs B cell switching to IgG1 and IgE [5, 7, 8], interferon- γ to IgG2a and IgG3 [5, 8], and transforming growth factor- β (TGF- β) to IgA and IgG2b [5, 8]. In humans, IL-4 drives B cell switching to IgE, IgG4 [5, 8], IgG3, and IgG1 [9], IL-10 to IgG3 and IgG1 [10], IL-13 to IgG4 and IgE [11], and TGF- β , vasoactive intestinal peptide, and dendritic cells to IgA [12–14]. Cytokine-driven isotype switching directly correlates with

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the ability of the given cytokine to selectively induce GT from a specific IgH locus [14], and the induced GT precedes CSR [15]. Based on these facts, an Ig CSR “accessibility model” has been proposed [5, 15]: A given cytokine, by inducing transcription through a specific IgH locus, opens the locus so as to be “accessible” to the putative, pre-existing Ig-CSR machinery for CSR.

The general accessibility model for CSR has been tested using mutant mice, where the ability to induce GT is disrupted. Targeted replacements of the Ig germline promoters, which contain cytokine-response elements that initiate and regulate the corresponding Ig GT with a *neo* gene cassette in opposite orientation, selectively block GT and CSR to those specific loci but not other IgH loci [16, 17]. These results indicate that IgH germline promoters are required for GT and CSR. S region DNA transcription per se is not sufficient to render S region DNA for efficient CSR [18]; the spliced products of GT, e.g., the germline transcripts themselves, are implicated in CSR [19]. How the germline transcripts are involved in the regulation of CSR process is not known, as they do not code for any known proteins. Although the spliced “germline” transcripts

are critical, the I exon sequences in these transcripts are not important for GT and CSR, as replacement of I exon with other sequences does not appear to affect CSR [20]. These results indicate that the Ig germline promoters play a control role in determining the accessibility for the specific IgH loci, supporting the hypothesis of accessibility model for CSR. As a result of the possible insertion effects that could be mediated by the drug-resistant genes in the targeting vectors, however, the explanation and conclusion drawn based on these experiments should be cautious, as the inserted drug-resistant genes themselves sometimes are able to mediate the regulatory effects on the targeting genes [21, 22]. To exclude the possible effects contributed by the inserted drug-resistant genes, the deletion-targeting approach [23], instead of the replacement approaches with the drug-resistant genes, should be used to evaluate the function of targeting genes or elements.

The original accessibility model for CSR postulates that the cytokine-induced accessible configurations of the specific IgH loci are attacked by the putative, pre-existing switch recombinase for CSR. Hence cytokine(s) are thought to function primarily as inducers of chromosomal remodeling to determine

the IgH locus accessibility control. The fact that cytokine(s), for example, IL-4 and/or TGF- β , are also required for activation of switch recombinase activities for switch recombination in artificial switch constructs reveals that they might function beyond the accessibility control in CSR, and some components of the putative switch recombinase activities are also dependent on the appropriate induction instead of pre-existing as originally proposed. In these artificial switch constructs, the transcription through the switch regions is controlled by the constitutively activated promoters (virus promoters) [24, 25] that are not subject to the control of cytokines. IL-4 and/or TGF- β are able to induce the expression of AID (refs. [24, 26], and our unpublished results), which is absolutely required for CSR but not required for Ig GT [27], and suggest that some cytokines capable of promoting AID induction function in CSR by affecting accessibility control and induction of switch recombinase activity. Therefore, the switch recombinase activity is, at least in part, not fully preformed as suggested by the original accessibility model. To reflect the advanced knowledge, the original accessibility model should be modified accordingly.

