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Repair of Chromosomal RAG-Mediated DNA Breaks by Mutant RAG Proteins Lacking Phosphatidylinositol 3-Like Kinase Consensus Phosphorylation Sites

Eric J. Gapud,*†1 Baeck-Seung Lee,*† Grace K. Mahowald,* Craig H. Bassing,‡§ and Barry P. Sleckman*

Ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunits (DNA-PKcs) are members of the phosphatidylinositol 3-like family of serine/threonine kinases that phosphorylate serines or threonines when positioned adjacent to a glutamine residue (SQ/TQ). Both kinases are activated rapidly by DNA double-strand breaks (DSBs) and regulate the function of proteins involved in DNA damage responses. In developing lymphocytes, DSBs are generated during V(D)J recombination, which is required to assemble the second exon of all Ag receptor genes. This reaction is initiated through a DNA cleavage step by the RAG1 and RAG2 proteins, which together comprise an endonuclease that generates DSBs at the border of two recombining gene segments and their flanking recombination signals. This DNA cleavage step is followed by a joining step, during which pairs of DNA coding and signal ends are ligated to form a coding joint and a signal joint, respectively. ATM and DNA-PKcs are integrally involved in the repair of both signal and coding ends, but the targets of these kinases involved in the repair process have not been fully elucidated. In this regard, the RAG1 and RAG2 proteins, which each have several SQ/TQ motifs, have been implicated in the repair of RAG-mediated DSBs. In this study, we use a previously developed approach for studying chromosomal V(D)J recombination that has been modified to allow for the analysis of RAG1 and RAG2 function. We show that phosphorylation of RAG1 or RAG2 by ATM or DNA-PKcs at SQ/TQ consensus sites is dispensable for the joining step of V(D)J recombination.


lymphocyte Ag receptor genes are assembled by the process of V(D)J recombination, whereby different V, D, and J gene segments are appended to generate the second exon of all Ag receptor genes (1). The V(D)J recombination reaction can be divided into DNA cleavage and joining steps. The DNA cleavage step is carried out by the RAG1 and RAG2 proteins, which together form the RAG endonuclease that introduces DNA double-strand breaks (DSBs) at the borders of two recombining gene segments and their associated RAG recognition sites, termed recombination signals (RSs) (2). Proteins belonging to the non-homologous end-joining pathway of DNA DSB repair process and join the resulting pair of hairpin-sealed coding ends and blunt phosphorylated signal ends to generate a coding joint and a signal joint, respectively (3, 4).

The ataxia telangiectasia mutated (ATM) and DNA dependent protein kinase catalytic subunit (DNA-PKcs) proteins are members of the phosphatidylinositol 3-like family of serine/threonine kinases and are activated early in the DSB response (5–7). Once activated, ATM and DNA-PKcs phosphorylate and regulate a host of downstream proteins that function in DNA damage responses and DSB repair (5–10). ATM and DNA-PKcs specifically phosphorylate serine and threonine residues that are directly followed by glutamine (SQ/TQ motifs). Both kinases are activated by RAG DSBs and are integrally involved in the processing and joining of coding and signal ends (3, 4, 11–19). ATM functions to stabilize coding ends in postcleavage complexes until they can be joined (16). DNA-PKcs promotes the hairpin-opening activity of the Artemis nuclease (3, 4, 20, 21). ATM and DNA-PKcs also have overlapping activities that are critical for the efficient repair of signal ends (12, 13).

A majority of the known functions attributed to ATM and DNA-PKcs during the process of V(D)J recombination depend on their kinase activities, suggesting that they modulate downstream targets in DSB repair pathways (3, 4, 12, 13, 16). In this regard, many proteins involved in the repair of RAG DSBs can be phosphorylated by ATM or DNA-PKcs either in vitro or in vivo. In addition to ATM and DNA-PKcs themselves, these proteins include Ku70, Ku80, XRCC4, DNA Ligase IV, Artemis, XLF, H2AX, and the components of the MRN complex (Mre11, Rad50, and Nbs1) (3, 4, 9, 19, 22–36).

In addition to their central role in DNA cleavage, the RAG proteins also have been implicated in repairing the DSBs generated by their endonuclease function (37–39). After DNA cleavage in vitro, the RAG proteins remain closely associated with signal ends in postcleavage complexes (40, 41). Subsequent dissociation

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Abbreviations used in this article: abl, ablelson; ATM, ataxia telangiectasia mutated; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; RS, recombination signal; SQ/TQ, serine and threonine residues directly followed by glutamine; VDJ, VK oligonucleotide.

