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MutSα Binds to and Promotes Synapsis of Transcriptionally Activated Immunoglobulin Switch Regions

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Summary

Immunoglobulin class switch recombination joins a new constant (C) region to the rearranged and expressed heavy chain variable (VDJ) region in antigen-activated B cells (Figure 1A) (reviewed in [1, 2]). Switch recombination is activated by transcription of intronic, G-rich and repetitive switch (S) regions and produces junctions that are heterogeneous in sequence and position in the S regions. Switch recombination depends upon the B cell-specific cytidine deaminase, AID, and conserved DNA repair factors, including the mismatch repair heterodimer, MutSα (MSH2/MSH6). In mice, ablation of *Msh2* or *Msh6*, but not *Msh3*, decreases levels of switch recombination and diminishes heterogeneity of switch junctions [3–7]. Here, we demonstrate that MSH2 associates with transcribed S regions in primary murine B cells activated for switch recombination. Electron microscopic imaging reveals that MutSα binds in vitro to DNA structures formed within transcribed S regions and mediates their synapsis. MutSα binds with high affinity to G4 DNA formed upon transcription of the S regions and also binds to U·G mismatches, initial products of DNA deamination by AID. These results suggest that MutSα interacts with the S regions in switching B cells to promote DNA synapsis and recombination.

Results and Discussion

MSH2 Associates with Transcribed S Regions in Switching B Cells

We assayed association of MSH2 with S regions in vivo by chromatin immunoprecipitation (ChIP), monitoring recovery of Sγ3 switch regions by duplex PCR relative to a triosephosphate isomerase (TPI) control. Primary murine splenocytes were cultured with lipopolysaccharide (LPS) to induce switching from IgM to IgG3 (Figure 1B). In chromatin prepared from these switching B cells, normalized enrichment of Sγ3 sequences after immunoprecipitation with anti-MSH2 antibodies was 1.5-fold (Figure 1C, top); and there was no enrichment of Sγ3 sequences after immunoprecipitation from the murine pre-B cell line PD31, which proliferates robustly but does not switch (Figure 1C, bottom).

Specific association of MSH2 with transcriptionally activated Sγ3 regions was assayed by serial immunoprecipitation or "ChIP/reChIP" of chromatin from LPS-cultured B cells. The first IP was with an anti-acetylated histone H4 antibody to enrich for transcribed genomic regions, and the second was with anti-MSH2 antibodies. The normalized enrichment of Sγ3 sequences was 1.8-fold (Figure 1D). These enrichment levels, measured in a heterogeneous population of primary mammalian cells, compare well with observations in other systems. For example, after induction of HO endonuclease cleavage in a synchronized *S. cerevisiae* culture, a 3.5-fold maximal enrichment of recombination factors including Rad51p is evident at the HO cleavage site [8]. We conclude that MSH2 associates with transcribed Sγ3 regions in B cells switching to IgG3, consistent with participation of MSH2 in the recombination mechanism.

MutSα Binds In Vitro to G4 DNA and U·G Mismatches

To identify potential DNA targets for MutSα at activated S regions in vivo, we assayed binding of purified, recombinant human MutSα (see Figure S1A available with this article online) to substrates representing activated S regions. Transcription of S regions causes formation of G loops in which a stable, cotranscriptional RNA/DNA hybrid formed on the C-rich strand prevents reannealing of the DNA duplex after transcription, and the G-rich strand includes regions of G4 DNA interspersed with single-stranded DNA [9]. In G4 DNA, four strands associate, stabilized by Hoogsteen bonding between planar arrays of guanines, or G-quartets. MutSα bound tightly to G4 DNA formed from the TP oligonucleotide, which carries a consensus sequence from a murine S region (apparent K_D = 1 nM) (Figures 2A and 2B), and to G4 DNAs formed from other G-rich sequences (Figure S1B). MutSα binding to G4 DNA is therefore structure specific not sequence specific.

AID converts C to U in transcribed regions, thereby creating U·G mismatches (reviewed in [10, 11]). MutSα bound to a synthetic heteroduplex carrying a U·G mismatch with affinity (apparent K_D = 32 nM) greater than its affinity for homoduplex DNA (Figures 2A–2C). Human MutSα has also been shown to bind UU·GG double mispairs (apparent K_D = 24 nM) [12]. MutSα did not specifically bind other predicted intermediates in the AID/UNG pathway, including duplex oligonucleotides carrying an abasic site opposite G or duplexes cleaved at an abasic site produced by combined action of UNG and APE-1 (not shown).

