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Ku80 is required for immunoglobulin isotype switching

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Isotype switching is the DNA recombination mechanism by which antibody genes diversify immunoglobulin effector functions. In contrast to V(D)J recombination, which is mediated by RAG1, RAG2 and DNA double-stranded break (DSB) repair proteins, little is known about the mechanism of switching. We have investigated the role of DNA DSB repair in switch recombination in mice that are unable to repair DSBs due to a deficiency in Ku80 (Ku80$^{-/-}$). B-cell development is arrested at the pro-B cell stage in Ku80$^{-/-}$ mice because of abnormalities in V(D)J recombination, and there are no mature B cells. To reconstitute the B-cell compartment in Ku80$^{-/-}$ mice, pre-rearranged V_{B1-8} DJ_H2 (μ^i) and $V_{3-83}J_K2$ (κ^i) genes were introduced into the Ku80$^{-/-}$ background (Ku80$^{-/-}$ $\mu^{i/+}\kappa^{i/+}$). Ku80$^{-/-}\mu^{i/+}$ $\kappa^{i/+}$ mice develop mature mIgM$^+$ B cells that respond normally to lipopolysaccharide (LPS) or LPS plus interleukin-4 (IL-4) by producing specific germline Ig constant region transcripts and by forming switch region-specific DSBs. However, Ku80$^{-/-}$ $\mu^{i/+}\kappa^{i/+}$ B cells are unable to produce immunoglobulins of secondary isotypes, and fail to complete switch recombination. Thus, Ku80 is essential for switch recombination *in vivo*, suggesting a significant overlap between the molecular machinery that mediates DNA DSB repair, V(D)J recombination and isotype switching.

Keywords: class switch recombination/DNA repair/immunoglobulin genes/Ku80

Introduction

During an immune response, B lymphocytes maintain their antigen-binding specificity but can change the antibody constant region subclass they produce by a DNA recombination process known as class switching (reviewed by Lorenz and Radbruch, 1996; Stavnezer, 1996). Switching occurs between highly repetitive DNA sequences, known as switch regions, which are located 5′ of the μ, γ, α and ε constant region (C_H) genes (Shimizu *et al.*, 1982). Switch recombination is preceded by DNA demethylation, increased deoxyribonuclease I hypersensitivity and germline transcription of the implicated C_H genes (Lorenz and Radbruch, 1996; Stavnezer, 1996).

Like V(D)J recombination (Tonegawa, 1983), switching involves DNA deletion (Honjo and Kataoka, 1978; Coleclough *et al.*, 1980; Cory *et al.*, 1980; Davis *et al.*, 1980a; Rabbitts, 1980; Sakano *et al.*, 1980; Yaoita and Honjo, 1980) by a mechanism whereby intervening sequences are excised in the form of circular DNA (Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; von Schwedler *et al.*, 1990). In addition, switching resembles V(D)J recombination in that a double-stranded break (DSB) intermediate appears to be part of the switch reaction (Wuerffel *et al.*, 1997). However, isotype switching is a unique process which differs from V(D)J recombination in several respects. V(D)J recombination is characterized by site-specific recombination between short well-defined recombination signal sequences (RSSs) (Tonegawa, 1983). In contrast, the precise signals that mediate switching are unknown, and switch regions range in size from 2.5 kb for Sε to 10 kb for Sγ1. Unlike the RSSs which are shared by all V, D and J gene segments, the switch regions are composed of tandem nucleotide arrays that vary both in sequence and in length (Davis *et al.*, 1980b; Dunnick *et al.*, 1980; Sakano *et al.*, 1980; Kataoka *et al.*, 1981; Nikaido *et al.*, 1981, 1982; Obata *et al.*, 1981). Furthermore, RAG1 and RAG2, the enzymes that recognize the RSSs and activate V(D)J recombination, are not required for switching (Lansford *et al.*, 1998).

Mechanistically, V(D)J recombination involves a transesterification reaction that results in blunt-ended DNA DSBs and hairpin intermediates (van Gent *et al.*, 1996). Resolution of the broken DNA ends requires several non-lymphoid-specific proteins that were first defined by transfection and genetic studies in mutant cell lines and mice. These experiments showed that V(D)J recombination required proteins implicated in DNA DSB repair, including the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs}), Ku and XRCC4 (Bosma *et al.*, 1983; Schuler *et al.*, 1986; Hendrickson *et al.*, 1991; Pergola *et al.*, 1993; Taccioli *et al.*, 1993, 1994; Rathmell and Chu, 1994; Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996).

