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DNA-dependent Protein Kinase Mediates V(D)J Recombination via RAG2 Phosphorylation

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V(D)J recombination, a site-specific gene rearrangement process occurring during the lymphocyte development, begins with DNA double strand breaks by two recombination activating gene products (RAG1/2) and finishes with the repair process by several proteins including DNA-dependent protein kinase (DNA-PK). In this report, we found that RAG2 was specifically phosphorylated by DNA-PK at the 365th serine residue, and this phosphorylated RAG2 affected the V(D)J recombination activity in cells in the GFP expression-based assay. While the V(D)J recombination activity between wild-type RAG2 and mutant S365A RAG2 in the assay using a signal joint substrate was undistinguishable in DNA-PK deficient cells (M059J), the activity with wild-type RAG2 was largely increased in DNA-PK proficient cells (M059K) in comparison with mutant RAG2, suggesting that RAG2 phosphorylation by DNA-PK plays a crucial role in the signal joint formation during V(D)J recombination.

Keywords: DNA-dependent protein kinase, Protein phosphorylation, Recombination activating gene 2, V(D)J recombination

Introduction

DNA-dependent protein kinase (DNA-PK), a member of phosphatidylinositol-3-kinase (PI-3-kinase) family, responds to the DNA double strand breaks induced by DNA damage reagents such as UV, IR or reactive oxygen species (Poltoratsky *et al*., 1995; Rahman, 2003). In particular, DNA-PK plays a crucial role in the repair responses to DNA breaks derived from V(D)J recombination, a cellular process for providing the diversity of antigen receptors during the lymphocyte development (Blunt *et al*., 1995; Jackson and Jeggo, 1995). Thus, loss of DNA-PK function causes severe combined immunodeficiency (SCID) phenotypes and radio-sensitivity (Peterson *et al*., 1995).

DNA-PK consists of three components. DNA-PK catalytic subunit (DNA-PKcs) is a large 350 kDa polypeptide that possesses a kinase activity. DNA-PK requires DNA molecules to activate its kinase activity, and its activity is greatly enhanced by two DNA end binding proteins: Ku heterodimer of 70 kDa (Ku70) and 80 kDa (Ku80) (Giffin *et al*., 1996; Nussenzweig *et al*., 1996; Gu *et al*., 1997). Upon induction of DNA double strand breaks, Ku heterodimer quickly recognizes free DNA ends to prevent DNA loss from nucleases and recruits DNA-PKcs for further processing. DNA-PK has been known to phosphorylate a serine or threonine residue that immediately followed by a glutamine, so-called "S/Q or T/Q motif". Many DNA damage-related proteins such as Ku heterodimer, XRCC4 and Artemis can be phosphorylated by DNA-PK (Leber *et al*, 1998; Chan *et al*., 1999; Ma *et al*., 2002). In addition, several cell cycle check point proteins and apoptotic proteins, including p53, CHK1 and CHK2, are controlled by DNA-PK through their phosphorylation (Lees-Miller *et al*., 1992; Goudelock *et al*., 2003; Li and Stern, 2005).

During the V(D)J recombination process, DNA is specifically cleaved by two recombination activating gene products (RAG1/RAG2), and the broken DNA ends are processed and sealed by the non-homologous end-joining (NHEJ) system (Kim *et al*., 2000). DNA-PK, one of the NHEJ components, has been suggested that it is specifically involved in the V(D)J recombination (Blunt *et al*., 1995; Jackson and Jeggo, 1995). The lymphocyte development in *scid* mice possessing the defective DNA-PK activity failed because of unsuccessful

Abbreviations: DNA-PK, DNA-dependent protein kinase; RAG, recombination activating gene; RSS, recombination signal sequence; GFP, green fluorescence protein; MBP, maltose binding protein

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V(D)J recombination. In this mouse, the intermediate hairpin products generated from the V(D)J cleavage were accumulated, suggesting that DNA-PK might be involved in the hairpin process (Peterson *et al*., 1995). Recently, DNA-PK also phosphorylates Artemis nuclease. This phosphorylation switched its catalytic property from exonuclease to endonuclease that is necessary for processing hairpins (Ma *et al*., 2002). On the contrary, some other work suggested that joint formation at the signal ends was also impaired in the DNA-PKcs defective cells (Errami *et al*., 1998; Fukumura *et al*., 1998). Therefore, the exact role of DNA-PK in V(D)J recombination still remains unclear.

