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Defective Signal Joint Recombination in Fanconi Anemia Fibroblasts Reveals a Role for Rad50 in V(D)J Recombination

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V(D)J recombination of immunoglobulin loci is dependent on the immune cell-specific Rag1 and Rag2 proteins as well as a number of ubiquitously expressed cellular DNA repair proteins that catalyze non-homologous end-joining of DNA double-strand breaks. The evolutionarily conserved Rad50/Mre11/Nibrin protein complex has a role in DNA double-strand break-repair, suggesting that these proteins, too, may participate in V(D)J recombination. Recent findings demonstrating that Rad50 function is defective in cells from patients afflicted with Fanconi anemia provide a possible mechanistic explanation for previous findings that lymphoblasts derived from these patients exhibit subtle defects in V(D)J recombination of extrachromosomal plasmid molecules. Here, we describe a series of findings that provide convincing evidence for a role of the Rad50 protein complex in V(D)J recombination. We found that the fidelity of V(D)J signal joint recombination in fibroblasts from patients afflicted with Fanconi anemia was reduced by nearly tenfold, compared to that observed in fibroblasts from normal donors. Second, we observed that antibody-mediated inhibition of the Rad50, Mre11, or Nibrin proteins reduced the fidelity of signal joint recombination significantly in wild-type cells. The latter finding was somewhat unexpected, because signal joint rejoining in cells from patients with Nijmegen breakage syndrome, which results from mutations in the Nibrin gene, occurs with normal fidelity. However, introduction of anti-Nibrin antibodies into these cells reduced the fidelity of signal joint recombination dramatically. These data reveal for the first time a role for the Rad50 complex in V(D)J recombination, and demonstrate that the protein product of the disease-causing allele responsible for Nijmegen breakage syndrome encodes a protein with residual DNA double-strand break repair activity.

Keywords: V(D)J recombination; Fanconi anemia; Rad50; fibroblast; Nijmegen breakage syndrome

Introduction

V(D)J recombination is a selective break/rejoining process that cleaves and rejoins chromosomal DNA to create novel recombinant immunoglobulin and T-cell receptor genes. This developmentally regulated and cell-type specific pathway is responsible for the generation of immunoglobulin diversity in vertebrates.[1–3] The process is initiated by the products of the recombination activating genes 1 and 2 (Rag1, Rag2), which together form a site-specific recombinase that binds to recombination signal sequences (RSS) present in the immunoglobulin

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Abbreviations used: RSS, recombination signal sequence; NHEJ, non-homologous DNA end-joining; RMN, Rad50/Mre11/Nibrin; FA, Fanconi anemia.

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and T-cell receptor genes, and introduces DNA double-strand breaks into chromosomal DNA of pre-B and pre-T cells.[4] The Rag1/Rag2 complex generates a pair of DNA double-strand breaks in the chromosome, thereby generating four new DNA ends. Two of these consist of 5′ phosphate, 3′ hydroxyl blunt ends and are referred to as signal ends. The other two consist of sealed hairpins and are called coding ends. The hairpins are opened by a nuclease called Artemis[5] before modification and rejoining to reconstitute the immunoglobulin or T-cell receptor locus.[6]

A fascinating aspect of the V(D)J recombination reaction is its dependence on the combined action of an enzyme that is selectively expressed in pre-B and pre-T cells, i.e. the heterodimeric site-specific nuclease comprised of the Rag1 and Rag2 proteins, as well as on a host of ubiquitously expressed enzymes that catalyze the non-homologous DNA end-joining (NHEJ) pathway of DNA double-strand break-repair.[7] These latter enzymes include DNA ligase IV,[8] and its binding partner Xrcc4,[9] the DNA end-binding heterodimer of Ku70 and Ku86,[10,11] Artemis,[5] the catalytic subunit of the DNA-dependent protein kinase,[12] and a more recently identified component identified as XLF[13] or Cernunnos.[14]

The NHEJ end-joining pathway is evolutionarily conserved, and *Saccharomyces cerevisiae* possess homologues of DNA ligase IV,[15] Xrcc4,[16] Ku70[17] and Ku86.[18] Recent work provides evidence[19] that Cernunnos is a homologue of the yeast DNA end-joining protein Nej1.[20] Interestingly, however, NHEJ in yeast functions in the apparent absence of homologues of Artemis and the catalytic subunit of DNA-dependent protein kinase. In addition, NHEJ in yeast displays an absolute requirement for a heterotrimeric complex of the Rad50, Mre11 and Xrs2 proteins.[21]