Ku is a heterodimer composed of Ku70 and Ku80 subunits that binds to DNA DSBs, nicks, gaps and hairpins in a sequence-independent manner (Mimori *et al.*, 1986; Morozov *et al.*, 1994). DNA-bound Ku recruits and activates DNA-PK_{cs}, which is then thought to phosphorylate a series of factors which are themselves implicated in mediating DNA DSB repair (Gottlieb and Jackson, 1993; Jeggo *et al.*, 1995). The absence of either DNA-PK_{cs},

Ku80 or Ku70 results in a deficiency in both DNA DSB repair and V(D)J recombination (Bosma *et al.*, 1983; Schuler *et al.*, 1986; Hendrickson *et al.*, 1991; Pergola *et al.*, 1993; Taccioli *et al.*, 1993, 1994; Rathmell and Chu, 1994; Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Ouyang *et al.*, 1997). However, the absence of Ku does not appear to affect DNA repair mediated by homologous recombination, which remains intact both in Ku-deficient yeast and mammalian cells (Boulton and Jackson, 1996a,b; Liang and Jasin, 1996; Liang *et al.*, 1996; Milne *et al.*, 1996; Siede *et al.*, 1996).

In contrast to V(D)J recombination, the role of DNA DSB repair in switch recombination in mature B cells has not been evaluated. Developing B cells in mice that carry mutations in DSB repair genes fail to complete V(D)J recombination, and lymphocyte development is arrested at early precursor stages (Bosma *et al.*, 1983; Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Ouyang *et al.*, 1997). Therefore, the cell type that normally undergoes class switching is absent, and this process cannot be assessed. Recombination between switch regions has been studied in DNA-PK_{cs} (SCID) mutant pro-B cells deprived of interleukin-7 (IL-7) (Rolink *et al.*, 1996). In the absence of IL-7, pro-B cells undergo programmed cell death and differentiate into pre-B cells (Grawunder *et al.*, 1993). When cell death is delayed in the IL-7-deprived pro-B cells by overexpression of Bcl-2, they undergo DNA recombination between Ig switch regions in a reaction that is independent of the assembly of a functional Ig transcription unit (Rolink *et al.*, 1996). Recombination between switch regions in pro-B cells *in vitro* requires DNA-PK_{cs}, but the requirements for this reaction may or may not be the same as authentic switching in mature B cells (Rolink *et al.*, 1996).

Here we report on switch recombination in mature $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells isolated from mice that carry pre-rearranged immunoglobulin heavy [$V_{B1-8}DJ_H2$ (Sonoda *et al.*, 1997)] and light [$V_{3-83}J_K2$ (Pelanda *et al.*, 1996)] chain genes. We find that B cells that are deficient in Ku80 are unable to complete switch recombination. These data implicate the DNA DSB repair machinery in class switch recombination.

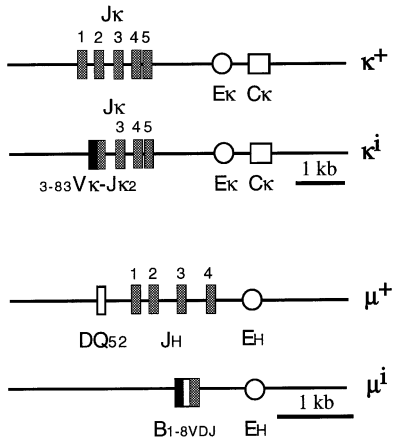

Fig. 1. Map of the wild-type (+) Igκ and Igμ loci and their predicted structure after insertion (i) of the rearranged constructs. The 3′ κ and H enhancers (E) are depicted as open circles, and the constant (C) κ region as open squares. Variable, diversity and joining regions are represented as black, open and gray bars respectively.

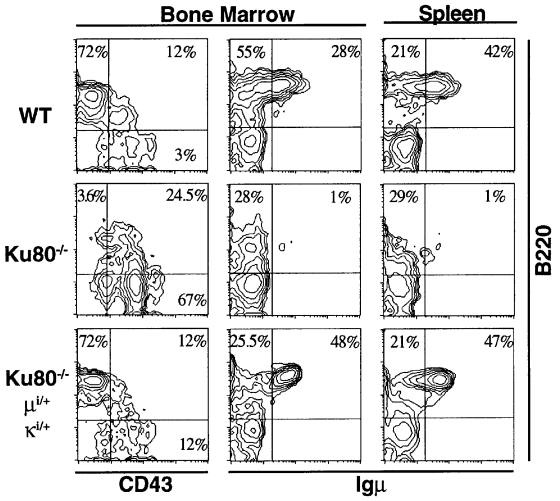

Fig. 2. Reconstitution of B-cell development in $Ku80^{-/-}$ mice. Bone marrow and spleen samples from 6- to 10-week-old wild-type, $Ku80^{+/-}$ and $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ mice analyzed for B-cell maturation. Cell percentages were calculated from total gated populations. Bone marrow cells were stained with anti-B220 and anti-CD43, and splenocytes with anti-B220 and anti-IgM antibodies.

