

Novel insights into class switch recombination

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The discovery of activation-induced cytidine deaminase, a putative RNA editing enzyme essential for both class switch recombination and somatic hypermutation, marked the field of isotype switching studies in the year 2000. More recent work from the same group now highlights some essential mechanistic aspects of the switch recombination process. In particular, much has been learnt about the relationship between transcription and recombination and the transcriptional competence of intronic promoters on looped-out circular DNA uncoupled from the proximal and distal enhancers. These findings have far-reaching implications, particularly for studies of class switch recombination in tissues. Although these important advances do not directly relate to interleukin-4-dependent immunoglobulin E switching, the conceptual and experimental tools developed through these studies are certain to foster progress in the immunoglobulin E field. *Curr Opin Allergy Clin Immunol* 2:147–151. © 2002 Lippincott Williams & Wilkins.

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Abbreviations

AID activation-induced cytidine deaminase
CSR class switch recombination

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Introduction

After several years of conceptual and technical struggle – neither of which is surprising in view of the remarkable complexity and uniqueness of the issues under investigation – the field of class switch recombination (CSR) has recently made tumultuous progress. The discovery of activation-induced cytidine deaminase (AID), a putative RNA editing enzyme essential for both CSR and somatic hypermutation [1], was due to Tasuku Honjo's group, and marked the year 2000 (for a review in this journal, see [2]). Capitalizing on the insights provided by AID discovery, as well as on novel rigorous approaches to dissect individual steps in CSR, the same group has now highlighted some essential mechanistic aspects of the CSR process. In particular, much has been learnt about the relationship between transcription and recombination and the transcriptional competence of intronic promoters on looped-out circular DNA uncoupled from the proximal and distal enhancers. These recent findings are discussed herein, together with their implications for CSR in tissues. While the field of interleukin-4-dependent CSR to IgE did not specifically advance, it will certainly benefit from an increased understanding of the mechanisms that dictate the choice of the immunoglobulin isotype expressed during the immune response.

Molecular mechanisms of class switch recombination: targeting of recombination through transcription

The ability of B cells to express immunoglobulins with identical antigen specificity but different effector functions results from their capacity to undergo CSR. This process, also known as isotype switching, is accompanied by looping-out deletion of constant heavy chain genes by recombination between the switch (S) μ region ($S\mu$) and one of the downstream S regions located 5' to each constant heavy chain gene. Thus CSR results in the juxtaposition of different downstream constant heavy chain genes to the expressed V(D)J transcription unit (reviewed in [3]). CSR can be divided into three steps: selection of a target S region to be recombined with the $S\mu$ region, cleavage of both $S\mu$ and the targeted S region, and repair/joining to generate a deleted chromosomal IgH locus and a looped-out circular DNA. The repair step of CSR appears to be mediated by the nonhomologous end-joining repair mechanism which is also involved in V(D)J recombination (reviewed in [4]).

The target commitment of CSR to a particular S region is determined by transcription from an intronic promoter

located 5' to each S region, which gives rise to synthesis of germline transcripts. All germline transcripts share the same overall structure, and initiate from TATAA-less promoters a few kilobases upstream of the S region, proceed through the S regions and constant heavy chain exons, and are polyadenylated near the normal poly(A) site for mature IgH mRNAs. The intronic exon is then spliced to the normal acceptor site in the first exon of the constant heavy chain gene. During CSR, the region containing the germline promoter and the intronic exon is deleted as part of a switch circle. Germline transcripts are 'sterile', that is they do not appear to be translated into protein, because they contain stop codons in all reading frames. The efficiency of CSR is severely affected by mutations that abolish germline transcription (reviewed in [5]).

Since stimulation of B lymphocytes with different cytokines induces activation of specific intronic promoters, the choice of the S region targeted by CSR is dictated by cytokines provided by T cells [6•]. Thus, human B lymphocytes activated by CD40 crosslinking in the presence of interleukin-4 or interleukin-13 switch from IgM to IgE [7,8], either directly or sequentially, through $\gamma 4$ [9,10]. The accessibility model proposes that transcription of the S region enhances its accessibility to a putative cleaving enzyme (reviewed in [3]). Despite intense efforts, however, the molecular basis for accessibility or target selection remains largely unknown. It has been speculated that germline transcripts *per se* may have some functional roles in CSR, although they cannot encode any proteins. One hypothesis is the formation of transient DNA/RNA heteroduplexes or R-loops with the S region, providing the specific structure for recognition by the cleaving enzyme [11,12]. Splicing of germline transcripts has also been claimed to be required for CSR [13,14]. Actual breakpoints of CSR have been clustered in S regions and their flanks, which are spliced out from the germline transcripts [4]. These results suggest that transcription and cleavage may be coupled by an unknown mechanism.