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of the RAG proteins from signal ends is required for the joining of these DNA ends in vitro (42). In contrast, the role of the RAG proteins during coding joint formation remains unclear. RAG2 possesses 3 SQ/TQ motifs, and RAG1 has 10, any of which could be phosphorylated by ATM and/or DNA-PKcs to modulate RAG function during the repair steps of V(DJ) recombination. In this regard, DNA-PKcs has been shown to phosphorylate RAG2 on a conserved SQ motif (Ser356) in vitro (43). However, no significant effects on V(DJ) recombination were observed in cells expressing RAG2 containing a Ser356 to alanine mutation (44). In addition, cells expressing a mutant form of RAG1 with two conserved SQ motifs (S479 and S913) mutated to alanine also exhibited no defects in V(DJ) recombination (44). Because only a subset of RAG1 and RAG2 SQ/TQ motifs were analyzed, it remains possible that phosphorylation of the RAG proteins by ATM or DNA-PKcs at other SQ/TQ motifs is required for the normal repair of RAG-mediated DSBs. Moreover, in this study, V(DJ) recombination was analyzed on extrachromosomal plasmid substrates in which the requirements for repair may be different from RAG DSBs generated within the context of the chromosome. Indeed, although neither ATM nor Mre11 deficiency leads to defects in the repair of RAG DSBs generated on extrachromosomal plasmid substrates, repair of chromosomal RAG DSBs is defective in both mutant backgrounds (14–16, 18, 29, 30, 45, 46).

We have previously developed an experimental approach that allows for the induction of chromosomal V(DJ) recombination in abl-onc-transformed pre-B cells, hereafter referred to as abl pre-B cells (16). Treatment of abl pre-B cells with the abl kinase inhibitor STI571 leads to: 1) G1 cell-cycle arrest; 2) induction of B cells (16). Treatment of abl pre-B cells with the abl kinase inhibitor STI571 (Novartis Pharmaceuticals) for the indicated times at 10^9 cells/ml as previously described (16).

The mutant RAG2 cDNA with the two SQ and lone TQ motifs converted to AQ (RAG2 10AQ) was generated using a PCR-based mutagenesis approach. This was done through sequential overlapping PCR using complementary oligonucleotide pairs that contained the three mutations. These sense and antisense pairs included S165A, T264A, and S365A oligonucleotides (Supplemental Table I). A mutant BstXI/SacI 0.9-kb fragment was generated using these oligonucleotides coupled with the 5’ RAG2-HindIII and the PSP72DS oligonucleotide (Supplemental Table I). pSP72-FLAG-RAG2 WT and pSP72-FLAG-RAG2 3AQ-GFP were generated by replacing the 0.9-kb BstXI/SacI fragment in pSP72-FLAG-RAG2 WT and pSP72-FLAG-RAG2 WT-GFP with the mutant PCR fragment.

The mutant RAG1 cDNA with the six SQ and four TQ motifs changed to AQ (RAG1 10AQ) was also generated using a sequential overlapping PCR mutagenesis approach. The overlapping pairs of oligonucleotides included sense and antisense oligonucleotide pairs T76A, T136A, S191A, T250A, S479A, S635A, S738A, T859A, S913A, and S1034A oligonucleotides. These mutations were clustered in three fragments: Bmpl/SpI 1.5-kb fragment (T76A, T136A, S191A, T250A, and S479A), Splh/MluI 1.5-kb fragment (S635A, S738A, T859A, and S913A), and an MnlII/BglII 0.2-kb fragment (S1034A). The oligonucleotides used to flank the Bmpl/SpI 1.5-kb fragment were Bmpl-BmtI and S635A antisense. The oligonucleotides used to flank the Splh/MluI 1.5-kb fragment were S479A sense and PSP72DS. The oligonucleotides used to flank the Splh/MluI 1.5-kb fragment were S479A sense and PSP72DS. The oligonucleotides used to flank the Splh/MluI 1.5-kb fragment were S479A sense and PSP72DS. The oligonucleotides used to flank the Splh/MluI 1.5-kb fragment were S479A sense and PSP72DS. The oligonucleotides used to flank the Splh/MluI 1.5-kb fragment were Splh/SpI 0.2-kb fragment were S913A sense and PSP72DS. These fragments were shuttled into pSP72-FLAG-RAG1 WT and pSP72-FLAG-GFP-RAG1 WT to generate pSP72-FLAG-RAG1 10AQ and pSP72-FLAG-GFP-RAG1 10AQ. Conditions for all PCR reactions were 95°C for 5 min followed by 15 cycles of 92°C for 1 min. 57°C for 1.5 min, and 72°C for 1.5 min. The different RAG1 and RAG2 cDNAs were then subcloned into the pcST-iThyl.1 retroviral vector and then introduced into abl pre-B cells by retroviral transduction as previously described (16, 49).