Results

$V_{B1-8}DJ_H2$ and $V_{3-83}J_K2$ replacements reconstitute B-cell development in $Ku80^{-/-}$ mice

$Ku80^{-/-}$ mice are unable to repair DNA DSBs and show a phenotype that includes proportional dwarfism, and a profound disruption in both T- and B-lymphocyte development (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). In the absence of Ku80, developing lymphocytes cannot repair the DNA breaks produced by RAG1 and RAG2 during V(D)J recombination. The result of the DSB repair deficiency is that both T- and B-cell development are arrested at early precursor stages in $Ku80^{-/-}$ mice (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996).

To determine whether the absence of B cells in $Ku80^{-/-}$ mice was due solely to impaired V(D)J recombination, we introduced pre-rearranged targeted Igμ (Sonoda *et al.*, 1997) and Igκ (Pelanda *et al.*, 1996) genes into the $Ku80^{-/-}$ background (Figure 1). The mice resulting from these crosses are referred to as $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ mice. Like $Ku80^{-/-}$ controls, $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ mice were proportional dwarfs and had no mature T cells (not shown). However, B-cell development in the bone marrow of $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ mice differed from that of $Ku80^{-/-}$ mice in that B cells progressed beyond the pro-B cell stage (Figure 2). FACS analysis of $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ bone marrow showed that in the presence of pre-rearranged μ and κ Ig genes, Ku80-deficient B cells progressed to the $B220^+CD43^-$ pre-B cell stage and developed into immature and mature $B220^+IgM^+$ B cells (Figure 2). Mature B cells expressing surface IgM were also found in peripheral lymphoid organs such as spleen, but in 6- to 8-week-old mice the total number of B cells in $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ was only 15–25% ($n = 10$) of that found in wild-type and $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ littermate controls. This low number of mature B cells in the periphery of the reconstituted mice may be secondary to the absence of T cells and T $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ B cell clonal expansion. Alternatively, the relative B

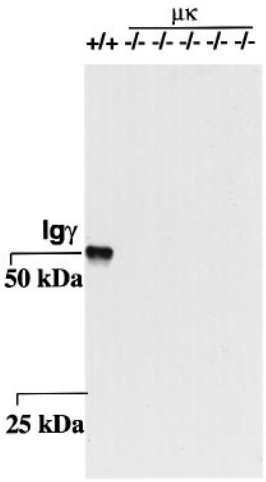

Fig. 3. IgG heavy chain (50 kDa) expression in Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ mice and controls. Two ml of serum from wild-type (+/+) and Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ (–/–) mice was analyzed by PAGE, and blotting with goat anti-mouse IgG visualized with alkaline phosphatase.

lymphopenia could be due to the documented inability of Ku80$^{-/-}$ cells to repair DNA DSBs incurred during normal proliferative responses (Nussenzweig *et al.*, 1996).

We conclude that pro-B cell arrest in Ku80$^{-/-}$ mice is a function of impaired resolution of DNA breaks resulting from V(D)J recombination and that Ku80 is not essential for other aspects of antigen-independent B cell development.

Ku80$^{-/-}$ B cells are deficient in switch recombination

Consistent with the lower than normal number of peripheral B cells in Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ mice, the level of circulating IgM was 62% of that found in wild-type mice as measured by an IgM-specific enzyme-linked immunosorbent assay (ELISA) (1.1 mg/ml in wild-type versus 0.68 in Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ $n = 5$ mice of each type). In contrast to IgM, secondary Ig isotypes, which are normally found in the serum of un-immunized animals in the mg/ml range, were not detectable in Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ mice. Western blotting with specific goat anti-mouse IgG showed no IgG heavy chains in the serum of the reconstituted mice (Figure 3).

The absence of secondary Ig isotypes in the serum of Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ mice could be due to a deficiency in switch recombination or might be a consequence of the absence of T cells in these mice. To determine whether the absence of secondary antibody isotypes in serum was due to a cell-autonomous defect, we isolated B cells and stimulated them with either lipopolysaccharide (LPS), or the combination of LPS plus IL-4 to activate switch recombination in cell culture.