Activation of germline promoters

Activation of Ig germline promoters is selectively directed by cytokines, among which IL-4 and TGF- β are best characterized. IL-4 activates the responsible Ig germline promoters via Janus kinase/signal transducer and activation of transcription (JAK/STAT) signaling pathways [28]. IL-4, through binding to its receptor, induces tyrosine phosphorylation of the nonreceptor tyrosine kinases, JAK1 and JAK3, which are constitutively associated with IL-4-receptor α -chain (IL-4R α). Activation of JAK kinases results in tyrosine phosphorylation of IL-4R α , which recruits and phosphorylates STAT6. The phosphorylated STAT6 homodimerizes via reciprocal phosphotyrosine-Src homology 2 interaction, translocates to nucleus, binds to IL-4 response elements located in those Ig germline promoters, and subsequently activates them [28]. TGF- β , conversely, activates Smad3 via phosphorylation. The phosphorylated Smad3 complexed with Smad4 is translocated to nucleus, where the complex binds, in cooperation with the TGF- β -induced polyomavirus enhancer-binding protein 2/core-binding factor α 3 [29], to the TGF- β responsive elements in the Ig α germline promoters and drives their activation and transcription [29, 30].

Cytokine-initiated activation of Ig germline promoters is synergized by other transcriptional factors. Nuclear factor (NF)- κ B activation through the CD40 signaling pathway [31] plays an important role in synergizing with IL-4-mediated activation of germline promoters through interaction between NF- κ B and STAT6 [32]. Other transcription factors, such as C/EBP, Ap-1, Sp-1, Pu.1, B cell-specific activating protein (BSAP), and Ets, are also differentially required for activation of various germline promoters [5, 8, 33].

As JAK/STAT signal pathways are critical for activation of IL-4-dependent germline promoters, negative regulators for these signal pathways play a role in limiting the cytokine-mediated activation of Ig germline promoters. These negative regulators include nuclear and cytoplasmic phosphatases, protein inhibitors of the activated STAT family of proteins, suppressors of the cytokine signaling family of proteins (reviewed

in ref. [28]), and JAK phosphatase of CD45 [34]. Recently, we identified CD45 as a JAK phosphatase that is able to negatively regulate CSR to IgE through inhibition of ϵ germline transcription in human B cells [35].

Role of the 3' Ig α enhancers in GT and CSR

The 3' IgH α enhancers are located $\sim$ 16 Kb and 20 Kb downstream of the IgH C α gene and in the mouse [36] and human [37] span $\sim$ 30 Kb and 15 Kb, respectively. Humans have two 3' IgH α enhancers, one 3' to each IgH cluster. The importance of the 3' Ig α enhancer in controlling Ig gene expression was originally identified from the observation that IgH gene expression was markedly reduced in a myeloma cell line that lost a segment of DNA downstream from the murine IgH C α gene [38]. Further studies revealed that the potential physiological function of these 3' Ig α enhancers includes regulation of the IgH locus rearrangement, transcription, somatic mutation, and Ig CSR [39–44].

The strongest evidence for the requirement of the 3' Ig α enhancer in GT and CSR comes from the gene-targeted deletions of the 3' Ig α enhancer in mice. Those experiments confirm that only the HS3b and HS4 regions but not the HS3a and HS12 regions of the murine 3' Ig α enhancer play an important, controlling role in multiple Ig GT and CSR [23]. These results suggest that the 3' Ig α enhancer-dependent Ig GT and CSR processes are differentially controlled by different regions in the 3' Ig α enhancers. The CSR deficiency in the 3' Ig α enhancer mutant mice correlates with their inability to produce the corresponding GT and germline transcripts [23], indicating that the 3' Ig α enhancer affects CSR through regulating the accessibility of the specific IgH promoters. These results also indicate that the germline promoters are not sufficient to drive efficient GT and CSR in the endogenous locus without collaboration with 3' Ig α enhancers.

Action mode of 3' Ig α enhancer in regulation of Ig GT

The 3' Ig α enhancers most likely exert their roles in regulating IgH locus accessibility via their LCR functions [43–45]. It has been shown that the 3' Ig α enhancer major DNase I hypersensitivity sites (HS), designated HA3A, HA12, HS3B, and HS4 in mice and HS3, HS12, and HS4 in humans [37], are able to synergize with promoters to enhance the promoter-directed transcription activity. The individual HS fragments tested are able to synergize, although differentially, with the V κ promoter, V μ promoter, IgH germline promoters, including the germline promoter for γ 2b, γ 3, α , and ϵ , and non-Ig promoters (such as the *c-myc* promoter) [37, 43, 44, 46] so as to enhance promoter-directed transcription activity in transient transfection assays while demonstrating cell-type and developmental stage specificity. In stable, transfected cell lines [47] and transgenic mice [48], HS fragments show position-independent transcription-enhancing activity, indicating that the HS fragments confer, at least in part, the LCR function of the 3' Ig α enhancers.

