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Spatio-Temporal Regulation of RAG2 Following Genotoxic Stress

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Abstract

V(D)J recombination of lymphocyte antigen receptor genes occurs via the formation of DNA double strand breaks (DSBs) through the activity of RAG1 and RAG2. The coexistence of RAG-independent DNA DSBs generated by genotoxic stressors potentially increases the risk of incorrect repair and chromosomal abnormalities. However, it is not known whether cellular responses to DSBs by genotoxic stressors affect the RAG complex. Using cellular imaging and subcellular fractionation approaches, we show that formation of DSBs by treating cells with DNA damaging agents causes export of nuclear RAG2. Within the cytoplasm, RAG2 exhibited substantial enrichment at the centrosome. Further, RAG2 export was sensitive to inhibition of ATM, and was reversed following DNA repair. The core region of RAG2 was sufficient for export, but not centrosome targeting, and RAG2 export was blocked by mutation of Thr⁴⁹⁰. In summary, DNA damage triggers relocalization of RAG2 from the nucleus to centrosomes, suggesting a novel mechanism for modulating cellular responses to DSBs in developing lymphocytes.

Keywords

V(D)J recombination; RAG1; RAG2; DNA damage response; centrosome; genotoxic stress

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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1. Introduction

V(D)J recombination consists of cycles of DNA breakage and repair toward the assembly of antigen receptor genes (1). The recombinase complex that initiates these events consists of RAG1 and RAG2, which together generate DNA double strand breaks (DSBs) at the border of V, D, or J gene segments and their flanking recombination signal sequences (1–4). While the active site of the recombinase complex is limited to RAG1, both RAG1 and RAG2 are necessary for DNA cleavage. Functions of RAG2 in this reaction include assisting RAG1 towards recognition of the recombination signal sequence (5), and activation of DNA cleavage activity (1).

RAG-mediated DNA cleavage activity is critical for lymphocyte development, yet the generation of DNA DSBs is potentially deleterious to the cell, since they increase the likelihood of recombination events that disrupt the genome (6). Consequently, a DNA damage response (DDR) is rapidly activated when cells detect the presence of DNA DSBs (7), such as during V(D)J recombination. Specifically, ATM and DNA-PKcs are activated by and/or participate directly in repair of RAG-mediated breaks during V(D)J recombination (8). ATM is recruited to RAG-generated DSBs, and its absence results in increased aberrant recombination events (9,10). DNA-PKcs is a key factor in nonhomologous end joining, the DNA repair pathway essential for the DNA joining phase of V(D)J recombination (11).

DNA DSBs in early lymphocytes are not restricted to those generated from V(D)J recombination, but will also occur through exogenous and endogenous sources, such as ionizing radiation (IR) and by-products of cellular metabolism (12). Cells containing excess DNA DSBs formed by genotoxic stressors are likely to be particularly vulnerable to errors in V(D)J recombination, as the greater availability of DNA ends can increase the frequency of erroneous joining to ends formed by RAG activity (11). When DNA DSBs occur during the G1 phase of the cell cycle, an ATM-mediated arrest in the cell cycle takes place to allow DNA repair prior to replication (7). Coinciding with this arrest will be expression of the RAG complex (13), thereby providing an opportunity for continued mistakes in joining as repair coincides with recombination activity.

RAG expression is maximal during the G0 and G1 cell cycle phases. Interestingly, RAG2 is phosphorylated and degraded at the G1-S border (14,15), and evidence suggests that this coincides with transport of RAG2 from the nucleus to the cytoplasm (16). However, it is not known whether other conditions cause RAG2 export from the nucleus to regulate its expression, such as following genotoxic stress.

Here, we asked how the presence of DNA damage, which will arrest G1-S cell cycle progression, impacts the cellular localization of RAG2. To determine the effect of DSBs introduced in early B cells on RAG2 localization, we measured the effect of DSB generation on RAG2 localization using cell imaging and subcellular fractionation. Our findings show that introduction of DNA damage leads to a rapid relocalization of RAG2 from the nucleus, which is abrogated by inhibition of ATM. Interestingly, upon export, RAG2 enriched at the centrosome, placing it in a position to affect the cell cycle, since the initiation of centrosome duplication is coordinated with the progression of G1 to S (17). Following time sufficient for

DNA repair, RAG2 re-established localization in the nucleus to pre-DNA damage levels, indicating that RAG2 spatial regulation is a function of the extent of DNA damage.