Mice

Animals were housed in a specific pathogen-free animal facility at Washington University. Animal protocols were approved by the Washington University Institutional Animal Care and Use Committee.

**Generation and culture of abl pre-B cell lines**

V-abl–transformed pre-B cells were generated by culturing bone marrow from 3–5-wk-old mice with the pMSCV v-abl retrovirus as described previously (16). To generate RAG2 1/2−/−:INV and RAG1 1/2−/−:INV cell lines, RAG2 1/2−/− and RAG1 1/2−/− abl pre-B cells were infected with pMX-INV, and cells that had integrated the recombination substrate were sorted based on CD4 expression. These cells were then subcloned, and individual subclones were analyzed by Southern blotting to identify cells with single pMX-INV integrants. Cells that underwent robust pMX-INV rearrangement when reconstituted with RAG1 (for RAG1 1/2−/−:INV abl pre-B cells) or RAG2 (for RAG1 1/2−/−:INV abl pre-B cells) were chosen for analysis. RAG2 1/2−/−:DELSI and RAG1 1/2−/−:DEL cell lines were made identically, except cells were infected with pMX-DEL 3SI. Retroviral transduction and maintenance of abl pre-B cell cultures was carried out as previously described (16). Cells were treated with 3.0 μM STI571 (Novartis Pharmaceuticals) for the indicated times at 10^6 cells/ml as previously described (16).

**Southern blotting and PCR analysis**

Southern blot analysis of pMX-INV and pMX-DEL 3SI rearrangements were carried out using the C4 and C4h probes as previously described (13, 16). Quantitative PCR analyses were carried out for VJκ rearrangements using the Vκ oligonucleotide (VκD) and Jκ2–3′ oligonucleotides (Supplemental Table I). PCR products were hybridized with the probe JK2 (Supplemental Table I). Quantitative loading control PCR for the H2.2 gene was performed as described previously (16). Conditions for both PCR reactions were 95°C for 5 min followed by 30 cycles of 92°C for 1 min, 60°C for 1.5 min, and 72°C for 1.5 min. Densitometric measurements of band intensities in Fig. 2D were determined using ImageJ software (National Institutes of Health; http://rsweb.nih.gov/ij/).

**Northern blotting analysis**

Northern blot analysis was carried out as previously described using a 0.5-kb EcoRV/SphI RAG1 cDNA fragment or an 0.8-kb PstI RAG2 cDNA fragment (16).

**Immunoprecipitation and Western blot analyses**

Immunoprecipitation was carried out from 1 × 10^8 abl pre-B cells using anti-FLAG mAb (F3040; Sigma-Aldrich) using a previous protocol, except that anti-FLAG Ab (1:500) was prebound to protein A-Agarose resin beads.
Flow cytometry

Flow cytometric analyses were carried out using a BD FACSCalibur (BD Biosciences) and data analyzed using FlowJo 4.6.2 for Macintosh (Tree Star). Flow cytometric cell sorting was carried out using a BD Aria Cell Sorter (BD Biosciences). Rat anti-Thy1.1–PE Ab, 1:2000 (#551401; BD Biosciences), was used to detect cells expressing Thy1.1.

Results

Experimental approach to study RAG function in chromosomal DSb repair

Our strategy to reconstitute inducible chromosomal V(D)J recombination in RAG2−/− abl pre-B cells is shown in Fig. 1. A similar strategy was used for reconstituting inducible chromosomal V(DJ) recombination in RAG1−/− abl pre-B cells. Initially, several independently derived RAG1−/− and RAG2−/− abl pre-B cell lines were generated with single integrants of the pMX INV retroviral recombination substrate (RAG1−/−:INV and RAG2−/−:INV abl pre-B cells, respectively) (Fig. 2A) (16). pMX-INV has a single pair of RSs that flank an antisense GFP cDNA (Fig. 2A). Recombination of pMX-INV occurs by inversion, placing the GFP cassette in the sense orientation, which permits GFP expression as an indicator of successful rearrangement. Full-length wild-type RAG1 and RAG2 cDNAs with single N-terminal FLAG tags were introduced into the pCST-FLAG-RAG1WT-Thy1.1 and pCST-FLAG-RAG2WT-Thy1.1, respectively (Supplemental Fig. 1A). pCST-FLAG-RAG1WT-Thy1.1 has an internal ribosome entry site-Thy1.1 cassette, permitting flow cytometric purification of cells with retroviral integrants based on Thy1.1 expression (Fig. 1, Supplemental Fig. 1A, 1B).