LPS induces murine B cells to switch from μ to γ3 and γ2b, whereas LPS plus IL-4 activates switching to γ1 and, to a lower extent, ε. In all cases, switch recombination is preceded by sterile transcription of the switch-targeted C_H genes (see Figure 4A). To determine whether Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells could respond to either LPS or LPS plus IL-4 by activating switch transcription, we measured germline sterile mRNAs by a semi-quantitative RT–PCR assay using Iγ3-, Iγ1- (Snapper *et al.*, 1996) and Iγ2b-specific

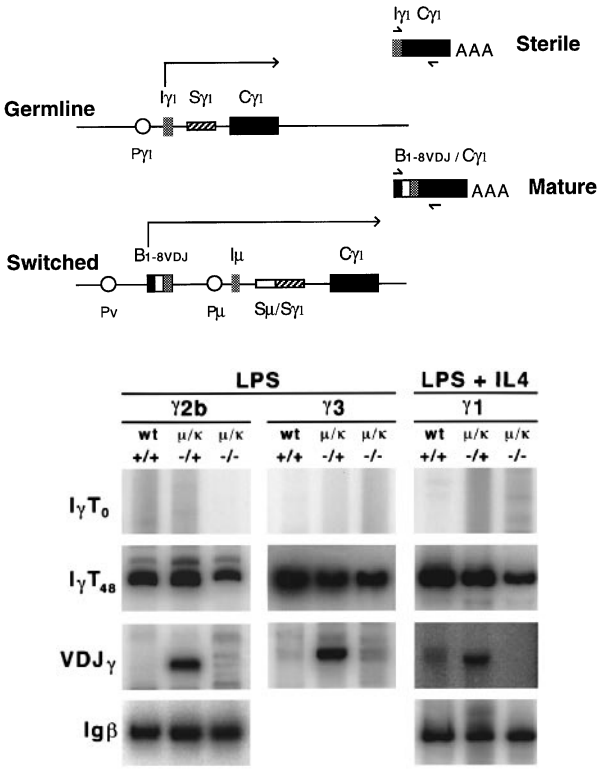

Fig. 4. Ig germline and mature switch transcription. (**A**) Schematic representation of the Igγ1 locus before and after switching. The I exon (I), switch (S) and constant (C) regions for μ and γ1 are shown. Their respective promoters are depicted as open circles. Switch recombination is preceded by the generation of sterile transcripts initiated at the I exons. Sterile transcripts were detected using I- and C-specific primers. (**B**) cDNA was prepared from wild-type (+/+), Ku80$^{+/-}\mu^{i/+}\kappa^{i/+}$ (–/+ μ/κ) and Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ (–/– μ/κ) unstimulated B cells (T_0) or B cells that were cultured with LPS or LPS plus IL-4 for 48 h (T_{48}). Germline (Iγ) and mature (VDJγ) transcripts for γ2b, γ3 and γ1 were detected by RT–PCR using 30 cycles of amplification. Under the experimental conditions, the signal intensity was proportional to the amount of input cDNA. B cell-specific Igβ mRNA was used to normalize each RT–PCR reaction.

primers. Igβ mRNA was used as a B cell-specific mRNA loading control. Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ resembled control B cells in that Iγ2b, Iγ3 and Iγ1 germline transcripts were induced specifically by LPS and LPS plus IL-4 respectively, although the levels of sterile transcripts found in reconstituted mice were 10–30% lower than those found in wild-type mice, as assayed by phosphorimaging (Figure 4B, and data not shown). We conclude that Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells are competent to respond to signals that induce switch recombination *in vitro*.

To determine whether Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells can produce secondary antibodies in response to switch signals, we first measured cell surface expression of γ3 and γ1 after stimulation with LPS or LPS plus IL-4, respectively. B cells from Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ mice were compared with wild-type and Ku80$^{+/-}\mu^{i/+}\kappa^{i/+}$ B cells after staining with isotype-specific antibodies (Figure 5). As expected, wild-type and Ku80$^{+/-}\mu^{i/+}\kappa^{i/+}$ B cells expressed cell surface γ3 and γ1 following culture with LPS or LPS plus IL-4. In contrast, Ku80$^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells showed no secondary isotype expression when cultured under the same conditions (Figure 5).

To enhance the sensitivity of the assay for secondary

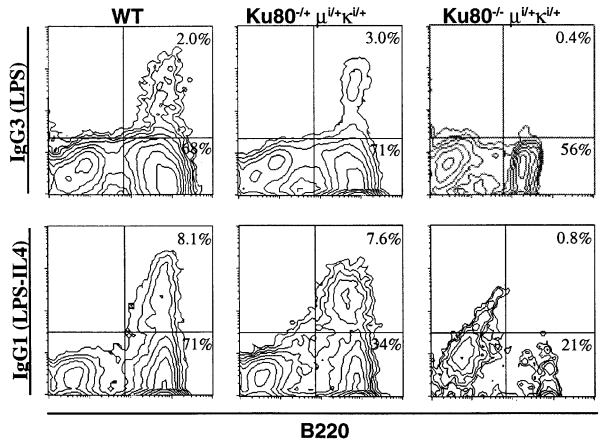

Fig. 5. Cell surface expression of secondary Ig isotypes. Flow cytometry analysis of splenocytes from wild-type, $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$, and $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ after LPS or LPS plus IL-4 stimulation. Cells were cultured for 3 days and stained with anti-B220 and anti-IgG3 (LPS) or anti-IgG1 (LPS-IL4). Percentages from total gated populations are shown.