2. Materials and Methods

2.1. Gene construction

The gene encoding full length (FL) RAG2 fused to enhanced green fluorescent protein (GFP) was generated by amplifying the murine RAG2 gene using the following primers: AACACAACAACAACGCTAGCCTGCAGATGGTA (coding), and TTAATCAAAGAGTCTTCTAAG (noncoding). The RAG2 PCR product was then subcloned into the *Sma*I site of the GFP-expression vector pWay21, downstream of the sequence encoding enhanced GFP. RAG2 fused with fluorescent protein was active in V(D)J recombination based on extrachromosomal activity assays (not shown), using methods previously described (18). The gene encoding core RAG2 (residues 1 to 378) with GFP fused to the N-terminus was generated with QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) through introduction of a stop codon in place of the codon for residue 379 using the following primer and its complement: GGGACTCCACTCCCTTTTAAGACTCAGAGGAATTT. Similarly, the gene encoding the T490A mutation of GFP fused to RAG2 FL was generated using the following primer and its complement: GCAAGAGCATTGCAAGCTCCCAAAGAAACCCC.

2.2. Cell culture

The v-abl pre-B (A70) and v-abl *ATM*^{-/-} pre-B cells were a gift from Barry Sleckman (Washington University, St. Louis, MO) and are previously described (19). v-abl *RAG2*^{-/-} pro-B cells (63-12) were a gift from Mark Schlissel (Brown University, Providence, RI), and have been previously described (20). The cells were maintained in complete media containing RPMI with 10% FCS, 0.1% 2-mercaptoethanol, 2% sodium pyruvate, 1% nonessential amino acids and 10% fetal bovine serum. To induce RAG1 and RAG2 expression, the cells were cultured overnight at a density of 10⁶ cells/ml in media containing 3 μM STI-571 (Cayman Chemical, Ann Arbor, MI) that was added from a 100x stock solution in DMSO (19). Induction of RAG expression by these conditions is indicated as STI-571⁺. Expression of RAG2 was evidenced by increased immunostaining with clone 39 rabbit monoclonal antibody specific to RAG2 (21) (Supplemental Figure 1A), and the appearance of a 60 kDa band in RAG2 immunoblots of whole cell lysates specific to samples grown in STI-571 (Supplemental Figure 1B).

Stable clones expressing GFP-RAG2 fusion proteins were generated by limiting dilution of transfected cells in complete media containing G418 at a final concentration of 1.5 mg/ml, followed by flow cytometry to enrich for GFP⁺ cells, as previously described (22). Following selection, the cells were maintained in complete media containing 0.5 mg/ml G418.

2.3. Generation of DSBs

DSBs generated by irradiation was done by exposing cells at room temperature to 4 Gy IR using a Cs¹³⁷ gamma irradiator (Gammacell-40 exactor) applied at a dose of 1.1 Gy/min.

Following irradiation, the samples were immediately transferred to 37°C and maintained for 30 min before seeding and fixation. Alternatively, cells were treated with etoposide for 1 hr at 37°C at a final concentration of 40 µg/ml, or with acetaldehyde (Sigma-Aldrich, St. Louis, MO) for 4 hr at 37°C at a final concentration of 4 mM. Etoposide and acetaldehyde were added from a 100x stock solution in DMSO. Equivalent amounts of vehicle (DMSO) were added to untreated control cells.

2.4. Inhibition of the DDR

Inhibition of ATM was done by treating cells with 10 µM KU-55933 (Tocris Biosciences, Bristol, United Kingdom); DNA-PK was inhibited using 15 µM NU7026 (Tocris Biosciences). For each treatment, inhibitor was added from a 100x stock solution in DMSO, using cells seeded at a density of 10^7 /ml in RPMI and containing 50 mM HEPES (pH 7.4). The samples were incubated at 37°C for 1 hr, followed immediately by treatment with genotoxic stressor.