Many transcription-binding sites have been identified in the 3' Ig α enhancer region, especially in enhancer HS regions. These include E1, E5, E3, Id3, BSAP, NF- κ B, Oct, NF- α P,

activated protein-1 (AP-1), and NF- κ B (Ets/AP-1) sites [37, 44, 49–52]. These sites are differentially involved in the regulation of the 3' Ig α enhancer function and are responsible for cell-type and differentiation-stage specificity of the 3' Ig α enhancer activity. Id3, a helix-loop-helix protein, expressed in early B lineage cells but down-regulated in plasma cells, represses 3' Ig α enhancer function [53]. BSAP, which is abundantly expressed in early-to-mature B cell stage but disappears as B cells mature to plasma cells, exerts an active, repressive influence on transcription, as well as HS12 activity on early differentiation-stage B cells [44, 49–51]. However, HS12 activity is not blocked by BSAP in mature B cell lines and primary B cells [44]. NF- κ B has been demonstrated to be important for the enhancer activity of HS4, as the HS4 fragment with the NF- κ B mutation blocks the enhancer activity for *c-myc* promoter-directed transcription [54, 55]. OCA-B, which is a coactivator for Oct-1 and Oct-2 also involved in the regulation of 3' Ig α enhancer activity [51]. Some of the transcription factor-binding sites (e.g., BSAP, NF- κ B, OCA-B) confer the inducible activity of the HS enhancers by the stimuli, such as LPS, IL-4, and α CD40, which activate the germline promoter and drive germline transcription [51, 56, 57]. A DNA looping process is postulated to deliver transcription factors, bound to the transcription factor-binding sites in the enhancer regions, to the promoter region for activation of the promoter-directed transcription [45].

Promoter competition model

To explain the possible interactions of multiple Ig germline promoters with one 3' Ig α enhancer, a “promoter competition model” has been proposed. This postulates that a given Ig germline promoter competes with other promoters for the limited 3' Ig α enhancer availability for efficient germline promoter activation and germline transcription [21, 22]. The result is the activation of the “successful” promoter accompanied by the inhibition of promoters that fail to compete successfully for the enhancer [21, 22]. Insertion of a PGK-*neo*^r cassette into the IgH locus short-circuits the ability of the 3' IgH regulatory region to facilitate germline transcription of dependent IgH genes upstream but not downstream of the insertion [22]. This observation supports the existence of a long-range 3' IgH regulatory region required for Ig germline transcription and CSR to multiple IgH genes [22, 58].

Role of histone acetylation in accessibility control

The amino acid residues in histone tails (most frequently, the lysine residues) can be post-translationally modified through multiple mechanisms, including acetylation/deacetylation, phosphorylation/dephosphorylation, and methylation/demethylation [59]. A specific pattern of one or more of these types of modification in histone tails determines whether the histone-associated DNA is accessible [59]. Histone hyperacetylation (and phosphorylation) in a given gene is frequently associated with the gene activation, whereas histone deacetylation (and methylation) in the corresponding region has been linked with transcriptional silencing [59, 60]. Studies on the function of the 3' Ig α enhancer reveal that up-regulation of transcriptional activity in a linked *c-myc* gene by the murine 3' Ig α enhancer

is accompanied by a widespread increase in histone acetylation along the linked gene [61], indicating that the 3' Ig α enhancer may regulate gene expression through histone hyperacetylation. These findings support the concept that histone acetylation/deacetylation of a given Ig gene controlled by IgH germline promoters or the LCR function of the 3' Ig α enhancer is a mechanism for regulating the accessibility and CSR. Histone acetylation has recently been shown to play a critical role in controlling the accessibility of the T cell receptor (TCR) loci to recombination-activating gene (RAG) protein cleavage for VDJ recombination, in which the TCR locus enhancer functions as a long-range, developmental regulator of histone acetylation [62, 63].