Because the genes encoding Rad50,[22] Mre11,[23] and Nibrin,[24] the mammalian homologue of the yeast Xrs2 protein, are essential, it has proven difficult to address directly whether these proteins have a role in DNA repair in mammalian cells. However, several recent lines of evidence are consistent with the interpretation that they do. First, Donahue and Campbell showed recently that antibody-mediated inhibition of the Rad50/Mre11/Nibrin (RMN) protein complex rendered mammalian somatic cells hypersensitive to the cytotoxic effects of induced DNA damage, and inhibited extrachromosomal plasmid end-joining dramatically *in vivo*.[25] Second, it has been shown recently that conditional inactivation of the Nibrin gene in murine cells reduced chromosome stability and enhanced cellular sensitivity to ionizing radiation-induced cell death.[26,27] Third, biochemical evidence supports a role for the RMN protein complex in DNA end-joining *in vitro*. For example Zhong *et al*. showed that anti-Rad50 antibody blocked plasmid end-joining in mammalian nuclear cell extracts.[28] In addition, studies performed using yeast proteins revealed that the purified Rad50 complex stimulated DNA ligase IV-dependent plasmid end-joining.[29] Furthermore, analysis of plasmid end-joining catalysed by fractionated protein extracts derived from mammalian cells revealed that plasmid end-joining was enhanced by the addition of a fraction enriched for the Rad50 complex.[30] Fourth, a recent description of a plasmid DNA end-joining defect in cells from patients suffering from Nijmegen breakage syndrome, which is associated with partial loss-of-function alleles of the gene encoding Nibrin.[31] Fifth, Chen *et al*. showed that Nibrin localized within freshly harvested thymocytes at sub-nuclear foci.[32] Their data indicated that Nibrin foci formed in a V(D)J recombination-dependent manner, and these authors proposed that the foci represented newly formed signals ends.

Interestingly, analysis of cells from mice harbouring a conditional inactivation of the Nibrin gene highlighted a previously unknown role of the RMN complex in immunoglobulin rearrangements. Kracker *et al*. showed that Nibrin has a role in immunoglobulin class-switch recombination.[26] Reina-San-Martin *et al*. reported similar observations.[27] These results are consistent with the finding that immunoglobulin class switch recombination occurs aberrantly in cells from patients with hypomorphic mutations in the Mre11 gene.[33] Over-expression of Nibrin is associated with increased frequency of somatic hypermutation and gene conversion in human and chicken somatic cells.[34] Finally, Clatworthy *et al*. showed recently that signal joint V(D)J recombination, which can be induced in transgenic yeast strains expressing Rag 1 and Rag 2,[35] was dependent on the RAD50, MRE11 and XRS2 genes.[36] Taken together, these findings provide support for the hypothesis that the RMN complex participates in V(D)J recombination in mammalian somatic cells.

Intriguing support for this hypothesis comes from two separate studies of cells from the cancer predisposition/DNA repair defect disorder Fanconi anemia (FA). First, Smith *et al*. showed that V(D)J recombination in immortalized lymphoblasts from FA patients is associated with abnormal rearrangements, primarily involving deletions at the sites of rejoining.[37] Second, Donahue and Campbell showed that Rad50-dependent DNA end-joining, which has a dominant role in plasmid end-joining *in vivo* is deficient in fibroblasts from FA patients.[25] This is consistent with an earlier study documenting aberrant Rad50 function in cells from FA patients.[38]

To shed further light on this question, we performed three series of experiments. First, we used a plasmid recombination assay to compare V(D)J recombination in diploid fibroblasts from FA patients to that observed in fibroblasts from normal donors. Second, we used this same system to evaluate the consequence of antibody-mediated inhibition of RMN function on V(D)J recombination in normal cells. Third, we examined V(D)J recombination in an immortalized fibroblast strain from a patient afflicted with Nijmegen breakage syndrome. The results presented below demonstrate that the rejoining of coding ends occurs aberrantly in