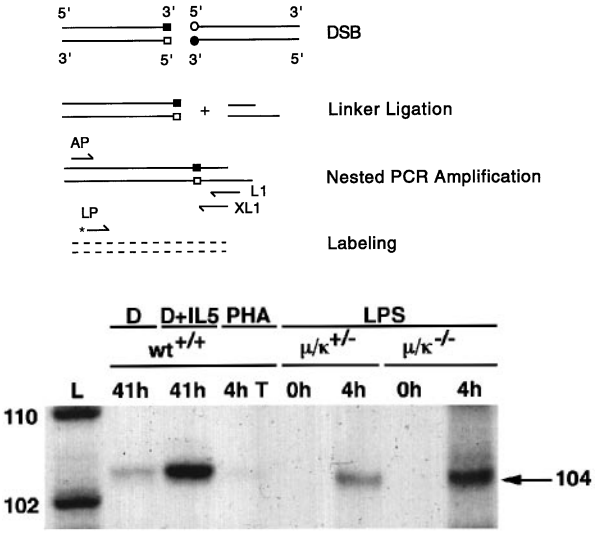

Fig. 6. γ3 switch breaks in stimulated B cells. (**A**) Schematic diagram of the DSB assay. A linker is ligated to 5′-phosphorylated blunt DNA ends in genomic DNA. DNA tagged with the linker is then PCR amplified for 15 cycles using the linker primer (L1) and a locus-specific primer (AP) (Wuerffel *et al.*, 1997). A second round of 15 cycle PCR amplification using AP and a nested linker primer (XL1) is then performed. The amplified products are then radioactively labeled and the precise positions of the DSBs are determined by denaturing PAGE. (**B**) $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ ($\mu/\kappa^{-/-}$) and control $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ ($\mu/\kappa^{+/-}$) splenocytes were stimulated with LPS for the indicated times and assayed for DNA breaks (Wuerffel *et al.*, 1997). Switch breaks are 104 nucleotides long and are found in live B cells undergoing switch recombination (Wuerffel *et al.*, 1997). As control, wild-type ($wt^{+/+}$) B cells were stimulated for 41 h with anti-δ-dextran (D) antibodies, and anti-δ-dextran plus IL-5 (D+IL5). L = DNA ladder.

isotypes and to determine whether $Ku80^{-/-}\mu^{i/}\ \kappa^{i/+}$ B cells could produce mature secondary Ig mRNAs, we devised an isotype-specific PCR assay. $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ and $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ mice show a highly homogeneous B-cell compartment in which the great majority of the cells express the $V_{B1-8}DJ_H2$ replacement gene (Papavasiliou *et al.*, 1997). Primers specific for $V_{B1-8}DJ_H2$ combined with primers designed to hybridize Cγ3, Cγ2b or Cγ1 regions can therefore be used to measure mature γ2b, γ3 or γ1 mRNAs (see Figure 4A). As expected, mature γ2b, γ3 or γ1 mRNAs were abundant in the control $Ku80^{+/-}\mu^{+/-}\kappa^{i/+}$ B cells stimulated with LPS or LPS and IL-4 (Figure 4B). In contrast, $V_HB1–8$-Cγ2b or -Cγ3 mRNAs were detected at very low levels, and $V_HB1–8$-Cγ1 was not detected in $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells. Dilution analysis and quantitation by phosphorimaging showed that the levels of $V_HB1–8$-Cγ2b or -Cγ3 were at least 10-fold lower than those found in $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ B cells.

When B cells are cultured with appropriate mitogens and cytokines, DNA DSBs are induced in the targeted switch regions (Wuerffel *et al.*, 1997). These breaks have been determined to be specific by several criteria including: (i) sequence; (ii) absence in T cells; (iii) absence in mitogen-stimulated T cells; and (iv) absence in resting B cells (Wuerffel *et al.*, 1997). To determine whether these DNA breaks were present in $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells, we made use of this γ3 switch region-specific DNA DSB assay (Wuerffel *et al.*, 1997) (Figure 6A). Figure 6B shows that DNA DSBs are generated in γ3 switch regions from $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ B cells and $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ controls upon activation with LPS for 4 h. These γ3 switch-specific breaks were not detected in unstimulated B cells or T cells stimulated with phytohemagglutinin (PHA). Further, B cells stimulated with anti-δ-dextran alone, which induces B-cell proliferation but little switch recombination, show only low levels of the γ3 switch-specific breaks (Figure 6B). In contrast, B cells treated with the combination of anti-δ-dextran plus IL-5, which induces high levels of γ3 switching, show high levels of γ3 switch-specific breaks. Thus, the γ3 switch breaks are seen in B cells stimulated with specific inducers of γ3 switching, and the breaks are seen in the presence and absence of Ku80 (Figure 6).