Thy1.1-expressing RAG2−/−:INV abl pre-B cells infected with pCST-FLAG-RAG2WT-Thy1.1 (referred to as RAG2−/−:INV/R2WT abl pre-B cells) constitutively express wild-type RAG2 transcripts from the retrovirus (Supplemental Fig. 1C). Treatment of these cells with STI571 leads to G1 cell-cycle arrest and induction of RAG1 gene expression, which, when coupled with retroviral RAG2 expression, leads to rearrangement of pMX-INV (Fig. 2B–D, Supplemental Fig. 1). Rearrangement of pMX-INV is evidenced by GFP expression in 18% and 49% of RAG2−/−:INV/R2WT abl pre-B cells treated with STI571 for 48 and 96 h, respectively (Fig. 2B). Moreover, Southern blot analyses revealed a 3-kb EcoRV/NcoI C4 probe hybridizing fragment, indicative of pMX-INV coding joint formation in STI571-treated RAG2−/−:INV/R2WT abl pre-B cells, but not in RAG2−/−:INV abl pre-B cells (Fig. 2C, 2D). Rearrangement of pMX-INV in RAG2−/−:INV/R2WT abl pre-B cells approached levels observed in wild-type abl pre-B cells (WT:INV) (Fig. 2B–D). Using an identical approach, RAG1−/−:INV abl pre-B cells were reconstituted with wild-type RAG1 (RAG1−/−:INV/R1WT abl pre-B cells) using the pCST-FLAG-RAG1WT-Thy1.1 retroviral vector. Treatment of RAG1−/−:INV/R1WT abl pre-B cells with STI571 also led to inducible rearrangement of pMX-INV (Fig. 2B–D).

The abl pre-B cells undergo inducible rearrangement of the endogenous IgLk locus upon inhibition of the abl kinase with STI571. The murine IgLk locus contains ~250 Vκ gene segments and 4 functional Jk gene segments. To determine if IgLk gene rearrangement is also inducible in RAG2−/−:INV/R2WT and RAG1−/−:INV/R1WT abl pre-B cells treated with STI571, genomic DNA from these cells was assayed by PCR using a degenerate VκD and an oligonucleotide downstream of Jk2 (Jk2–3). The combination of these two oligonucleotides detects Vκ rearrangements to Jk1 and Jk2 (Fig. 3A). VκJk rearrangements were readily detected in STI571-treated RAG2−/−:INV/R2WT and RAG1−/−:INV/R1WT abl pre-B cells (Fig. 3B, 3C). Prior to STI571 treatment, these cells exhibit only low levels of VκJk rearrangements (Fig. 3B, 3C). Finally, RAG2−/−:INV and RAG1−/−:INV abl pre-B cells had no detectable IgLk rearrangements regardless of whether they were treated with STI571 (Fig. 3B, 3C). Together, these findings demonstrate that chromosomal V(DJ) recombination at either pMX-INV or the endogenous IgLk locus is readily inducible in RAG2−/−:INV or RAG1−/−:INV abl pre-B cells reconstituted with wild-type RAG2 or RAG1, respectively.