S region-binding proteins

Late SV40 factor (LSF), a ubiquitously expressed protein, is found to directly bind to S μ and S α sequences [64]. Its binding activity to S μ decreases upon induction of CSR, and overexpression of a dominant negative version of LSF increases the CSR capacity to IgA, implying that LSF negatively regulates CSR. Immunoprecipitation shows LSF is able to interact with chromatin modify factor Sin3A, histone deacetylase-1 and -2, suggesting that LSF might affect CSR through regulating the accessibility of the S region to CSR machinery [64]. In addition to LSF, several other proteins, including Pax-5/BSAP [65], LR1 [66], NF- κ B/p50 [67], switch nuclear A-site protein [68], S μ BP-2 [68], Endo-SR [69], Rad51 [70], and SWAP-70 [71], are found to directly bind to S region sequences. However, whether these S region-binding factors indeed play roles in controlling or regulating the S region accessibility for switch recombination remains to be determined.

S REGION DNA RECOGNITION AND CLEAVAGE

DNA structure requirement for CSR

CSR is mediated through region but not site-specific recombination occurring in the specific S region DNA. One of the remarkable characterizations of S region DNA is its GC-rich repeat sequences [72]. It is believed that the GC-rich repeat S DNA might serve as the recombination substrate as a result of the GC-rich DNA forming the special three-dimensional structures upon transcription. Transcription of supercoiled plasmid-containing murine S α sequences in vitro leads to the formation of DNA/RNA hybrids that lose the superhelical turn of S α [73]. Such structures are also observed in S γ sequence-containing plasmids under in vitro transcription [74], and such transcription-induced structures in S regions are susceptible to cleavage by nucleases [75]. These findings lead to the hypothesis that transcription-induced R-loops in S regions might be a recognition target for CSR machinery [76]. However, failure to block CSR in an artificial experiment system from the overexpression of RNase H, an enzyme that is able to specifically digest the RNA in RNA/DNA hybrids, does not support such a hypothesis [76]. The G-rich S sequence-dependent, four-stranded DNA structure has been proposed to be the potential target as the substrate for CSR [77].

Recent results obtained using artificial switch constructs indicate that S region primary sequences might not be critical for CSR [78]. Thus, the palindromic nature, rather than the GC-rich or repetitive sequence, of the S region sequence may be key as the appropriate substrate for efficient CSR. Palindromic sequences, including those AT-rich *Xenopus* S region, multiple cloning sequences with palindromic sequences, are efficient substrates for CSR in switch constructs [78]. In contrast, telomeric DNA, which is G-rich and repetitive but not palindromic, cannot be served as efficient substrate for CSR [78]. These results lead to the proposal that the stem-loop structure formed by the palindromic sequences, which is also abundantly present in S regions, could serve as the recognition targets for CSR. Stem-loop structures are predicted to form transiently in S regions during transcription-induced, transient S DNA denature. This proposal gains support from the data showing CSR breakpoints are frequently in or close to the neck of the predicted stem-loop formation [78].

CSR does occur in the mutant mice with a μ deletion [79], albeit with much lower frequencies, indicating that S region is not absolutely required as the recombination substrate for CSR. In this mouse, CSR occurs without the tandemly repetitive core μ sequences [79]. Similar data, where CSR could occur in the nontypical GC-rich S region, have also been observed in the artificial switch recombination models [24, 25]. Overall, these results favor the notion that the primary GC-rich DNA sequence is not absolutely required as the recognition target for CSR.

Role of AID in CSR

Recognition of the requirement of AID for CSR is one of the most important recent advances in CSR. AID is homologous to that of apolipoprotein B mRNA-editing enzyme APOBEC-1 and thought to be a putative RNA-editing enzyme [26]. AID expression is strictly localized to germinal center B lymphocytes and is required for CSR [26, 27], V_H somatic hypermutations (SHM) [27, 80, 81], and V_H gene conversion [82]. Genetic disruption of the AID gene completely abolishes the CSR to IgG, IgE, and IgA in vivo and in vitro [27], SHM in V_H regions [27], and gene conversion of V gene segments [82]. In humans, mutations in the AID gene cause type II hyper-IgM immunodeficiency [80]. Therefore AID is the first defined protein that is involved in CSR and SHM [5, 27]. The direct linkages of Ig CSR with Ig V_H hypermutation point to the existence of common pathways dependent on AID for Ig V_H SHM and Ig CSR.