2.5. Subcellular fractionation

3×10^6 cells were washed twice in 0.5 ml chilled PBS (4°C), and then suspended in 400 µl chilled RSB buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 4°C) containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF). Next, the samples were incubated on ice for 10 min, then homogenized using 10 strokes with a Dounce homogenizer. Nuclei were sedimented by centrifuging for 5 min at $1,450 \times g$ at 4°C. The pellet was washed once with 250 µl chilled PBS, then resuspended in 30 µl SDS-PAGE sample buffer containing 1% 2-mercaptoethanol. Following incubation at 100 °C for 5 min, the samples were separated by SDS-PAGE, then transferred to PVDF membrane. Membranes were blocked using 5% milk in PBS for 1 hr at room temperature, then probed using anti-RAG2 antibody (clone 39), followed by biotinylated anti-rabbit from goat (Vector Laboratories, Burlingame, CA), and streptavidin-labeled HRP (Vector Laboratories). After measurement of RAG2, membranes were re-probed with antibody to either SP-1 (rabbit polyclonal, Santa Cruz, Biotechnology, Santa Cruz, CA) or actin (mouse monoclonal antibody, Sigma-Aldrich). Detection was done using enhanced chemiluminescence (ECL Prime, GE Healthcare Life Sciences, Piscataway, NJ) exposed on HyBlot CL (Denville Scientific Inc., Metuchen, NJ).

In experiments with the cytoplasmic fraction, protein was concentrated by TCA precipitation. TCA was added to a final concentration of 10% to supernatant collected after removal of nuclei by centrifugation of Dounce homogenate. Next, the samples were incubated on ice for 30 min, and then centrifuged for 10 min at $2,000 \times g$. The pellet was collected and washed twice with chilled acetone (−20°C). After the final wash, the samples were suspended in SDS-PAGE sample buffer and measured by Western blot using the anti-RAG2 antibody as described above.

2.6. Fluorescence labeling and cell imaging

For immunostaining, 10^6 cells were seeded on to a glass coverslip coated with poly-L-lysine. Following seeding, the samples were fixed by incubating at room temperature for 30 min in PBS containing 4% paraformaldehyde, then permeabilized using 0.1% TX-100 in PBS containing 10 mM glycine (PBS-glycine). For staining, the samples were first blocked

by incubating for 2 hr in PBS-glycine containing 10% normal goat serum. The samples were then incubated overnight at 4°C in PBS-glycine containing 10% goat serum and anti-RAG2 antibody (clone 39). Immunostaining of γ H2AX and γ -tubulin was done using mouse monoclonal antibody clone JBW301 (Millipore, Billerica, MA) and clone GTU-88 (Sigma-Aldrich), respectively, each with incubation for 1 hr at room temperature. Secondary antibodies for staining consisted of IgG from goat that was species-specific to the respective primary antibody, and conjugated with either Alexa Fluor 488 (Alexa488) or Texas Red (TR). Following immunostaining, the samples were stained with DAPI by incubating in a 300 nM solution in PBS-glycine for 5 min at room temperature. Imaging was performed using a Zeiss 710 multi-photon confocal microscope equipped with a Zeiss Axio Observer Z1 microscope and 63X objective (NA = 1.4).

Image processing and quantification were performed using iVision (BioVision Technologies). Background was removed by subtracting the average intensity of a blank region of interest from the intensity value of each pixel in the image. Thresholding to increase contrast in the images was linear, and restricted to between the largest and smallest intensity values within the image.

Calculation of the RAG2 localized to the nucleus was performed by first using the DAPI image of the cell to draw a mask that defined the nucleus. The RAG2 that lay within the mask was assigned as the nuclear pool. Total RAG2 signal was determined by drawing a region of interest that included the entire cell. The fraction of RAG2 that was nuclear (Fraction Nuclear) was calculated using the ratio of total RAG2 fluorescence signal within the nuclear mask divided by the total fluorescence signal of RAG2 in the cell. By using total fluorescence signal in each compartment for this calculation, we eliminate the contribution of nuclear and cell morphology on this parameter, since change in morphology will affect average fluorescence intensity rather than total fluorescence intensity in the respective compartments.

Centrosome localization was quantified using the image from γ -tubulin staining to define a mask that covered the centrosome. The relative enrichment of RAG2 at the centrosome was calculated using the average fluorescence signal of RAG2 within the centrosome mask divided by the average RAG2 signal in an adjacent region of interest of equal size as the mask within the cytoplasm.