To determine whether the deficiency in switch transcription and protein production was due to a failure to complete switch recombination at the DNA level, we assayed for μ–γ1 switched DNA directly using a previously described γ1 digestion–circularization PCR (DC–PCR) assay. This assay, which detects recombination between μ and γ1 switch regions, was modified to increase sensitivity by including a second round of amplification with nested primers (Chu *et al.*, 1992) (see Figure 7A). We used the non-rearranging acetylcholine receptor (Ach) gene as a positive control for our digestion and ligation reactions (Chu *et al.*, 1992). All of the DNA samples were positive in the Ach DC–PCR reaction. In addition, μ–γ1 rearrangement was present in DNA from both wild-type and $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ control B cells stimulated with LPS and IL-4. In contrast, $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ showed a complete absence of μ–γ1 DNA recombination (Figure 7B). We conclude that there is no DNA recombination between μ and γ1 switch regions in Ku80-deficient B cells stimulated with LPS and IL-4.

Discussion

Reconstitution of Ku80-deficient mice

As previously reported, $Ku80^{-/-}$ mice exhibit impaired V(D)J recombination and B-cell maturation is arrested at the $B220^+CD43^+$ stage (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). We bypassed this early block in development by introducing heavy and light chain variable region gene

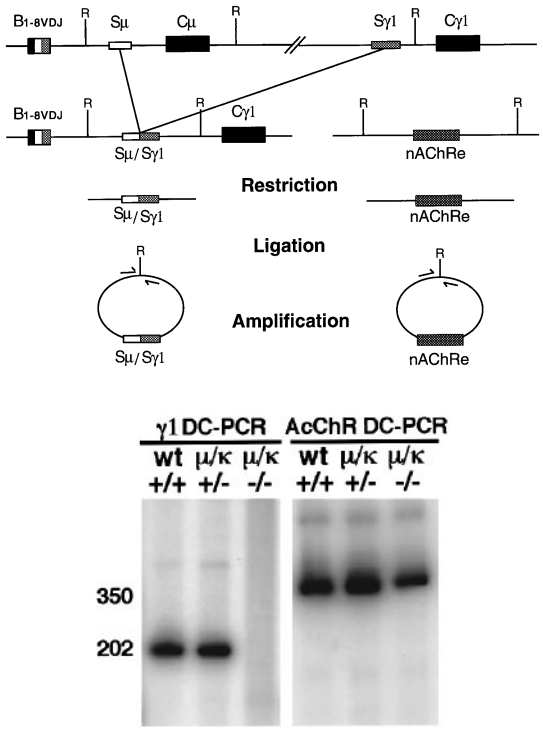

Fig. 7. Digestion–circularization-mediated PCR assay for switch recombination. (**A**) Strategy for the DC–PCR adapted from Chu *et al.* (1992). Schematic map (not to scale) of the non-rearranged B1–8VDJ locus depicting the μ and γ1 switching (S) and constant (C) regions. *Eco*RI digestion, ligation and PCR amplification results in a product only after Sμ–Sγ1 recombination. A control PCR product from the nAChR is found regardless of chromosomal rearrangement. R represents *Eco*RI sites. (**B**) Semi-quantitative nested DC–PCR was performed on DNA extracted from wild-type, $Ku80^{+/-}\mu^{i/+}\kappa^{i/+}$ and $Ku80^{-/-}\mu^{i/+}\kappa^{i/+}$ splenocytes that had been stimulated with LPS and IL-4 for 3 days. The absence of switch DNA rearrangement in Ku80-deficient B cells was confirmed by overexposure or by using higher concentrations of genomic DNA (up to 100 ng per reaction, data not shown). The size of the amplified products is indicated.

replacements that create fully functional Ig loci in the appropriate genomic context (Pelanda *et al.*, 1996; Sonoda *et al.*, 1997). These gene replacements were sufficient to reconstitute the peripheral B-cell compartment, indicating that, in early B-cell development, Ku80 is required solely for processing of V(D)J recombination intermediates. This result is consistent with previous reconstitution experiments using randomly integrated Ig transgenes in mice deficient for other components of the V(D)J recombinase (Spanopoulou *et al.*, 1994; Young *et al.*, 1994; Chang *et al.*, 1995). However, it was not possible to study switch recombination in mice with randomly integrated transgenes since switching normally occurs *in cis* on any given chromosome (Wabl *et al.*, 1985). In contrast to these studies, targeted V_H gene replacements provide physiologic substrates in mature B cells to study isotype switching (Lansford *et al.*, 1998).