Here we report that DNA-PK can phosphorylate RAG2 at the 365th serine residue and modulate V(D)J recombination activity in the cell-based assay using signal or coding joint substrates containing a green fluorescence protein (GFP) gene.

Materials and Methods

Materials. Core RAG2 proteins were obtained from purification previously described (Kim and Oettinger, 1998). DNA-dependent protein kinase (DNA-PK) catalytic subunit and Ku heterodimer (Ku70/Ku80) were purified from human placenta as described (Chan *et al*., 1996). Two cell lines M059J (DNA-PK deficient) and M059K (DNA-PK proficient) were purchased from American type culture collection (ATCC).

Bacterial RAG2-MBP-expressing constructs and protein purification. RAG2 gene (1-438 amino acids) was isolated from the plasmid pDRK541 by the digestion with two *Nco*I an *Sal*I restriction enzymes and subcloned into the MBP-fusion plasmid (pDRK573) cut with the same enzymes to construct a bacterial expression vector pDRK624 producing MBP-fused wild-type RAG2 proteins tagged by eight histidines at the carboxyl terminus. RAG2 mutant constructs (S365A, T165A, T264A, and S365A/T264A) were produced from the plasmid pDRK624 by site-directed mutagenesis using a pair of oligonucleotide primer as previously described (Kim *et al*., 1999). All constructs were confirmed by DNA sequencing.

MBP-fused RAG2 proteins were basically purified by the method described before (Kim *et al*., 1999; Kim, 2003). In brief, *E. coli* DH5 containing each RAG2 expressing-construct was cultured in 1L LB broth with 100 μg/ml ampicillin at 37°C until OD_{600} reached about 0.7 and induced by the addition of IPTG (1 mM final). Cells were further incubated for 3 h in the presence of 50 μg/ml ampicillin. After lysis by sonication, RAG2 proteins were purified by Ni-NTA agarose chromatography. Each protein was stored at −70°C after dialysis in the buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 2 mM DTT).

***In vitro* DNA-PK kinase assay.** *In vitro* RAG2 phosphorylation by DNA-PK was initiated by incubation with 100 ng DNA-PK catalytic subunit (DNA-PKcs), 50 ng Ku 70/80, 100 ng RAG2 protein in the 20 μl reaction buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM EGTA and 51 mM KCl) containing 1.66 pmole of ^{32}P-ATP and 10 ng calf thymus DNA. The reaction mix was incubated at 37°C for 30 min and stopped by addition with the same volume of 2X SDS sample buffer. Reaction samples were analyzed on a 12.5% SDS-polyacrylamide gel unless indicated. The phosphorylated proteins were visualized by autoradigraphy.

Construction of V(D)J recombination substrates. For the measurement of V(D)J recombination activity in cells, the new substrates using EGFP expressing system have been constructed. pEGFP-N1 vector (Clonetech) was modified by inserting a recombination signal sequence (RSS) isolated from pDVG42 plasmid (*Sal*I-*BamH*I fragment; 12RSS (▶)-*Xho*I-1kb DNA spacer-*Mlu*I-23 RSS (◀)) between CMV promoter and GFP-coding region at the *Xho*I and *BamH*I, resulting in non-digestive *Xho*I at the pEGFP-N1 plasmid. This resulting plasmid was further modified by inserting a DNA fragment containing a SV40 polyA site derived from pEGFP-C1 between 12 RSS and 23 RSS at *Xho*I and *Mlu*I sites. This results in the production of a coding joint substrate pEGFP-CJ. The signal joint substrate pEGFP-SJ was made by a sequential replacement of pEGFP-CJ with two inverted signal sequences, each of which was annealed from two synthetic oligonucleotides: *Bgl*II-12RSS (◀)-*Xho*I and *Mlu*I-23RSS (▶)-*Age*I.