2.7. Statistical Analysis

Analysis was performed using Prism 5 software (GraphPad Software, La Jolla, CA). Means and SDs are indicated by lines and error bars, respectively, in the plots, and were calculated using data accumulated from three or more independent trials, each trial consisting of measurement of approximately 50 separate cells. ***, $p < 0.001$, **, $p < 0.01$, and *, $p < 0.05$, ns, $p \geq 0.05$ determined by One-way ANOVA, using a Dunnett's test to determine significance relative to the control sample in each experiment. For experiments that consisted of only two separate conditions, analysis was by a two-tailed Student's t-test.

3. Results and Discussion

3.1. Nuclear export of RAG2 in pre-B cell lines by genotoxic stress

To determine the effect of genotoxic stress on RAG2 localization, the Abelson-transformed (v-abl) pre-B cell line, A70, was cultured overnight in media containing 3 μ M STI-571. These conditions lead to cell cycle arrest in G1, and induction of endogenous RAG1 and RAG2 expression (19). Following RAG expression, A70 cells were exposed to either 4 Gy IR, or the topoisomerase II inhibitor etoposide. Each of the treatments was followed by fixation and immunostaining, and the labeled RAG2 was measured using laser scanning confocal microscopy. Consistent with findings from previous studies (23–25), RAG2 localized in the nucleus, which we identified by staining cells with DAPI (Figure 1A and Supplemental Figure 2A).

Interestingly, genotoxic stress that formed DSBs, shown by staining with antibody to γ H2AX, caused a significant reduction of the RAG2 in the nucleus, and an increase in cytoplasmic RAG2. These properties are evidenced in Figure 1A and Supplemental Figure 2A by decreased colocalization of RAG2 with DAPI-stained nuclei, and enrichment of RAG2 in the cytoplasm (white arrows). We quantified this effect by plotting the relative fluorescence intensity (Rel. Fluor. Inten.) values of RAG2 staining along a line across the cell, and these data accompany the respective imaging data. The plots show that cytoplasmic RAG2 intensity values were approximately 2–3 fold greater than the average RAG2 intensity in the nucleus in cells with DSBs. The finding that export of a fraction of nuclear RAG2 was sufficient to cause its enrichment in the cytoplasm may be related to the relative size of each compartment, since measurements of equatorial confocal images showed the area of the cytoplasm averaged one-third that of the nucleus.

The decrease in nuclear RAG2 following genotoxic stress was quantified in many cells by calculating the Fraction Nuclear (Section 2.6). These data, accumulated from measurement of multiple fields of cells over several independent trials, are plotted in Figure 1B. Our results show a significant reduction in nuclear RAG2 in populations of labeled cells using either irradiation or etoposide to generate DSBs. In separate measurements of nuclear area, we found no significant change in average size following etoposide treatment (Supplemental Figure 2B). Thus, the redistribution of RAG2 is not due to a change in the size of the nucleus.

A change in cellular localization following genotoxic stress was also evidenced in v-abl RAG2^{-/-} pro-B cells expressing GFP-labeled full length (FL) RAG2 (GFP-RAG2 FL) (Figure 2). Treatment with either irradiation (Figure 2A through C), or acetaldehyde (Figure 2D), a metabolic genotoxic stressor (26,27), decreased nuclear GFP-RAG2 FL, and caused its enrichment in the cytoplasm (white arrow). Nuclear depletion of GFP-RAG2 FL also occurred in cells that were not grown in STI-571 before treatment (Figure 2C and D), suggesting that RAG1 expression was not necessary for changes in nuclear pools of RAG2. Significantly, leptomycin B blocked an etoposide-mediated decrease in nuclear GFP-RAG2 FL (Figure 2E), indicating that the reduction in nuclear RAG2 following genotoxic stress is an exportin 1-dependent nuclear export process (28).