Binding to G4 DNA resulted in formation of higher-order complexes, even at very low MutSα concentrations (≤2 nM) (Figure 2A). Higher order complexes were also observed upon binding of MutSα to T·G and U·G heteroduplexes at higher protein concentration (Figure 2A). Homologs of MutSα can oligomerize in vitro [13, 14] but at concentrations three orders of magnitude greater than those used in the binding assays shown. Thus, the higher order complexes are unlikely to reflect

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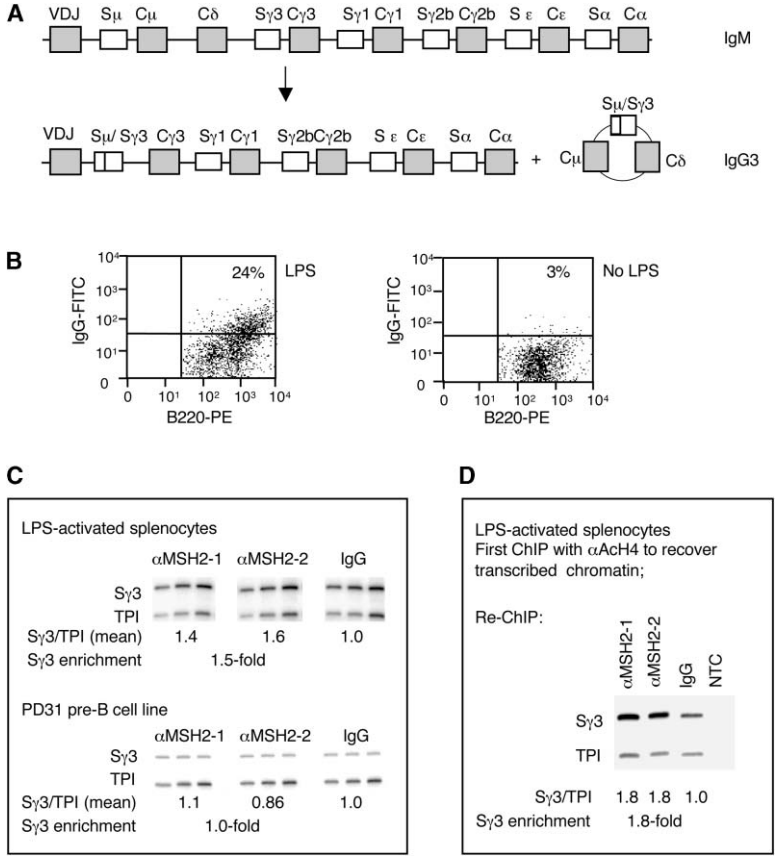

Figure 1. MSH2 Associates with the S Regions in Switching B Cells

(A) Class-switch recombination from μ to γ3. The rearranged murine heavy chain locus is shown, including the rearranged variable region (VDJ) and proximal switch (S) and constant (C) regions. Recombination joins a new constant region (Cγ3) to the expressed variable region, rejoining chromosomal DNA and generating a circle containing the excised sequences (bottom).

(B) Flow cytometry of LPS-activated murine splenocytes. Splenocytes were cultured for 72 hr with (left) or without (right) LPS, stained with anti-B220 antibodies (X-axis), specific for B cells, and anti-IgG (y axis), and analyzed. The percentage of cells that have switched is indicated in the upper right quadrant of each plot.

(C) Enrichment of Sγ3 regions in MSH2 bound chromatin. Native gel electrophoresis of duplex PCR products amplified with primers specific for the Sγ3 switch region (Sγ3) or the triosephosphate isomerase gene (TPI) from immunoprecipitated chromatin templates. Left, products of amplification from immunoprecipitations with anti-MSH2 antibodies (αMSH2-1, Oncogene, and αMSH2-2, Santa Cruz Biochemicals) or polyspecific rabbit IgG (IgG) of chromatin from splenocytes cultured for 72 hr with LPS (top) or PD31 pre-B cells (bottom); serial 2-fold dilutions of template are shown. Sγ3 enrichment was calculated as the recovery ratios of Sγ3/TPI amplicons normalized to the IgG control for nonspecific immunoprecipitation: anti-MSH2 (Sγ3/TPI)/IgG (Sγ3/TPI). Mean enrichment is shown below the gel; recovery is tabulated in Table S1.

(D) Enrichment of transcribed Sγ3 regions in MSH2 bound chromatin. Products of amplification of chromatin from LPS-activated splenocytes immunoprecipitated first with anti-acetylated H4 antibodies followed by anti-MSH2 antibodies. NTC, no template control. Other notations as in (C).

nonspecific aggregation, which raised the possibility that they might be relevant to the physiological function of MutSα. The observed higher order complexes (Figure 2A) could represent multiple MutSα heterodimers bound to one DNA molecule or interactions between MutSα heterodimers bound to different DNA molecules.