Chromosomal V(D)J recombination catalyzed by RAG1 and RAG2 GFP fusion proteins

To assay the effects of different RAG1 and RAG2 mutations on V(DJ) recombination, we required cells with equivalent levels of wild-type and mutant RAG proteins. To this end, we generated a version of RAG2 with an N-terminal FLAG tag and a C-terminal GFP fusion and introduced this cDNA into the pCST-iThy1.1 retroviral vector (Supplemental Fig. 1D, 1E). RAG2WT-GFP expression was readily detected by flow cytometric analyses of pMX-INV abl pre-B cells infected with the pCST-RAG2WT-GFP-iThy1.1 retroviral vector (RAG2−/−:INV/R2WT-GFP) (Supplemental Fig. 1D). Moreover, treatment of RAG2−/−:INV/R2WT-GFP abl pre-B cells with STI571 led to inducible rearrangement of both pMX-INV and the IgLk locus (Figs. 2C, 2D, 3B). In contrast, the RAG1WT-GFP C-terminal fusion was not expressed at significant levels and did not rescue rearrangement in RAG1−/−:INV cells (data not shown). Accordingly, we generated and expressed a FLAG-tagged N-terminal GFP-RAG1WT fusion protein in RAG1−/−:INV abl pre-B cells (RAG1−/−:INV/GFP-R1WT) (Supplemental Fig. 1E). The N-terminal GFP-RAG1 fusion variant rescued STI571-inducible rearrangement, albeit at lower levels than those observed in wild-type or RAG1−/−:INV/R1WT abl pre-B cells (Figs. 2C, 2D, 3C, Supplemental Fig. 1E). Together, these findings demonstrate that retroviral introduction of the relevant RAG/GFP fusion into RAG-deficient abl pre-B cells rescues STI571-inducible rearrangement of pMX-INV and the IgLk locus. Importantly, the levels of wild-type GFP-RAG1WT, RAG2WT-GFP, and their corresponding mutants can be compared by flow cytometry.

Chromosomal V(D)J recombination in cells expressing an SQ/TQ RAG2 mutant

The murine RAG2 protein is 527-aa long and contains two SQ (S165 and S365) motifs and one TQ (T264) motif (Fig. 4A). These residues do not overlap with the plant homeo domain, which functions to tether RAG2 to methylated histone H3 present in chromatin (Fig. 4A) (2, 51). A version of FLAG-tagged RAG2 was generated in which the three SQ/TQ motifs were mutated to AQ (RAG23AQ). This mutant was introduced retrovirally into RAG2−/−:INV abl pre-B cells, yielding a cell line that expresses RAG23AQ (RAG2−/−:INV/R23AQ) at levels equivalent to wild-type RAG2 in RAG2−/−:INV/R2WT abl pre-B cells (Fig. 4B). After treatment with STI571, robust rearrangement of pMX-INV was observed in both RAG2−/−:INV/R2WT and RAG2−/−:INV/R23AQ abl pre-B cells, as evidenced by both flow cytometric analyses and Southern blotting (Fig. 4C, 4D). Analyses of another independently generated RAG2−/−:INV abl pre-B cell line that expressed either
RAG2 or RAG2^3AQ yielded similar findings (Supplemental Fig. 2A, 2B). Moreover, STI571-treated RAG2^−/−:INV abl pre-B cells were then infected with retroviruses encoding wild-type RAG2 (or RAG2 mutants) with Thy1.1 as an indicator of retroviral transduction to generate RAG2^−/−:INV/R2 abl pre-B cells. These retroviruses permit constitutive expression of RAG2 (shown in gray). Treatment with STI571 leads to G1 cell-cycle arrest and induction of transcription at the RAG1 and RAG2 loci. As the RAG2 gene has been replaced by the neomycin resistance gene, treatment with STI571 leads only to RAG1 protein expression (shown in gray). This coupled with RAG2 expression from the retrovirus leads to rearrangement of the pMX-INV and the endogenous IgLk locus (gray arrow).

During chromosomal V(D)J recombination, deficiency of ATM leads to a 10–20% loss of coding ends from postcleavage complexes (16). This dissociation results in diminished coding joint formation, an accumulation of unrepaired coding ends, and the formation of pMX-INV hybrid joints, the latter of which are produced by aberrant ligation of chromosomal pMX-INV coding and signal ends (Figs. 2A, 4D, 4E). To test whether these defects are linked to a loss of ATM-mediated RAG2 phosphorylation, we performed Southern blot analysis of genomic DNA from RAG2^−/−:INV/R2^WT abl pre-B cells treated with STI571. We did not detect unrepaired pMX-INV coding ends or pMX-INV hybrid joints, which were both readily apparent in ATM-deficient abl pre-B cells (Fig. 4D, 4E). Similar results were observed when analyzing another independently derived RAG2^−/−:INV abl pre-B cells reconstituted with RAG2WT or RAG2^3AQ (Supplemental Fig. 2B). Finally, V(D)J recombination was similar in RAG2^−/−:INV abl pre-B cells that express RAG2^3AQ-GFP (RAG2^−/−:INV-R2^3AQ-GFP) or RAG2WT-GFP (RAG2^−/−:INV-R2^WT-GFP) (Fig. 4D, 4F).
Together, these data demonstrate that phosphorylation of RAG2 at SQ/TQ motifs is dispensable for efficient formation of chromosomal coding joints.

**Coding joint formation in cells expressing SQ/TQ mutant RAG1**

The mouse RAG1 protein is 1040-aa long and contains four TQ (T76, T136, T250, and T859) and six SQ (S191, S479, S635, S738, S789, and S906) motifs.