We also measured the effect of genotoxic stress on cellular RAG2 pools using subcellular fractionation. This approach also showed that etoposide decreased the amount of RAG2 in the nuclear fraction, and increased the amount in the cytoplasm so that it was now detectable by TCA precipitation of cytosol (Figure 1C). Significantly, measuring the RAG2 in whole cell lysates (WCL) showed no change in the amount of total RAG2 by treatment, indicating that the decrease in nuclear pools of RAG2 was not due to protein degradation (Figure 1C). Consistent with this interpretation, a decrease in nuclear RAG2 levels following genotoxic stress was observed in samples pre-treated with the proteasome inhibitor MG-132 (Supplemental Figure 2C). Moreover, the enrichment of RAG2 in the cytoplasm was not due to accumulation of newly synthesized RAG2, as total RAG2 was unchanged when cells were pre-treated with cycloheximide (Supplemental Figure 2D). A decrease in the nuclear pool of RAG2 by IR similar to that in A70 cells was also observed in primary pre-B cells (Supplemental Figure 2F), which were cultured and subsequently withdrawn from IL-7 as previously described (29). These data indicate that our results with v-abl pre-B cells showing nuclear export of RAG2 following genotoxic stress are representative of events in primary pre-B cells. Future studies will be necessary to show whether genotoxic-dependent RAG2 localization properties in primary cells occurs regardless of cell lineage (pre-B versus pre-T cells), or the origin of the cells (mouse versus human).

Collectively, the reduction of labeled RAG2 in the nucleus coinciding with its enrichment in the cytoplasm in a leptomycin B-sensitive manner, together with the lack of effect by MG-132 and cycloheximide, show that a rapid export of RAG2 occurs within 30 min of DSBs generated by separate genotoxic stressors. Furthermore, the similar amounts of RAG2 export following the separate genotoxic stresses suggests that either similar amounts of DSBs were generated in each of the conditions, or that RAG2 export becomes saturated and does not increase with increased numbers of DSBs. To discriminate these separate interpretations, we measured DSBs by flow cytometry of γ -H2AX staining. This showed a similar amount of DNA DSBs generated by IR and acetaldehyde, and approximately 4-times greater DSBs by treatment with etoposide (Supplemental Figure 3). The similar levels of RAG2 nuclear export despite the significantly greater number of DNA breaks in the etoposide-treated cells suggests that RAG2 export becomes saturated at a DSB frequency less than what was produced in our experiments.

3.2. Nuclear depletion of RAG2 is transient

To determine if the nuclear RAG2 loss by genotoxic stress was recovered following DNA repair, we compared cells that were fixed 4 hr post-IR with those fixed 30 min post-IR. Confocal imaging of RAG2 in A70 cells grown in STI-571 showed a significant export of RAG2 at 30 min post-IR, such as evidenced in Figure 1, yet there was no significant difference in the RAG2 nuclear content between the 4 hr post-IR and untreated control samples (Figure 3A and B). Similarly, subcellular fractionation showed that RAG2 in the nuclear fraction recovered at 4 hr, and was significantly greater than the RAG2 in the nuclear fraction at 30 min post-IR (Figure 3C). Imaging cells at 4 hr post-treatment showed minimal staining with antibody to γ H2AX (Figure 3A), indicating that sufficient time had elapsed to allow repair of most DSBs. Thus, the re-establishment of pre-DNA damage

RAG2 localization patterns, either through import of newly synthesized RAG2 and/or the reimport of previously exported RAG2 protein, coincided temporally with DNA repair.

3.3. Nuclear export of RAG2 is suppressed by inhibitors of the DNA Damage Response

DNA damage activates the DDR system. In G1, which is when the RAG proteins are expressed, ATM and DNA-PK mediate overlapping and redundant roles in DDR (30). To determine if DDR-associated signals affect RAG2 export following genotoxic stress, we measured the nuclear localization of RAG2 in A70 cells first grown in STI-571, then treated with the ATM inhibitor KU-55933, the DNA-PK inhibitor NU7026, or both, prior to generation of DSBs using etoposide. Measurement of multiple fields of immunostained cells showed that inhibition of either ATM or DNA-PK alone or together blocked export of RAG2, evidenced by a reduced change in the nuclear fraction of RAG2 by etoposide (Figure 4A). Similarly, subcellular fractionation showed that co-treating cells with both KU-55933 and NU7026 effectively blocked RAG2 export when DSBs were generated by IR (Figure 4B and C). Consistent with these results, RAG2 export was also inhibited in irradiated *ATM*^{-/-} pre B cells (Figure 4D and E). Altogether, these results are consistent with the notion that DDR repair pathways contribute to the regulation of RAG2 export following DNA damage. Future studies will be important to determine the specific pathways involving ATM and DNA-PK for RAG2 export.