Switching: a homology-based or an end-joining recombination mechanism?

At least two distinct biochemical pathways mediate DNA DSB repair in *Saccharomyces cerevisiae* and higher eukaryotes (Kramer *et al.*, 1994; Moore and Haber, 1996). The gene products of the RAD52 epistasis group function in DNA repair by homologous recombination, a process in which damaged chromosomes restore genetic integrity by physically pairing with a sister chromatid or homolog. Ku80 and Ku70 are part of a separate group of proteins that are essential for DSB repair by a mechanism that does not require DNA homology (Boulton and Jackson, 1996a,b; Liang *et al.*, 1996; Milne *et al.*, 1996; Siede *et al.*, 1996). Consequently, Ku-deficient yeast strains are unable to repair DSBs properly by end-joining (Boulton and Jackson, 1996a,b; Milne *et al.*, 1996; Siede *et al.*, 1996). In mammalian cells, Ku80 is similarly dispensable for repair by homologous recombination, but required for rejoining of endonuclease-induced DNA breaks (Liang *et al.*, 1996) and for V(D)J recombination (Taccioli *et al.*, 1993, 1994; Rathmell and Chu, 1994; Smider *et al.*, 1994).

Although isotype switching is known to proceed through looping out and deletion of DNA (Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; von Schwedler *et al.*, 1990), the molecular details of the reaction remain to be determined. The similarity between different switch regions has led to the suggestion that short DNA stretches of identity could align and participate actively in the recombination process (reviewed in Stavnezer, 1996). However, if switch recombination were to proceed through a homology-based DNA repair mechanism, it would not be expected to be disrupted in Ku-deficient mice. Indeed, the finding that Ku is required for switching suggests that switch recombination involves a non-homologous DNA DSB mechanism. This idea is also supported by the observation that the catalytic subunit of DNA-PK is required for recombination between switch regions in pro-B cells *in vitro* (Rolink *et al.*, 1996).

The conclusion that DSB repair is required for switch recombination is consistent with the recent finding of DNA DSB intermediates in switch regions undergoing recombination (Wuerffel *et al.*, 1997). In these experiments, ligation-mediated PCR was used to document switch-specific, blunt and 5′-phosphorylated DNA ends in switch regions targeted for recombination (Wuerffel *et al.*, 1997). These switch ends are thus similar to the signal end intermediates found in V(D)J recombination (Roth *et al.*, 1993; Schlissel *et al.*, 1993). The requirement for Ku in switching and the detection of blunt 5′-phosphorylated switch DNA ends suggest a model for switching that resembles V(D)J recombination. In this model, switch donor and acceptor DNA tandem repeats are first cleaved by a switch-specific endonuclease to produce DNA DSB (Wuerffel *et al.*, 1997). Cleavage may be either imprecise or followed by limited processing leading to a cluster of breakpoints within a subregion of the tandem repeat (Wuerffel *et al.*, 1997). DNA ends produced by the switch endonuclease would then be held together by Ku and possibly other proteins in a synaptic complex analogous to the one described for signal ends during the V(D)J recombination reaction (Sheehan and Lieber, 1993; Eastman *et al.*, 1996; Agrawal, 1997). It has been suggested that the role of Ku in such a complex is to stimulate DNA end joining (Ramsden and Gellert, 1998).

An interesting prediction of this model is that switch region tandem repeats would not function in the DNA recombination process *per se*, but could act as recognition sites for a switch-specific endonuclease, much as RSSs are targeted by RAG1 and RAG2 in V(D)J recombination. Alternatively, transcription of these tandem repeats could

create DNA structures that would be recognized by a switch-specific endonuclease.

In conclusion, Ku80 is essential for switch recombination in mature B cells. Further, reconstitution of the mature B cell compartment using targeted Ig genes in mice deficient in DNA repair genes such as Ku70, DNA-PK_{cs} and XRCC4 provides a general method for evaluating the role of these and other genes in switch recombination *in vivo*.

Materials and methods

Mice

Mice were bred and maintained under specific pathogen-free conditions. Screening for targeted genes was as previously described (Nussenzweig *et al*., 1996; Pelanda *et al*., 1996; Sonoda *et al*., 1997).