MutSα Binding to G4 DNA Is ATP Resistant

Mismatch repair by MutSα is governed by ATP binding and hydrolysis, and MutSα does not stably bind heteroduplex DNAs in the presence of ATP (reviewed in [15]). Mutation of the *Msh2* ATPase has only modest impact on class-switch recombination [16], prompting us to ask if G4 DNA binding is sensitive to ATP. MutSα binding to G4 DNA binding was stable in ATP concentrations as high as 1 mM, in contrast to heteroduplex binding (Figures 2D and S2A). MutSα binding to another DNA structure, the Holliday junction, was also resistant to ATP (Figures 2D and S2B) [17]. This difference in ATP sensitivity suggests that MutSα may have distinct modes of binding and responding to DNA structures and heteroduplexes. These results raise the possibility that the ATPase-independent component of MutSα function in switch recombination may involve binding to DNA structures.

MutSα Binds to G Loops within Transcribed S Regions

To study the interaction of MutSα with individual transcribed S regions, we used electron microscopy to image complexes of MutSα with transcribed Sμ or Sγ3 regions in plasmid templates. S region transcription resulted in formation of distinctive G loops (e.g., Figure 3A), as previously reported [9]. MutSα is very large (~250 kDa) and was clearly visible bound to the G loops (e.g., Figure 3B). The fraction of bound G loops was dependent upon the concentration of MutSα, reaching 42% in a typical reaction containing 5 nM MutSα and 17 nM DNA. MutSα bound only to the thinner filament, which corresponds to the G-rich strand [9], and not to the thicker filament, which contains the RNA/DNA hybrid, nor to the duplex region of the plasmid (e.g., Figure 3B). Inclusion of a 10-fold molar excess of synthetic G4 DNA decreased the percentage of molecules bound from 42% to less than 4% (1 of 27 molecules) (not shown). The high affinity of MutSα for G4 DNA (Figures 2 and S1B) suggests that G4 DNA is a binding target for MutSα within the G loops.

MutSα Promotes S Region Synapsis

Strikingly, MutSα not only bound to G loops but also promoted interactions between them, effectively allowing

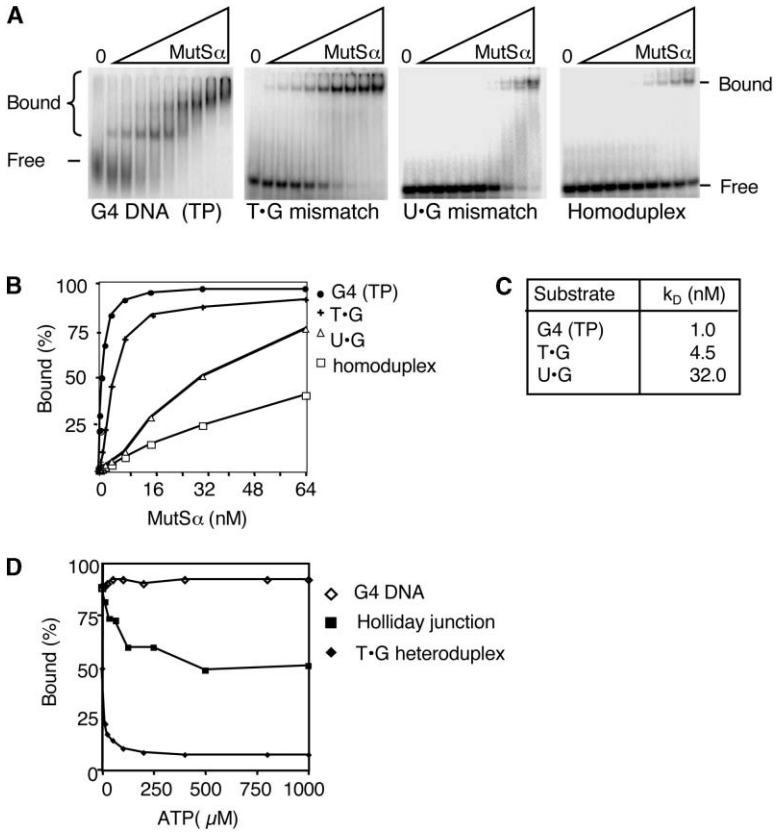

Figure 2. MutSα Specifically Recognizes G4 DNA, and Binding Is ATP Resistant

(A) Gel mobility shift analysis of hMutSα (0.25–64 nM) bound to ^{32}P-labeled oligonucleotides containing G4 DNA formed from the TP oligonucleotide (Sγ2b consensus sequence), heteroduplexes containing a single T·G or a single U·G mismatch, or a homoduplex. Free DNAs and MutSα bound complexes are indicated.