Over the past year novel insights into the relationship between S-region transcription and CSR were provided by experiments with artificial switch substrates stably integrated in the B cell genome. Plasmids containing the appropriate *cis* regulatory elements and transcribed in response to appropriate CSR-inducing stimuli have been shown to undergo CSR like their endogenous counterparts in the immunoglobulin locus [15]. Because of their smaller size and the presence of traceable markers, however, modifications occurring in these vectors are more readily detectable. The most recent experiments with these substrates used DNA constructs in which the downstream S region (S α) is regulated by a tetracycline-responsive promoter that allows for transcription of that S

region on demand, while S μ is transcribed constitutively [16•]. When these plasmids were transfected in CH12F3-2 cells (a well-validated model for CSR [6•,15]), transcription of the S regions was found to be quantitatively associated with CSR efficiency [16•]. By contrast, although opening of the chromatin structure may facilitate recognition of any DNA structure by CSR recombinase, transcription did not change the histone acetylation level in the S region of the artificial switch substrate. Furthermore, no evidence was obtained to support the possibility that germline transcripts *per se* are required for CSR [16•].

These results beg the question, what is the target of the CSR recombinase? That the primary sequence of the S region is not the recognition target of the recombinase is already known. Indeed, a multiple cloning site sequence containing palindromes could replace the S region [17•]. Furthermore, CSR junctions are preferentially clustered around the neck region, namely the border between double-stranded and single-stranded DNA predicted by a computer-generated analysis of S region secondary structure [18•]. Thus it appears more likely that the target of recognition is a secondary structure specifically associated with transcription and recombination. If so, efficient transcription of S regions may facilitate CSR by enhancing the formation of such secondary structures, which probably depend on transient and partial denaturation of the S region during transcription. Other possible recognition structures may include RNA transcripts that can hybridize with template DNA, a hypothesis that would be consistent with a mechanistic role of R loops in CSR [11]. Induced overexpression of RNaseHI in CH12F3-2 cells (to specifically snip the RNA in the DNA/RNA hybrids), however, failed to support the involvement of such hybrids in CSR [16•]. These results suggest that S-region transcription *per se*, rather than germline transcripts of defined sequence, may be critical to CSR. Because of the unusual properties of S-region DNA, transcription may induce the DNA to be transiently single stranded, thus allowing for the formation of secondary structure(s). Such structures may be the recognition targets of the recombinase. It is also possible, however, that in-situ processing of transcripts such as splicing may be important for CSR. Finally, CSR recombinase may require the RNA polymerase complex as adapter to bind the target DNA structure. These possibilities are not mutually exclusive, with transcription-induced formation of recombinase-recognizable secondary structures in S regions.

Novel molecular markers of class switch recombination

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. Until recently, however, it was difficult to pinpoint when and where CSR actually takes place, because of the lack of good molecular markers that appear at the moment of CSR and disappear quickly after CSR.

There are several candidate markers that are associated specifically with isotype switching. CSR is announced by the expression of germline transcripts initiated from cytokine-driven intronic promoters. AID has been shown recently to be induced specifically in activated B cells, and to be essential to both CSR and somatic hypermutation [1]. Because AID deficiency did not affect germline transcription [1], AID is most likely to act downstream of this molecular event. Indeed, evidence published at the end of 2001 places AID function upstream of the $S\mu$ -specific DNA lesions that initiate CSR, and indicate that AID is required for the co-localization of two DNA double-strand break repair factors, Nijmegen breakage syndrome protein and phosphorylated H2A histone family member X (γ -H2AX), to the IgH locus during CSR [19^{*}]. Finally, since CSR is accompanied by looping-out deletion of a DNA segment containing $C\mu$ and other constant heavy chain genes from the chromosome, the resultant circular DNA may be a good marker for active CSR, if it decays rapidly. Polymerase chain reaction amplification of DNA circles, however, generates products that are heterogeneous in size and thus appear smeary on gel electrophoresis unless single-cell polymerase chain reaction is undertaken. Furthermore, this method has limited sensitivity because only a single copy of circular DNA is generated in switched B cells.

A brilliant solution to these problems was recently provided by the finding that intronic promoters remain active even on DNA circles that have been looped out following CSR [6^{**}]. Isotype-specific IH- $C\mu$ circle transcripts are generated from intronic promoters located on excised circular DNA. Of note, these circle transcripts were produced only in cells that express AID and undergo CSR [6^{**}]. Kinetic analysis of circle transcripts showed that they disappear more quickly after removal of cytokine stimulation than germline transcripts, DNA circles, or AID itself. When stimulation was removed after 24 h of culture, the number of circle transcripts decreased quickly and fell below the limit of detection even by reverse transcriptase polymerase chain reaction coupled with Southern blotting. By contrast, induction of AID and germline transcripts increased rapidly after CSR-activating stimulation, and both remained detectable 48 h after removal of stimulation. DNA circles appeared as faint smeary bands 12 h after stimulation, and were still detectable 48 h later. Thus circle transcripts disappeared completely at a time when DNA circles, AID, and germline transcripts were still

detectable. These results indicate that circle transcripts driven by intronic promoters on looped-out circular DNA are a hallmark of active CSR [6^{**}]. The fact that circle transcripts disappear more quickly than DNA circles suggests that intronic promoters on circular DNA may remain responsive to cytokines even though they have lost links with both the $E\mu$ and 3' enhancers.