***In vivo* V(D)J recombination assay.** RAG1 (5 μg) and RAG2 (7.6 μg)-expressing vectors together with V(D)J recombination substrate plasmid (either 5 μg pEGFP-CJ or pEGFP-SJ) were transfected into 70% confluent DNA-PK deficient cells (M059J) or DNA-PK proficient cells (M059K) cultured in Dulbecco modified Eagle medium/F-12 (1 : 1) supplemented with 10% fetal bovine serum using lipofectamine. After 48 h incubation at 37°C and 5% CO_2, expression of EGFP in the cell for V(D)J recombination activity was assessed by measuring fluorescence (488 nm excitation and 530/30 bandpass filter) using flow cytometer (FACScalibur, BD Bioscience). RAG2 wild type and mutants used for this experiment were constructed by insertion of MBP-RAG2 fragments isolated from bacterial expression plasmids into a mammalian expression vector pCEP4 at the *BamH*I site. The orientation of RAG2 fragment was confirmed by restriction enzyme digestion.

Results and Discussion

RAG2 was phosphorylated by DNA-PKcs *in vitro*. DNA-PK, serine/threonine kinase, is a essential component for the V(D)J recombination process. Despite its functional significance in V(D)J recombination, its exact role during the V(D)J recombination is largely under veil. Recently, it has been suggested that DNA-PK modulates the activity of Artemis endonuclease which is required for the processing of hairpin structures produced during the V(D)J recombination (Ma *et al*., 2002). In this report, we investigated whether DNA-PK can directly phosphorylate and regulate the activity of RAG proteins, crucial initiators for the V(D)J recombination using *in vitro* phosphorylation of purified proteins and V(D)J recombination analysis in cells. The catalytically active RAG2 protein purified from mammalian cells using a recombinant

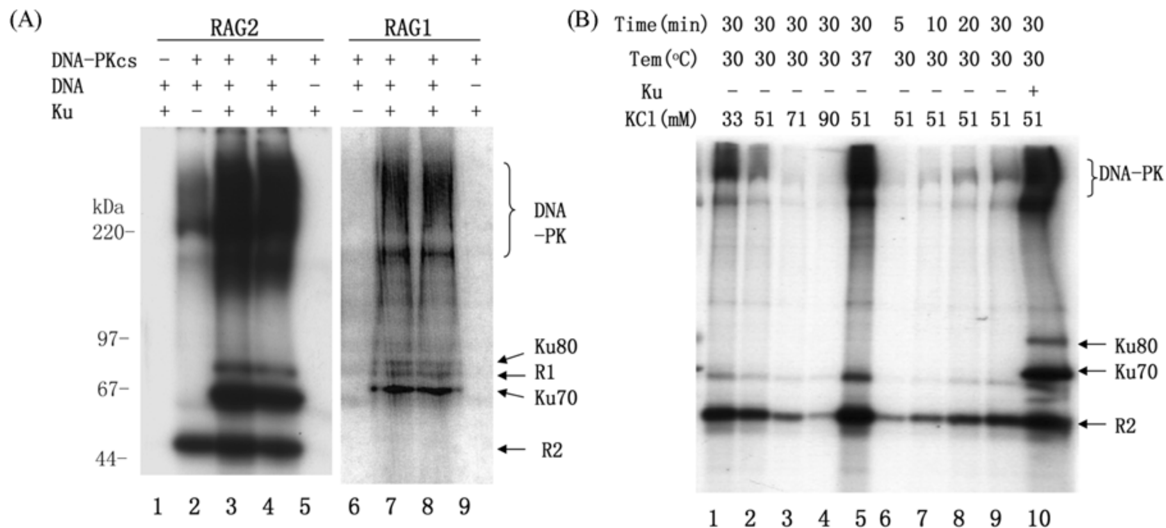

Fig. 1. RAG2 phosphorylation by DNA-PK. (A) *In vitro* kinase assay. RAG2 used for this kinase assay was purified from mammalian cells, and RAG1 was purified using the recombinant baculoviral expression system as described (Kim and Oettinger, 1998). The assay was carried out at 30°C, 30 min as described in "Materials and Methods". ^{32}P-phosphorylated proteins were separated on a 10% SDS-PAGE and visualized by autoradiography. DNA-PKcs and Ku represent DNA-PK catalytic subunit and Ku heterodimer (Ku70/Ku80), respectively. R1 and R2 indicate the phosphorylated RAG1 and RAG2, respectively. (B) Parameter variation in the kinase reaction. The kinase reaction was performed at the same condition as described previously except indicated terms, and proteins were analyzed on a 12.5% SDS-PAGE.