(B) Binding to G4 DNA, T·G and U·G mismatch heteroduplexes, and homoduplexes as a function of MutSα concentration, quantified by phosphorimager.

(C) Apparent affinities of MutSα binding to G4 DNA or heteroduplexes carrying T·G or U·G mismatches.

(D) ATP resistance of MutSα binding to G4 DNA, Holliday junctions, and T·G mismatch heteroduplexes, quantified by phosphorimager.

two DNA molecules to synapse (e.g., Figure 3C). In a representative reaction, 27% of molecules containing G loops were synapsed in the presence of MutSα (32 of 120 molecules). No synapsis was observed if BSA was substituted for MutSα (not shown). In all cases, the interactions between synapsed molecules localized to the G loops and involved the thinner filaments of the G loops, corresponding to the G-rich DNA strand (Figure 3C). Inclusion of a 10-fold molar excess of synthetic G4 DNA efficiently inhibited synapsis by MutSα so that only 1% (2 of 200) of molecules that contained G loops were synapsed (not shown). Competition by synthetic G4 DNA is consistent with the possibility that G4 DNA is the target for MutSα binding and synapsis within the G loops but does not eliminate the possibility that other structures are bound by MutSα. The synapsed structures imaged by electron microscopy may correspond to the higher order complexes evident in gel mobility shifts (Figures 2 and S1).

The plasmids pRX15F3 (Sμ) and pPH600 (Sγ3) differ in length by 0.5 kb, allowing them to be distinguished in electron micrographic images. When equal amounts of these two plasmids were transcribed, incubated with MutSα, linearized, and imaged, there were examples not only of Sμ-Sγ3 synapsis (e.g., Figure 3C, bottom), but also of Sμ-Sμ and Sγ3-Sγ3 synapsis (e.g., Figure 3C, top). Intra-S region synapsis (Sμ-Sμ or Sγ3-Sγ3) would produce intra-S region deletions, which are commonly found at switch junctions. Synapsed Sμ-Sγ3 regions (Figure 3C, bottom) are a predicted intermediate in switch recombination from μ to γ3 (see Figure 1). We conclude that MutSα can promote S region synapsis in vitro.

Model for MutSα Function in Recombination at the S Regions

We have shown that MSH2 is enriched at activated S regions in switching B cells and that MutSα can promote synapsis of transcriptionally activated switch regions in vitro. Based on our findings, we propose a model for one possible function of MutSα in class-switch recombination (Figure 4). Transcription of S regions causes formation of a stable RNA/DNA hybrid on the C-rich template strand and formation of G4 DNA interspersed with single stranded regions on the G-rich strand and allows AID to deaminate C to U on the G-rich strand. Colocalization of transcribed sequences to transcription factories may contribute to juxtaposition of S regions targeted for recombination. MutSα binds the activated S regions, and MutSα molecules interact to promote S region synapsis.

Our results identify two potential targets for MutSα at the activated S regions: G4 DNA and U·G mismatches produced by AID deamination. Genetic analysis suggests that both these targets may be used in activated B cells. Switch recombination is absolutely dependent upon *Msh2* in a strain of mice lacking a highly repetitive region of Sμ consisting of tandem reiterations of GAGTC, a hotspot for AID deamination [18]. This deletion leaves intact regions within Sμ that are G rich and may therefore form G4 DNA, and recognition of G4 DNA by MutSα could provide an alternative route to class switch recom-