Although AID deficiency causes a complete defect in Ig CSR, the process of Ig GT is not affected by AID deficiency [27]. Therefore, AID must act at the post-GT stage. Ectopic expression of AID initiates a transcription-dependent switch recombination in an artificial switch vector in fibroblasts [83], where the DNA repair and recombination machinery exist and function normally to repair the AID-dependent S region DNA DSB for efficient CSR. This suggests that AID is the only B cell-specific factor required for initiation of CSR process. However, the exact action mode of AID in CSR remains to be elucidated. It is postulated that AID may function as an RNA-editing enzyme to modify a putative DNA-cleaving nuclease

that eventually executes the required S DNA nicking and cutting. Alternatively, AID itself may function as a S region-specific DNA nuclease to directly attack S DNA [83] or as cytidine deaminase to deamination of a cytidine [84]. It may also be possible that AID is required to modify or edit a component that is involved in DSB repair processes, as there are evidences to show that DNA lesions and the induced DSB for SHM are AID-independent [85, 86].

Other factors

Several other factors are speculated to function as S region DNA-specific nucleases to cleave S DNA. Topoisomerase II, a DNA-nicking enzyme that normally does not cleave the S region DNA, could cleave G-rich tetraplex formed in S regions [87]. The excision repair nucleases XPF-ERCC1 and XPG, which can cleave R-looped structure induced from the transcription in vitro, are also proposed to be functioned in cleaving S region [75].

Isotype-specific factors that mediate the specific types of CSR in different types of B cell lines have been proposed based on the studies of CSR with transiently transfected artificial switch plasmids [88, 89]. These proposed isotype-specific factors showed a high degree of sequence specificity for S sequences, as the CSR activities for $\gamma 3$, $\gamma 1$, ϵ , and α were distinct and cell line-specific. However, the nature of the proposed isotype-specific factors, of which AID was not likely the candidate [89], has not been described. Furthermore, those results are in conflict with the data obtained from an independent artificial switch recombination system with stable transfection in the CH12F3-2 B cell line, which undergoes switch to IgA. This cell line could support CSR with $\Sigma 1$ and $\Sigma \epsilon$, as well as with $\Sigma \alpha$, with almost equal CSR efficiency [24].

Insights from inversional CSR: staggered double-strand cleavage in S regions

Deletional CSR generates a recombined switch region or hybrid switch region in the chromosome that is accompanied by the production of deleted circular DNA or "switch circle" [5, 90]. It is virtually impossible to simultaneously isolate and match the chromosomal recombined switch region and the deleted switch circle generated from the same CSR reaction from the single B cell. Thus, switch breakpoint sequence analysis, from the recombined switch regions or from the deleted switch circles, can only provide partial clues as to the exact CSR process. However, analysis of inversional CSR can provide direct information as to the action of the CSR machinery, as inversional CSR retains both sides of the cell switch breakpoints. Analysis of the switch junction ends, derived from the inversional CSR occurring in an artificial switch recombination assay, reveals that short deletions and duplications in addition to mutations frequently occur at the switch breakpoints [91]. These results suggest that staggered double-strand DNA cleavage is frequently mediated by CSR machinery. Short inversions, present in the inversional CSR junction end, suggest reinversions also take place during the CSR reaction [25].