Class switch recombination in tissues

Our advances in the understanding of the molecular markers of CSR promise to shed new light on CSR in tissues, an issue that is as potentially important as it is controversial. Classically, isotype switching to IgE has been considered to be restricted to lymphoid tissues, such as the regional lymph nodes and spleen [20]. IgE+ cells found within peripheral sites of allergic inflammation, such as the respiratory mucosa, have been thought to migrate from these centers. In some strong sense, the standard methods for the diagnosis of allergy (skin tests and serum levels of allergen-specific IgE) rely on this view. IgE, however, has been detected at sites of allergic inflammation even in the absence of positive skin tests or elevated serum IgE [21]. Thus it is possible that B cells residing within the tissue may also participate in production of IgE by local isotype switching.

Populations of IgM+ and IgG4+ B cells have been observed within nasal mucosa [22]. There are increased numbers of T cells expressing interleukin-4 and interleukin-13 mRNA+ [23,24] and the number of CD25+-activated T cells is also increased [25]. Mast cells, basophils and eosinophils have all been reported to express CD154 [26,27]; however their role in CSR remains unclear. Within the mucosa of sensitized individuals, these cells are present in ample number and are bound to IgE in anticipation of allergen exposure [28]. When the latter occurs, allergen cross-linking of surface-bound IgE induces mast cells to release interleukin-4 [29,30] and engage in interleukin-13 production [31]. In effect, following initial sensitization, these cells may be an important source of the signals required to induce B-cell isotype switching to IgE.

IgE protein [22] and mRNA have been identified in nasal lavage fluid of patients with symptoms of allergy [32,33]. Nasal challenge with allergen and diesel exhaust particles resulted in nasal lavage B cells that express $S\mu$ - $S\epsilon$ switch circle DNA [34]. For some time, however, it was not clear if these were truly resident B cells or simply those that infiltrated the nasal compartment after switching to IgE elsewhere. Recently, I ϵ RNA+ cells (those undergoing ϵ germline transcription) were demonstrated within the nasal mucosa of allergic rhinitis patients following allergen exposure [35,36] as well as bronchial biopsies from atopic asthmatics [37]. Furthermore, local ϵ germline transcription as well as CSR were

confirmed by the presence of $I\epsilon$ RNA as well as $S\mu$ – $S\epsilon$ and $S\epsilon$ – $S\gamma 4$ switch circle DNA in nasal mucosal tissue exposed to allergen *ex vivo*, by culturing the tissue in the presence of allergen (Cameron, in preparation). Overall this work suggests that in addition to the lymph nodes and spleen, isotype switching to IgE can also occur locally within the respiratory mucosa. These findings may provide an explanation for the presence of IgE within these tissues in the absence of positive skin tests and/or serum IgE.

The most compelling proof of principle for isotype switching in tissues was recently provided by IgA, rather than IgE, expression models in mice [38**]. A critical element of novelty in these studies is the use of a panel of molecular markers to define steps in $\mu \rightarrow \alpha$ CSR. This analysis looked at (1) α germline transcripts, the production of which is a prerequisite for CSR; (2) AID, which is essential for CSR and strictly induced in stimulated B cells undergoing class switching [1]; and (3) $I\alpha$ – $C\mu$ circle transcripts, the most decisive hallmark for active CSR to IgA, as discussed above. Lamina propria B220+IgA+ cells from the gut of BALB/c mice contained α germline transcripts, AID and $I\alpha$ – $C\mu$ circle transcripts, indicating that at least some of these cells had undergone CSR very recently [38**]. By contrast, lamina propria B220–IgA+ plasma cells expressed α germline transcripts, but none or very low amounts of AID and circle transcripts. All IgA+ populations contained $I\mu$ – $C\alpha$ transcripts that were expressed from the IgH locus after $\mu \rightarrow \alpha$ chain CSR [38**]. These findings indicate that CSR is likely to have occurred recently in lamina propria B220+IgA+ cells, and suggest that IgA+ plasma cells in the same tissue may be generated *in situ* by CSR in B220+IgM+ lymphocytes.

Conclusion

While the work discussed above focused on murine IgA switching in the gut, it is to be expected that the use of similar high-resolution molecular markers of CSR could provide more definitive information about the ability of human B cells to switch to IgE in tissues, and the potential involvement of local CSR in the pathogenesis of allergic inflammation.

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