**FIGURE 2.** Rescue of chromosomal V(D)J recombination in RAG1<sup>-/-</sup> and RAG2<sup>-/-</sup> abl pre-B cells. A, Schematic of the pMX-INV retroviral recombination substrate. Unrearranged (UR) pMX-INV and pMX-INV with a coding end intermediate (CE), completed coding joint (CJ), signal joint (SJ), and hybrid joint (HJ) products are shown. The approximate positions of the EcoRV (E) and NcoI (N) sites and C4 probe are indicated. B, Flow cytometric analysis of GFP expression at various times (h) after treatment with STI571 for wild-type (WT:INV) abl pre-B cells, (RAG1<sup>-/-</sup>:INV) pre-B cells transduced with pCST-FLAG-RAG1<sup>WT</sup>-iThy1.1 (RAG1<sup>-/-</sup>:INV:R1WT), and RAG1<sup>-/-</sup>:INV:R1WT<sup>aQ</sup> abl pre-B cells transduced with pCST-FLAG-RAG1<sup>WT</sup>-iThy1.1 (RAG1<sup>-/-</sup>:INV:R1WT<sup>aQ</sup>). DNA samples from abl pre-B cells from B and RAG1<sup>-/-</sup>:INV:R1WT<sup>aQ</sup> pre-B cells transduced with pCST-FLAG-GFP-RAG1<sup>WT</sup>-iThy1.1 (RAG1<sup>-/-</sup>:INV:GFP-R1WT<sup>aQ</sup>) were digested with EcoRV and NcoI and probed with the C4 probe. Relative quantification of coding joints (percent CJ) formed in C.
S913, and S1034) motifs (Fig. 5A). Two serines (S635 and S738) reside within a region that mediates RS heptamer binding and RAG2 interactions with one of these serines (S738) present in a zinc finger region (2). To investigate the potential function of these sites in coding joint formation, we developed mutant versions of FLAG-tagged RAG1 (RAG1T10AQ) and GFP-RAG1 (GFP-RAG1T10AQ) in which all 10 of these SQ/TQ motifs were mutated to AQ. These proteins were expressed in RAG1−/−:INV abl pre-B cells, and stable clones with equivalent levels of RAG1T10AQ (RAG1−/−:INV:R1T10AQ) and RAG1WT (RAG1−/−:INV:R1WT) or GFP-RAG1T10AQ (RAG1−/−:INV:GFP-R1T10AQ) and GFP-RAG1WT (RAG1−/−:INV:GFP-R1WT) were selected for analysis (Fig. 5B, SF).

After induction of V(D)J recombination, coding joint formation at pMX-INV and the IgLk locus proceeded with similar kinetics in RAG1−/−:INV:R1T10AQ and RAG1−/−:INV:R1WT abl pre-B cells (Figs. 3C, 5C, 5D). Similar results were obtained upon analysis of a second set of independently derived RAG1−/−:INV:R1T10AQ and RAG1−/−:INV:R1WT abl pre-B cell lines (Supplemental Fig. 2C, 2D). Moreover, neither unrepaird pMX-INV coding ends nor pMX-INV hybrid joints were detected in RAG1−/−:INV:R1T10AQ abl pre-B cells (Fig. 5D, 5E, Supplemental Fig. 2D). Similar results were obtained upon analysis of IgLk locus and pMX-INV rearrangement in RAG1−/−:INV:GFP-R1WT and RAG1−/−:INV:GFP-R1T10AQ abl pre-B cells (Figs. 3C, 5D and data not shown). Thus, we conclude that phosphorylation of RAG1 at any of the 10 SQ/TQ motifs is not essential for efficient coding joint formation.

**Function of RAG1 and RAG2 during signal joint formation**

ATM and DNA-PKcs have redundant functions during signal joint formation, suggesting that they phosphorylate common downstream targets important for the repair of signal ends (12, 13). To determine if RAG1 or RAG2 are important targets of ATM and DNA-PKcs during this process, we generated several RAG1−/− and RAG2−/− abl pre-B cell lines with single integrants of the pMX-DELSI retroviral recombination substrate (RAG1−/−:DELSI and RAG2−/−:DELSI). pMX-DELSI is identical to pMX-INV except that the RSs have been reoriented such that rearrangement results in the formation of a chromosomal signal joint (Supplemental Fig. 3A). Southern blot analyses can be carried out to detect unrepaird pMX-DELSI chromosomal signal ends. In this regard, inducing rearrangement in DNA Ligase IV-deficient abl pre-B cells containing pMX-DELSI (LigIV−/−:DELSI) leads to an accumulation of unrepaired signal ends due to the deficiency in DNA Ligase IV (Fig. 6, Supplemental Fig. 3).