3.4. Determinants within RAG2 required for nuclear export

The minimal region of RAG2 necessary to support V(D)J recombination is referred to as the core region. To determine whether either the core or non-core regions of RAG2 govern its export by genotoxic stress, we measured v-abl *RAG2*^{-/-} pro-B cells that expressed GFP-labeled core RAG2 (GFP-RAG2 Core). Measuring cells using confocal microscopy, we observed that irradiation produced a significant export of labeled GFP-RAG2 Core, and the export was similar in magnitude to that of GFP-RAG2 FL (Figure 5A and B). We also measured a GFP-labeled FL RAG2 construct containing a T490A mutation (GFP-RAG2 T490A), since Thr⁴⁹⁰ is phosphorylated to regulate RAG2 expression (14) and nuclear localization (16). Interestingly, although Thr⁴⁹⁰ lies outside the core region, the T490A mutation resulted in a complete inhibition of RAG2 export by genotoxic stress (Figure 5A and B). These trends were not affected by inducing expression of RAG1 with STI-571 (Supplemental Figure 4). Furthermore, inhibition of export by the T490A mutation was constitutive, since untreated cells showed significantly greater nuclear localization than either GFP-RAG2 FL or GFP-RAG2 Core (Figure 5B). The inhibition of export evidenced with the T490A mutation may occur by stabilization of associations between RAG2 and protein and/or nuclear complexes that also occur in wild type protein, but are less stable. This may occur through interactions between the RAG2 PHD finger that is proximal to Thr⁴⁹⁰, and modified histones (31). Further study is necessary to identify the mechanism by which Thr⁴⁹⁰ inhibits RAG2 export, and whether this is related to the proximal PHD finger.

3.5. Genotoxic stress causes RAG2 targeting to the centrosome

Nuclear proteins that function in DDR enrich in the centrosome following their export to the cytoplasm (32,33). Similarly, we noted in some images collected using confocal microscopy

that the labeled RAG2 enriched in a bright spot within the cytoplasm that was proximal to the nucleus. To determine if the exported RAG2 was targeted to the centrosome, we measured RAG2 in irradiated STI-571-treated A70 cells. Using γ -tubulin staining as a marker to identify the centrosome, we observed a frequent and significant enrichment of RAG2 at the centrosome (Figure 6A). Similar to the endogenous RAG2, GFP-RAG2 FL also enriched at centrosomes following genotoxic stress (Figure 6B).

We quantified the relative RAG2 enrichment (Rel. RAG2 Enrich.) at centrosomes using the ratio of RAG2 signal that colocalized with γ -tubulin divided by the RAG2 signal in an adjacent area of equal size. Our results are plotted in Figure 6C and D, where a value of 1.0 represents no enrichment, < 1.0 is depletion at the centrosome, and > 1.0 is enrichment. These data show that genotoxic stress caused an average 2–3-fold enrichment of RAG2 and GFP-RAG2 FL at centrosomes. Although the centrosome labeling by RAG2 and GFP-RAG2 FL significantly increased with DSBs, these values were conservative estimates. Specifically, we measured RAG2 and GFP-RAG2 FL labeling at centrosomes relative to the amount of each protein in the cytoplasm, which is also enriched with the RAG2 proteins in these conditions. We note the reduced amount of centrosome enrichment by endogenous RAG2 relative to GFP-RAG2 FL, and this may be due properties of the antibody used for detection, or effects of the fixation on antibody recognition of RAG2. Finally, future studies will be important to show whether RAG2 targeting to the centrosome becomes saturated, thus limiting the amount of protein that can associate with this structure.

Measurements in separate conditions showed that the centrosome enrichment was sensitive to inhibitors of DDR (Figure 6C), with the most robust inhibition occurring in cells that were co-treated with inhibitors to both ATM and DNA-PK. Interestingly, centrosome enrichment was abolished by removing the non-core region of RAG2 (Figure 6D), indicating this region contains a signal necessary for RAG2 targeting to the centrosome once export from the nucleus occurs.

4. Conclusions

Based on the combined results in this study, we propose that DSBs formed as a consequence of genotoxic stressors activates a DDR-regulated export, and subsequent centrosome-targeting, of nuclear RAG2 (Figure 6E). Importantly, our results using cellular imaging were obtained with both endogenously expressed RAG2 protein and GFP-labeled RAG2 expressed in RAG2-deficient pro-B cells. Thus, RAG2 export and centrosome targeting was not restricted to one method of protein labeling.