vaccinia viral system as previously described (Kim *et al*., 1999) was heavily phosphorylated by purified DNA-PK proteins in the presence of DNA (Fig. 1A, lanes 2-4). However, RAG1 was nearly not phosphorylated by DNA-PK in this assay (see Fig. 1A, lanes 7-8). Even in the absence of Ku proteins, RAG2 protein was phosphorylated well by DNA-PKcs if DNA was present in the reaction (Fig. 1A, lane 2). DNA-PKcs itself become autophosphorylation and also phosphorylated two auxiliary Ku proteins (Ku 70, Ku80). These two Ku proteins have already been known to be phosphorylated by DNA-PKcs (Chan *et al*., 1999). The extent of RAG2 phosphorylation by DNA-PK was very similar to that of Ku 70 (Fig. 1A, lanes 3-4). In the absence of DNA, DNA-PK was not activated, so it failed to phosphorylate not only Ku proteins but also RAG2 (Fig. 1A, lane 5 and 9).

We also investigated how KCl concentration, temperature and incubation time affect the RAG2 phosphorylation by DNA-PKcs in the absence of Ku heterodimer. KCl caused a gradual decrease of RAG2 phosphorylation in a concentration-dependent manner, suggesting that DNA-PK-dependent phosphorylation reaction is very sensitive to the concentration of KCl *in vitro* (Fig. 1B). Even, the presence of 90 mM KCl nearly blocked all RAG2 phosphorylation. Therefore, we used 51 mM KCl for the DNA-PK phosphorylation reaction at all later assays. Also, RAG2 phosphorylation was proportionally increased with respect to the incubation time, and reaction temperature largely affected *in vitro* RAG2 phosphorylation by DNA-PK (Fig. 1B, compare lane 5 and 9). In conclusion, RAG2, a key component of V(D)J recombination, is a good substrate for DNA-PK. And, this DNA-PK-dependent RAG2 phosphorylation might modulate the V(D)J recombination activity in cells.

Mapping of DNA-PK phosphorylation sites on RAG2. Generally, DNA-PK, a member of phosphoinositol-3 kinase (PI-3 kinase) family, can recognize a serine or threonine that places at the right front of a glutamine residue (SQ or TQ motif) and phosphorylate it. Here we scrutinized SQ/TQ motifs on the RAG2 amino acid sequences and found three motifs (165TQ, 264TQ and 365SQ). 165TQ was found only in the mouse and rat *rag*2 gene, two other motifs were conserved in all species including xenopus or chicken (Fig. 2A). These three serine or threonine residues were mutated to the alanine residue to search possible DNA-PK phosphorylation sites on RAG2. All MBP-fused RAG2 proteins: wild type or mutants were over-expressed in bacterial cells and purified to the near homogeneity. These purified RAG2 proteins were subjected to *in vitro* DNA-PK phosphorylation analysis. The threonine residue at the 165th of RAG2 was unlikely a target for DNA-PK. Its mutation to alanine did not cause decrease of the extent of phosphorylation by DNA-PK *in vitro* (Fig. 2B). However, the mutation at the 365th serine residue (S365A) greatly reduced RAG2 phosphorylation by DNA-PK (Fig. 2B). DNA-PK autophosphorylation and Ku 70 phosphorylation seemed to not be affected in these all assays. Since these RAG2 proteins were fused to maltose binding protein (MBP), we tested whether MBP itself can be phosphorylated by DNA-PK. As shown in Fig. 2B (lanes 10-12), MBP was not a target protein of DNA-PK, suggesting that MBP does not affect overall RAG2 phosphorylation by DNA-PK. Surprisingly,