RAG1−/−:DELSI abl pre-B cells were transduced with retroviruses encoding RAG1WT and RAG1T10AQ, and RAG2−/−:DELSI abl pre-B cells were transduced with retroviruses encoding RAG2WT-GFP, and RAG2T3AQ-GFP. Robust pMX-DELSI signal joint formation was observed after induction of V(D)J recombination in both RAG1−/−:DELSI, R1WT and RAG2−/−:DELSI, R2WT-GFP abl pre-B cells treated with STI571 (Fig. 6, Supplemental Fig. 3). Treatment of RAG1−/−:DELSI, R10AQ and RAG2−/−:DELSI, (D) or EcoRV (E) and probed with the C4 probe. Amn−/−:INV, RAG2−/−:INV, RAG2−/−:INV:R2WT, RAG2−/−:INV:R2AQ, RAG2−/−:INV:R2WT-GFP, and RAG2−/−:INV:R2AQ-GFP abl pre-B cells were treated with STI571 for 0, 48, or 96 h prior to harvesting DNA. Bands representing pMX-INV unrearranged (UR), coding joint (CJ), hybrid joint (HJ), and coding end intermediate (CE) are indicated. Molecular weight markers are also shown. F, Flow cytometric analysis of GFP expression in RAG2−/−:INV (dotted line), RAG2−/−:INV:R2WT-GFP (solid line), and RAG2−/−:INV:R2AQ-GFP (dashed line) pre-B cells.

**FIGURE 4.** SQ/TQ motifs in RAG2 are not required for efficient coding joint formation. A, Schematic showing positions of the two SQ motifs and the single TQ motif in the RAG2 protein. The plant homeo domain is also shown (black). B, Western blot analysis of RAG2 protein expression in RAG2−/−:INV, RAG2−/−:INV:R2WT, or RAG2−/−:INV:R2AQ abl pre-B cells. Samples were immunoprecipitated with an anti-FLAG Ab followed by immunoblotting with an anti-RAG2 Ab. C, Flow cytometric analysis of GFP expression after treatment of RAG2−/−:INV, RAG2−/−:INV:R2WT, and RAG2−/−:INV:R2AQ abl pre-B cells with STI571 for 0, 48, or 96 h. Southern blot analysis of genomic DNA digested with EcoRV and Ncol Abl pre-B cells treated with STI571 for 0, 48, or 96 h prior to harvesting DNA. Bands representing pMX-INV unrearranged (UR), coding joint (CJ), hybrid joint (HJ), and coding end intermediate (CE) are indicated. Molecular weight markers are also shown. F, Flow cytometric analysis of GFP expression in RAG2−/−:INV (dotted line), RAG2−/−:INV:R2WT-GFP (solid line), and RAG2−/−:INV:R2AQ-GFP (dashed line) pre-B cells.
R23AQ-GFP abl pre-B cells also led to efficient pMX-DEL SJ signal joint formation with no detectable accumulation of unrepaired signal ends (Fig. 6, Supplemental Fig. 3). Thus, chromosomal signal joining appears unimpaired in the presence of RAG1 or RAG2 proteins that are crippled for phosphorylation at their SQ/TQ motifs.

**Discussion**

In this study, we have shown that constitutive expression of wild-type RAG1 in RAG1−/− abl pre-B cells and wild-type RAG2 in RAG2−/− abl pre-B cells rescues STI571-inducible V(D)J recombination. Indeed, the level of pMX-INv and IgLk rearrangement in these cells after treatment with STI571 is similar to what is observed in wild-type abl pre-B cells treated with STI571. Thus, this approach can be used to assess the activity of mutant forms of RAG1 or RAG2 during chromosomal V(D)J recombination.

**FIGURE 5.** SQ/TQ motifs in RAG1 are not required for efficient coding joint formation. A, Schematic showing positions of the six SQ motifs and the four TQ motifs in the RAG1 protein. The zinc finger A (gray) and B (black) regions are shown. The heptamer-binding/RAG2 interacting region is also shown (bracket). B, Western blot analysis of RAG1 protein expression in RAG1−/−:INV, RAG1−/−:INV:R1WT, or RAG1−/−:INV:R10AQ abl pre-B cells. C, Flow cytometry of GFP expression at various times in culture with STI571 in RAG1−/−:INV, RAG1−/−:INV:R1WT, and RAG1−/−:INV:R10AQ pre-B cells.