S REGION DSB REPAIR AND RECOMBINATION

DNA-PK

DNA-PK consists of three different components: Ku70, Ku80, and DNA-PK catalytic subunit (DNA-PKcs). The Ku70 and Ku80 proteins bind to the ends of DNA DSB, recruit DNA-PKcs to form an active complex—a process believed to prevent the degradation of the generated DSB ends by exonucleases—and prevent such ends from being randomly integrated into the genome, resulting in DNA translocation [92]. Ku proteins are also required for the recruitment and activation of the ligase IV complex for the ligation step of DNA-DSB repair processes [93]. In addition, DNA-PKcs is able to regulate Artemis activity that is critical for DSB repair (see below). In Ku70- and Ku80-deficient mice, CSR cannot be induced in the transgenic IgH locus [94, 95]. DNA-PKcs deficiency shows a less severe phenotype, as this type of deficiency appears to affect Ig isotypes but not IgG1 [96, 97]. These results suggest that DNA-PKcs is required for CSR to most Ig isotypes, and CSR to IgG1 can occur via a DNA-PKcs-independent mechanism. Failure to undergo CSR in Ku70, Ku80, and DNA-PKcs mutant mice does not affect the generation of B cell precursors or production of Ig germline transcripts, suggesting that DNA-PK participates in the DNA recombination level rather than in accessibility control levels. Stimuli known to promote CSR, e.g., IL-4 plus CD40 signaling and dextran-conjugated anti-IgD antibody (Ab), up-regulate Ku expression in B cells [98]. Ku proteins have also been reported to directly bind to the cytoplasmic tail of CD40 [99]. The physiological importance of this observation is not clear at this time.

Artemis

Artemis is a protein involved in DNA-DSB processing and repair, including VDJ recombination [100]. A specific type of human severe combined immunodeficiency (SCID), e.g., radiosensitive SCID, is caused by the mutation in the Artemis protein [100]. Although the purified Artemis protein possesses single strand-specific 5' to 3' exonuclease activity, Artemis acquires endonuclease activity on 5' and 3' overhangs upon complex formation with DNA-PKcs and phosphorylation by the DNA-PKcs in the formed complex [101]. This endonuclease activity is able to nick and open hairpin structures generated *in vitro* or by the RAG complex and process the overhangs generated from the hairpin opening [101]. Thus, DNA-PKcs regulates Artemis activity through complex formation and phosphorylation to permit enzymatic activities that are critical for the hairpin-opening step in VDJ recombination and for the 5' and 3' overhang processing in a NHEJ pathway. As Ig CSR also requires the participation of the NHEJ pathway to process and repair the induced DSB in switch regions (Fig. 1), it will be interesting to know whether Artemis is also involved in Ig CSR.

MMR

MMR enzymes, including Msh2, Mlh1, and Pms2, are a group of proteins that correct mismatched bases resulting from errors caused by DNA polymerases. The role of MMR enzymes in Ig CSR has been shown through the analysis of CSR in MMR-

deficient mice [102, 103]. MMR-deficient mice displayed a 35–75% reduction in switching to IgG3, IgG1, IgG2b, and IgA, indicating CSR in MMR-deficient mice is only partially blocked. Therefore, MMR enzymes may not be absolutely required for switch recombination *in vivo* and may be functionally replaceable by other activities [102, 103]. MMR enzymes are also involved in DNA repair/recombination, as well as in the V_H gene SHM [102, 103].

Analysis of the switch recombination junctions generated in MMR-deficient mice reveals that the switch recombination in these mice is structurally distinctive from that in wild-type mice. In Pms2- and Mlh1-deficient mice, unusually long stretches of microhomology in switch junctions are observed [104, 105], indicating increased homology between two participating S regions is required for CSR in these mice. This type of microhomology, however, is required for switch recombination ending joints in the Msh2-deficient mouse, where decreased lengths of microhomology accompanied by increased insertions in the switch junctions are evident [105]. These results suggest that Msh2 and Pms2 use different pathways to resolve the switch breaks. This notion is further supported by the fact that Pms2 appears to affect only the CSR process, whereas Msh2 is able to alter CSR and SHM processes [104].