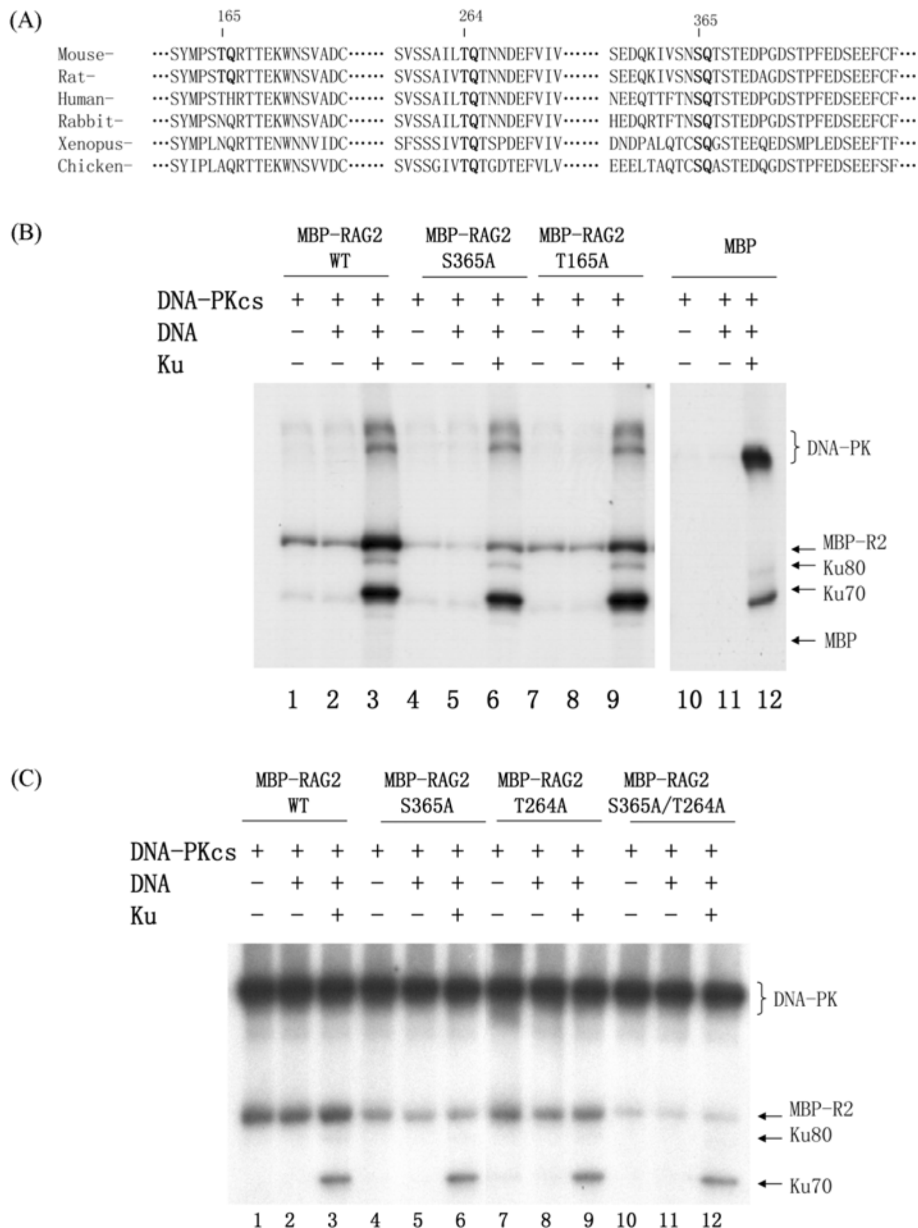

Fig. 2. Mutation analysis of RAG2. (A) Alignment of RAG2 amino acid sequences. Amino acid sequences of RAG2 derived from different species were aligned using a program (DNAstar). Amino acid number is based on the sequence of mouse RAG2. The candidates for DNA-PK phosphorylation were highlighted with bold. (B), (C) *In vitro* kinase assay of mutant RAG2. *In vitro* kinase assay was carried out as described in "Materials and Methods". All RAG2 proteins used here are fused to maltose binding protein (MBP).

DNA-PK catalytic subunit showed its intrinsic kinase activity even in the absence of DNA and Ku heterodimer (Fig. 2B. see lanes 1, 4, 7). This might be due to some DNA contamination during the MBP-RAG2 purification from bacterial cells. We purified MBP-RAG2 by one-step Ni^{2+} chromatography. In addition, when we used RAG2 proteins purified from mammalian cells using two-step affinity chromatography: Ni^{2+}-column and anti-flag antibody column, we did not observe any RAG2 phosphorylation in the absence of DNA (see Fig. 1A, lane 5 and 9).