**FIGURE 6.** Signal joint formation in abl pre-B cells expressing RAG1 or RAG2 SQ/TQ mutants. A and B, Southern blot analyses of EcoRV-digested genomic DNA probed with the C4b probe. RAG1−/−:DELJ abl pre-B cells and RAG1−/−:DELJ abl pre-B cells transduced with pCST-FLAG-RAG1WT-iThy1.1 (RAG1−/−:DELJ:R1WT) or a pCST-FLAG-RAG10AQ-iThy1.1 (RAG1−/−:DELJ:R10AQ) (A) and RAG2−/−:DELJ abl pre-B cells and RAG2−/−:DELJ abl pre-B cells transduced with pCST-FLAG-RAG2WT-GFP-iThy1.1 (RAG2−/−:DELJ:R2WT-GFP) or pCST-FLAG-RAG20AQ-GFP-iThy1.1 (RAG2−/−:DELJ, R20AQ-GFP) (B) pre-B cells were treated with STI571 for 0, 48, or 96 h. Bands representing unrearranged (UR) pMX-DEL SJ and normal signal joint (SJ) and unrepaired signal end (SE) are indicated. Molecular weight markers are shown.

R20AQ-GFP abl pre-B cells also led to efficient pMX-DEL SJ signal joint formation with no detectable accumulation of unrepaired signal ends (Fig. 6, Supplemental Fig. 3). Thus, chromosomal signal joining appears unimpaired in the presence of RAG1 or RAG2 proteins that are crippled for phosphorylation at their SQ/TQ motifs.
We have used this approach to determine whether phosphorylation of any serine or threonine residues in the 3 SQ/TQ motifs in RAG2 or the 10 SQ/TQ motifs in RAG1 may be responsible for any of the defects observed in signal and coding joint formation in cells deficient in ATM or DNA-PKcs. ATM-deficient abl pre-B cells exhibit defects in coding joint formation with an accumulation of unrepaird coding ends and significant levels of hybrid joint formation during inversional rearrangements (16). In contrast, these defects were not observed during V(D)J recombination in RAG2−/−:INV:R2αQ or RAG1−/−:INV:R1αQ abl pre-B cells. Additionally, although efficient repair of signal ends depends on the overlapping activities of ATM and DNA-PKcs, analysis of RAG2−/−:DEL5, R2αQ-GFP or RAG1−/−: DEL5, R1αQ abl pre-B cells revealed no defects in signal joint formation (12, 13). Together, these findings demonstrate that the observed defects in the repair of RAG DSBs in cells deficient in ATM and/or DNA-PKcs cannot solely reflect a requirement for these kinases to phosphorylate RAG1 or RAG2 at consensus SQ/TQ motifs. ATM or DNA-PKcs could phosphorylate other nonconsensus serines or threonines in RAG2αQ or RAG1αQ that exert either a direct or compensatory effect on RAG function during the joining step of the reaction. However, recent analyses of >700 ATM targets in response to ionizing radiation reveal that essentially all of the target serines and threonines were part of SQ/TQ motifs (9). Our studies are in agreement with previous analyses of mutants of the conserved SQ/TQ motifs in RAG1 and RAG2 and, importantly, extend these findings to show that none of the RAG1 and RAG2 SQ/TQ motifs is required for the normal repair of RAG DSBs (44).

There are several important features of the experimental approach described in this study that can be used to examine the biologic relevance of RAG1 or RAG2 mutations. Most notably, this method permits evaluation of RAG function during both the DNA cleavage and joining steps of chromosomal V(D)J recombination, method permits evaluation of RAG function during both the DNA cleavage and joining steps of chromosomal V(D)J recombination, and our approach described in this study that can be used to examine the biologic relevance of RAG1 or RAG2 mutations. Most notably, this method permits evaluation of RAG function during both the DNA cleavage and joining steps of chromosomal V(D)J recombination, and our approach described in this study that can be used to examine the biologic relevance of RAG1 or RAG2 mutations. Most notably, this method permits evaluation of RAG function during both the DNA cleavage and joining steps of chromosomal V(D)J recombination, and our approach described in this study that can be used to examine the biologic relevance of RAG1 or RAG2 mutations. Most notably, this method permits evaluation of RAG function during both the DNA cleavage and joining steps of chromosomal V(D)J recombination.

**References**