One interpretation of our results is that nuclear export of RAG2 functions to regulate V(D)J recombination activity within the nucleus. However, given that only a modest fraction of RAG2 undergoes export in these conditions, it is our opinion that this interpretation is incorrect. Rather we suggest the increase in cytoplasmic RAG2, accompanied by its targeting to the centrosome, coordinates the centrosome cycle with arrest of the cell cycle until DNA repair has been completed. Centrosomal localization of RAG2, possibly in association with other nuclear proteins, may signal ongoing V(D)J recombination in the nucleus, and in turn modulate both the centrosome cycle and the cell cycle until DNA repair

has occurred and RAG2 reassumes pre-DNA damage localization. Significantly, roles in modulating centrosome duplication have been noted for other nucleic acid-interacting proteins, including BRCA1 (34), Chk1 (35), and Che-1 (36). While we favor the notion that RAG2 functions as an intermediate in coordinating DNA repair with cell cycle control, further study is needed to distinguish between this interpretation and the possibility that export functions to control V(D)J recombination activity, such as by selective export of only a recombination-active form of RAG2.

Additional questions include the nature of the molecular events that lead to RAG2 nuclear export and centrosome association, the function of RAG2 at the centrosome, and if there is a role for RAG1 in this process. Altogether, DDR-induced nuclear export and centrosome association of RAG2 is an entirely novel model for regulation of V(D)J recombination upon genotoxic stress, and addresses fundamental questions regarding the needed balance between lymphoid development and maintenance of genomic stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations List

DDR	DNA damage response
DSBs	double strand breaks
GFP	green fluorescent protein
FL	full length
IR	ionizing radiation

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Highlights

- Genotoxic stress triggers nuclear export and centrosome targeting of RAG2.
- Inhibitors to DNA damage response factors blocked redistribution of RAG2.
- Following DNA repair, RAG2 relocates to its pre-DNA damage distribution pattern.
- The core region of RAG2 undergoes nuclear export, but not centrosome targeting.
- The RAG2 T490A mutation blocked nuclear export of full length RAG2.

Figure 1. Export of RAG2 from the nucleus following DSB generation

(A) Confocal images of STI-571⁺ A70 cells that were either untreated (Control) or irradiated (+ 4 Gy). γ H2AX and RAG2 staining were detected using secondary antibodies conjugated with Alexa488 and TR, respectively. The merge images were generated by combining Alexa488, TR, and DAPI channels. The plots represent the intensity values in the red (RAG2) and blue (DAPI) channels along the dashed lines in the merge images. The shaded box represents the region of the cytoplasm indicated by the arrows in the accompanying images. (B) Fraction Nuclear of RAG2 in STI-571⁺ A70 cells. Mean values were 0.75, 0.62, and 0.63 for untreated (U/T), IR (+ 4 Gy), and etoposide (Etop)-treated samples, respectively. Fraction Nuclear was calculated as described (Section 2.6). The blue lines and red error bars represent the mean and SD of each population, respectively. Statistical analysis was performed as described in Section 2.7 using data collected from 3 independent trials. (C) Immunoblots of RAG2 in the nuclear (*left*) and cytoplasmic (*middle*) fractions, and whole cell lysate (*right*), each prepared from STI-571⁺ A70 cells with the indicated treatments. The SP1 and actin blots were used to control for loading. SP1 was not detected in cytoplasmic fractions (not shown).

Figure 2. Export of GFP-labeled RAG2 by genotoxic stress

(A) Confocal images of untreated and irradiated STI-571⁺ RAG2^{-/-} cells stably expressing GFP-RAG2 FL. (B through D) Fraction Nuclear of GFP-RAG2 FL expressed in RAG2^{-/-} cells. DSBs were generated as indicated. The cells in (B) were pre-treated with STI-571 to induce RAG1 expression. Mean values in (B) were 0.90 and 0.77 for untreated and irradiated samples, respectively; in (C), 0.75 and 0.68; and in (D), 0.82 and 0.69. The mean values were averaged from 3 or more trials. (E) Nuclear Fraction measurements of GFP-RAG2 FL in RAG2^{-/-} cells treated with etoposide in the presence or absence of Leptomycin B (Lepto. B). Mean values were 0.78 (no etoposide, no leptomycin B), 0.65 (etoposide-treated, no leptomycin B), 0.76 (no etoposide, leptomycin B-treated), and 0.76 (etoposide-treated, leptomycin B-treated).