We also tested the 264th threonine residue for DNA-PK-dependent phosphorylation. The RAG2 protein mutated at this residue was not greatly phosphorylated by DNA-PK, but the extent of its phosphorylation showed some decrease in comparison with that of wild type RAG2 (Fig. 2C, lanes 7-9). Furthermore, The RAG2 protein which has been mutated at both 365th serine and 265th threonine residues revealed more severe defect on phosphorylation by DNA-PK (Fig. 2C. lanes 10-12). In conclusion, the 365th serine residue of RAG2 is a major target site for DNA-PK-dependent phosphorylation.

Effect of RAG2 mutants on the V(D)J recombination activity *in vivo.* RAG2 is a key catalytic subunit together with RAG1 in V(D)J recombination, so absence or catalytic defect of RAG2 causes loss of the V(D)J recombination activity in cells or *in vitro* assay system (Kim *et al*., 1999). Using RAG2 mutant proteins that were defective in DNA-PK-dependent phosphorylation, we tested how these mutant RAG2 proteins affect the V(D)J recombination activity in cells. We developed new substrates for the V(D)J recombination analysis in cells. Two signal sequences (12 RSS and 23 RSS) were placed upstream region of a gene encoding green fluorescence protein (GFP) regulated by a CMV promoter, and they were separated by a SV40 poly A sequence (Fig. 3A). Therefore, GFP protein can not be expressed unless a poly A sequence is removed by the V(D)J recombination reaction. GFP expression in the DNA-PK-deficient M059J or DNA-PK-proficient M059K cells carrying on V(D)J recombination was analyzed in a flow cytometer.

V(D)J recombination activity by wild type RAG2 using a signal joint substrate (pEGFP-SJ) was undistinguishable from that by S365A mutant RAG2 in the DNA-PK-deficient M059J cell. Both wild type and mutant RAG proteins showed the background level of activity in the absence of DNA-PK activity. However, wild-type RAG2 showed the largely increased V(D)J recombination activity in the DNA-PK-proficient M059K cell rather than S365A mutant RAG2 (Fig. 3B), suggesting that phosphorylation by DNA-PK at the 365 serine residue of RAG2 has influence on the signal joint formation during V(D)J recombination in cells. According to several FACS analyses, V(D)J recombination activity was more about 18 ± 5% increased in the reaction with wild-type RAG2 than with mutant RAG2. Typically the recombination efficiency has been observed at the range of 1-10% in previous cell-based V(D)J recombination assays using substrate plasmids with double drug markers (Kim *et al*., 1999; Hiom and Gellert, 1998). In addition, we carried out the same V(D)J recombination analysis using a coding joint substrate (pEGFP-CJ), but we did not detect any significant difference of V(D)J recombination activity between wild-type and S365A mutant RAG2 in DNA-PK proficient M059K cells although the recombination activity by these two RAG2 proteins revealed the background level in the absence of DNA-PK activity (Fig. 3C). About 13 ± 3% recombination activity in both reactions using either wild-type or S365A mutant RAG2 in M059K cells was observed over the reactions with only coding joint substrate plasmid. These data suggest that although DNA-PK-dependent phosphorylation at the 365th serine residue of RAG2 protein is not required for coding joining formation, DNA-PK activity is absolutely necessary for completion of V(D)J recombination at coding ends. The mutation at 264 threonine to alanine (T264A) RAG2 did not affect the V(D)J recombination activity in these assays (data not shown).

Previously, DNA-PK has been suggested that it is involved in the processing of coding joints during V(D)J recombination. The *scid* mouse, defective DNA-PK activity, tends to accumulate