Nucleases and polymerases

The staggered double-strand S DNA cleavage mediated by CSR machinery generates two S DNA breaks that are rarely compatible for ligation (Fig. 1). Therefore, processes to shape these primary generated ends are required for their ultimate repair and ligation. To accomplish this, nucleases and DNA polymerases are anticipated to participate, although they have not been specifically identified. There is evidence to support that DNA polymerase is involved in the NHEJ pathway [106]. It has been widely speculated that error-prone DNA polymerases are required for the S region DSB repair [4, 5], as mutations and insertions are frequently observed in or around switch recombination break junctions [107]. However, this view is less tenable in the wake of the determination of the functional role of AID, MMR, and Artemis in CSR and the NHEJ pathway, as all these components are potentially able to introduce mutations and/or insertions in the recombination junctions. A role in CSR for DNA polymerase ζ , thought to play a major role in Ig and Bcl-6 SHM [108], is currently untested.

XRCC4 and DNA ligase IV complex

DNA ligase IV, but not DNA ligase I, has been demonstrated to be the major pathway for final ligation process of the NHEJ pathway in eukaryotic cells [109]. XRCC4 is a polypeptide that forms a heteromultimer complex with DNA ligase IV *in vivo* [110]. XRCC4 is required for efficient ligation activity of DNA ligase IV, as it stabilizes and stimulates DNA ligase IV activity in the complex formed. DNA ligase IV-deficient patients exhibit immunodeficiency with impaired Ab production [111]. However, it remains to be formally determined if DNA ligase IV is indeed the DNA ligase that ligates the two participated S region DNA ends during the CSR process, although this complex has been demonstrated to be required for DNA end ligation for VDJ recombination [109].

Other proteins

The Nijmegen breakage syndrome protein (Nbs1) is required for the nuclear localization and function as a DNA repair complex ($M_{el}1$ -Rad50-Nbs1 complex) that is implicated in homologous recombination and NHEJ [112]. This complex is recruited to and colocalized with the sites of CSR as a DNA repair focus [111]. This process is AID-dependent, as such DNA repair foci are not formed in AID-deficient mice. Such a colocalization of Nbs1 with CSR sites suggests that Nbs1 may be involved in the DNA repair during CSR, although Nbs1 is not required for VDJ recombination [113]. Analysis of the CSR junctions from NBS patients whose Nbs1 proteins harbor mutations showed aberrant joints with a strong dependence on short sequence homology [114]. These results suggest that Nbs1 plays a role in the CSR process, although the mode of action is not clear.

The phosphorylated H2A histone family member X (γ -H2AX, also known as γ -H2afx) facilitates DNA-DSB repair and forms nuclear foci at CSR sites in cells undergoing CSR, and the localization of γ -H2AX to the IgH locus during CSR is also dependent on AID [112]. The fact that CSR is impaired in H2AX $^{-/-}$ mice further supports its role in CSR, possibly in S region repair during CSR.

Microhomology between two S DNA are also observed in the switch breakpoints derived from the patients with Ataxia-Telangiectasia [114]. This observation suggests that ataxia-telangiectasia mutation proteins are normally able to influence the DSB joint for CSR.

PERSPECTIVE

Elucidation of the action modes by which AID mediates the nicking and cleavage in S regions for CSR and VH regions for SHM will significantly advance our understanding of the molecular mechanisms of CSR and SHM. As the first defined component that was involved in CSR and SHM, AID unites the processes that carry out apparently different biological functions and are previously thought to be mediated by different mechanisms; therefore, the common features shared by CSR, SHM, and gene conversion will be particularly interesting. It will be important to define whether AID itself or AID-edited substrate(s) function as an endonuclease that nicks or cleaves the conformational changed S or VH regions or function as a component that is involved in the repair step of CSR and SHM. In this regard, understanding the mechanisms to control and regulate AID expression will also be considerably important and interesting.

Understanding the accessibility control for CSR in vivo is another potentially important issue to be answered in the near future. Currently, the knowledge regarding Ig CSR accessibility control is mainly obtained from the simplified experimental systems. Although limited progress has been made, the mechanisms for efficient Ig germline transcription in vivo and the processes required for the effective interactions and coordinations between the germline promoters and the 3' Ig α enhancers are still poorly understood. To further advance our knowledge about this issue, new approaches should be undertaken to

investigate and test the key questions and hypotheses, for example, to investigate the nature of the interaction between Ig germline promoters and 3' Ig α enhancers, to test the modified "accessibility model for CSR," and to examine the "promoter competition model" in vivo.

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