Figure 3. RAG2 reaccumulates in the nucleus upon DNA repair

(A) Confocal images of control (*top*) and irradiated (*middle, bottom*) STI-571⁺ A70 cells that were fixed either 0.5 or 4 hr following irradiation. The cells were maintained at 37° C in complete media following irradiation and prior to fixation. In (B) is the Fraction Nuclear of RAG2 measured in each of the conditions. Mean values were 0.76 (control), 0.58 (+ IR, 0.5 h), and 0.72 (+ IR, 4 h) (C) RAG2 immunoblots of the nuclear fraction of STI-571⁺ A70 cells treated with the indicated conditions.

Figure 4. RAG2 export following genotoxic stress is sensitive to inhibition of ATM
(A) The Nuclear Fraction of RAG2 measured in STI-571⁺ A70 cells treated with the indicated conditions. Mean values were 0.75, 0.66, 0.72, 0.70, 0.73 for control, etoposide-treated, and etoposide following pretreatment with KU-55933, NU7026, or KU-55933 + NU7026, respectively. **(B)** RAG2 and SP1 immunoblots of the nuclear fraction of A70 cells treated with the indicated conditions before irradiation. The values represent the intensity of the RAG2 band in the nuclear fraction of irradiated cells divided by the RAG2 band in the respective untreated sample, each normalized to the respective SP1 signal. The mean and SD calculated from 3 independent trials are plotted in **(C)**. **(D)** RAG2 in the nuclear fraction of A70 and *ATM*^{-/-} pre-B cells in the indicated conditions. These data are quantified in **(E)**, where plotted is the ratio of RAG2 in the nuclear fraction of irradiated cells divided by that in control cells (% Decrease in Nucl. Fraction), each corrected for loading using SP-1.

Figure 5. Export of GFP-labeled RAG2 by genotoxic stress requires Thr⁴⁹⁰

(A) Confocal images of GFP-RAG2 FL, Core, or T490A, expressed in *RAG2*^{-/-} pro-B cells and co-stained with DAPI. Samples were either untreated or irradiated before fixation and DAPI staining. Fraction Nuclear values measured for the respective samples are plotted in (B). Mean values were 0.76 and 0.64 (FL), 0.74 and 0.68 (core), and 0.90 and 0.90 (T490A), for untreated and irradiated samples respectively.

Figure 6. Cytosolic RAG2 enriches at the centrosome

Confocal images measuring RAG2 in STI-571⁺ A70 cells (**A**), and GFP-RAG2 FL expressed in RAG2^{-/-} cells (**B**). The samples were treated with either etoposide (**A**) or acetaldehyde (**B**). The arrows indicate areas of labeled RAG2 enrichment that co-localize with the γ -tubulin staining. (**C and D**) Relative enrichment values measured for RAG2 in STI-571⁺ A70 cells (**C**), and GFP-RAG2 FL and GFP-RAG2 Core in RAG2^{-/-} cells (**D**) at centrosomes labeled with antibody to γ -tubulin (Rel. RAG2 Enrich. at Centrosome). Centrosomal enrichment of RAG2 was measured as described in Section 2.6. The samples received the indicated treatments and genotoxic stresses immediately prior to fixation and staining. In (**C**), mean values are 1.3, 1.8, 1.6, 1.5, and 1.4 for untreated cells, cells with etoposide alone, and cells treated with etoposide following pre-treatment with KU-55933, NU7026, or KU-55933 + NU7026, respectively. In (**D**), mean values are 1.3, 3.0, and 1.3

untreated and treated GFP-RAG2 FL, and treated GFP-RAG2 Core, respectively. **(E)** Model for DNA damage-triggered relocalization of full length RAG2. Upon detection of DNA damage, full length RAG2, consisting of core and non-core (nc) regions, is exported from the nucleus in an ATM dependent and exportin 1-dependent mechanism. Upon export to the cytoplasm, full length RAG2 is targeted to the centrosome through its nc region.