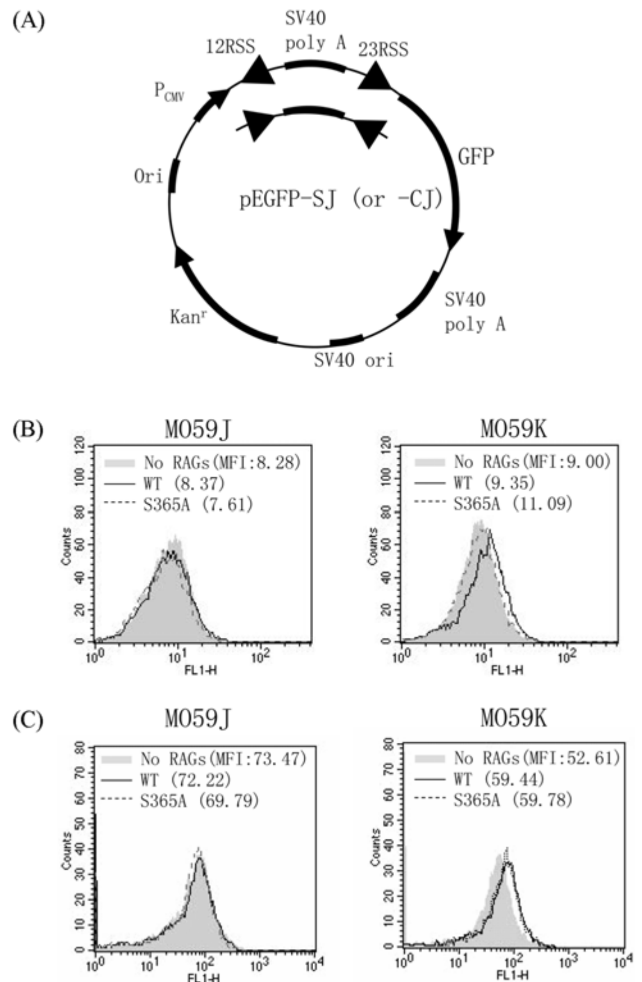

Fig. 3. V(D)J recombination assay in cells. (A) Recombination substrates. The detail of substrates for V(D)J recombination assay was described in "Materials and Methods". This substrate is for signal joint (SJ) formation. Inversion of two recombination signal sequences makes a coding joint (CJ) substrate as depicted inside. Triangles indicate either 12 RSS or 23 RSS. (B) Signal joint assay. The *rag*1, *rag*2 (wild type or S365A mutant) genes and substrate plasmid (pEGFP-SJ) were transfected into two different cell lines, and GFP expression was analyzed by FACS. The control with substrate only was indicated by grey color (SJ), and wild type and mutant *rag*2 genes were depicted by solid (WT) and dot line (S365A), respectively. FL1-H indicates fluorescence intensity. Numbers in parentheses represent mean fluorescence intensity (MFI) of each histogram. (C) Coding joint assay. The *rag*1, *rag*2 (wild-type or S365A mutant) genes and substrate plasmid (pEGFP-CJ) were transfected into two different cell lines, and GFP expression was analyzed by FACS. The grey area indicates the control experiment without *rag* genes (CJ), and the experiments with wild type *rag*2 gene (solid line) or S365A mutant *rag*2 gene (dot line) are represented.

coding hairpin products because of defective coding joint. In fact, activated DNA-PK can phosphorylate Arthemis nuclease and switch its exonuclease activity to endonuclease activity,

allowing hairpins to be processed during the V(D)J recombination reaction. In addition, in this study we showed that DNA-PK can phosphorylate RAG2 at the 365th serine residue and its mutant to alanine S365A is defective in the signal joint formation in the DNA-PK proficient cell, suggesting that RAG2 phosphorylation by DNA-PK at the 365th serine residue might also be involved in regulation of the joining process of signal ends during V(D)J recombination. In fact, some previous reports showed that DNA-PK activity is absolutely required for not only coding joint process but also signal joint in V(D)J recombination (Fukumura *et al*., 1998; Errami *et al*.,, 1998).

Since V(D)J recombination occurs in the developing lymphocytes, this process requires an accurate regulation to prevent loss of genomic DNA or malignant recombinant DNA formation from DNA double-strand breaks. Therefore, a tight protein complex formation with four broken DNA ends (two coding ends and two signal ends) throughout the process is essential. In fact, RAG1/2 proteins form a stable synaptic complex *in vitro* with two signal substrates (12 RSS and 23 RSS) in the presence of HMG1, a chromatin binding protein (Hiom and Gellert, 1998). In addition, these proteins can form a postcleavage synaptic complex with four pieces of DNA fragments (Agrawal and Schatz, 1997). That is, a whole process of V(D)J recombination including cleavage and joint at both signal and coding ends should be coordinately carried out in a large protein complex, presumably composed of at least RAG1/2 and DNA-PK, to keep genomic stability. In conclusion, DNA-PK is not exclusively involved in coding joint formation, rather it might be required for the V(D)J recombination.

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