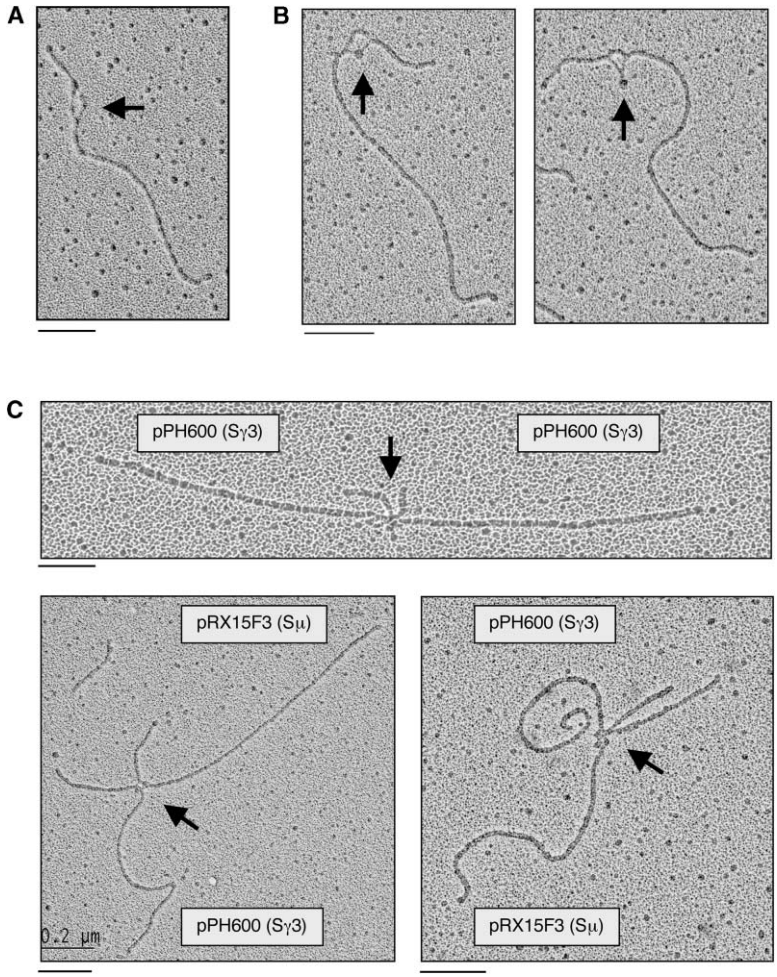

Figure 3. MutSα Binds to the Nontranscribed Strand of G Loops

(A) Example of G loop formed upon transcription, indicated by arrow. Bar, 200 nm.

(B) Representative EM image of MutSα bound to transcribed Sγ3 switch region in pPH600. Transcribed molecules were incubated with purified recombinant hMutSα and cross linked. MutSα bound to G loops is indicated by arrows. Bar, 200 nm.

(C) Representative EM images of MutSα-mediated synapsis between transcribed Sμ and Sγ3 sequences. Transcribed plasmids were incubated with MutSα, cross linked, and linearized to distinguish the arms of plasmids containing Sμ (pRX15F3) and Sγ3 (pPH600) sequences. Top, Sγ3-Sγ3 synapsis; bottom, Sμ-Sγ3 synapsis. Bar, 200 nm.

bination involving the intact G-rich regions. Conversely, a low level of switch recombination can occur in *Ung*$^{-/-}$ mice [19], but switching is completely abolished in *Ung*$^{-/-}$ *Msh2*$^{-/-}$ double mutants [20]. It is an intriguing possibility that in the *Ung*$^{-/-}$ background, MutSα bound to U·G mispairs may recruit factors with downstream functions in recombination.

Supplemental Data

Supplemental Data include two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/5/470/DC1/.

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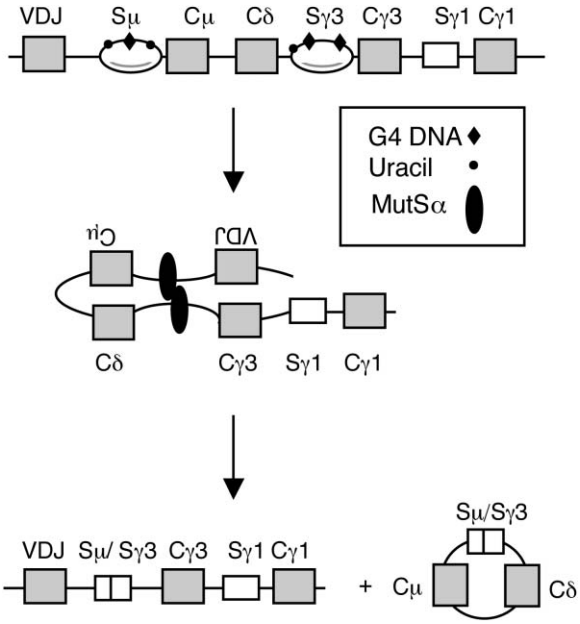

Figure 4. Model for MutSα Function in Class-Switch Recombination

Switch recombination is initiated by transcription, which produces a stable RNA:DNA hybrid (RNA, gray line); G4 DNA forms on the nontranscribed strand (diamonds) and within the transcribed region; AID deaminates cytidines to generate uracil (circles). MutSα (ovals) synapses the activated S regions. Resolution of DNA breaks generates a chromosomal Sμ/Sγ3 junction and a switch circle.

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