Cell culture and FACScan analysis

Bone marrow and spleen cells from 6- to 8-week-old mice were cultured for 3–4 days in complete RPMI medium [RPMI 1640 (Gibco-BRL) with 10% fetal calf serum (Sigma), 1% antibiotic–antimycotic (Gibco-BRL), 1% L-glutamine (Gibco-BRL), 2% minimal essential medium amino acids solution, 1% sodium pyruvate solution (Cellgro), 10 mM HEPES buffer (Gibco-BRL) and 53 μM β-mercaptoethanol (Fisher Scientific)] with the addition of 25 μg/ml LPS (*Escherichia coli* 0111:B4; Sigma) or LPS and IL-4 (50 U/ml; Gibco-BRL). Bone marrow or spleen cell suspensions were stained with phycoerythrin-labeled anti-B220, and fluorescein isothiocyanate-labeled anti-CD43 or anti-IgM antibodies respectively (Pharmingen). LPS or LPS–IL-4-stimulated spleen cells were stained with phycoerythrin-labeled anti-B220 and biotin-conjugated anti-IgG3 and IgG1 antibodies and developed with streptavidin-Cy-Chrome (Pharmingen). Stained samples were gated according to standard forward- and side-scatter values and analyzed on a Becton-Dickinson FACSscan fluorescence-activated cell sorter with CELLQuest software.

DC–PCR

To increase the sensitivity of the DC–PCR assay, we added a nested amplification to the basic protocol (Chu *et al*., 1992). Following ligation, 5 ng of DNA were denatured at 94°C for 5 min followed by 30 cycles of amplification with the first primer set at (94°C for 15 s, 66°C 1.5 min, 72°C 1 min) and a final amplification at 72°C for 10 min. From each first cycle reaction, 2 μl was amplified further for 30 cycles with a second set of primers in the presence of [α-^{32}P]dCTP (4 μCi). The cycle conditions were 94°C for 15 s, 68°C 1.5 min, 72°C 1 min. PCR products were analyzed by 8% PAGE and the products visualized and quantitated with a phosphorimager. PCR primers were Sμ–Sγ1 first set: S1, 5′ GAGCAGCTACCAAGGATCAGGGA 3′ and S2, 5′ CTTCACG-CCACTGACTGACTGAG 3′; Sμ–Sγ1 second set: S3, 5′ GGAGACCA-ATAATCAGAGGGAAG 3′ and S4, 5′ GAGAGCAGGGTCTCC-TGGGTAGG 3′. For the nicotinic acetyl choline receptor (nAChR), primers used for the first set were: A1, 5′ GCAAACAGGGCTGGATG-AGGCTG 3′ and A2, 5′ GTCCCATACTTAGAACCCCAGCG 3′. For the second set, the primers were: A3, 5′ GGACTGCTGTGGGTTT-CACCCAG 3′ and A4, 5′ GCCTTGCTTGCTTAAGACCCTGG 3′.

RT–PCR

Total RNA was reverse transcribed with Superscript II (Life Technologies). Then 5–100 ng of cDNA was amplified in the presence of [^{32}P]dCTP (4 μCi; DuPont) in a 25 μl PCR reaction with 10 pmol of each primer. To detect germline sterile transcripts, the following primers were paired: (i) Iγ3 , γ3–5′ CAAGTGGATCTGAACACA and γ3–3′ GGCTCCATAGTTCCATT (expected product 350 bp); (ii) Iγ2b, γ2b–5′ CCTGACACCCAAGGTCACG and γ2b–3′ CGACCAGGCAA-GTGAGACTG (expected product 345 bp); (iii) Iγ1, γ1–5′ CAGCCT-GGTGTCAACTAG and γ1–3′ GCAAGGGATCCAGAGTTCCAG (expected product 341 bp); (iv) Igβ, Igβ–5′ GATGACGGCAA-GGCTGGGATGGAGGAA and Igβ–3′ CTCATTCCTGGCCTGGATGC (expected product 142 bp). Mature γ3 and γ2b transcripts were amplified by combining a V_HB1–8-specific primer V_HB1–8 CAAGGGCAA-GGCCACACTG with either Cγ3 CCACTGCTGCCTGAGCCATCTC (expected product 313 bp) or Cγ2b CAGGTGACGGTCTGACTTGG (expected product 414 bp). Mature γ1 transcripts were amplified by combining a second V_HB1–8-specific primer V_HB1–8′ CCAGCT-ACTGGATGCACTG 3′ with Cγ1 GGACAGCTGGGAAGGTGTG 3′ (expected product 440 bp). All reactions were performed for 30 cycles. Amplified samples were analyzed by 8% PAGE and visualized by phosphorimaging.

Western blotting

An aliquot of 2 μl of mouse serum was diluted in 100 μl of phosphate-buffered saline (PBS) and incubated with 20 μl of protein A–Sepharose beads (Pierce) for 1 h. The beads were washed three times with PBS and the bound proteins separated by 8% reducing PAGE before blotting and visualization with alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce).

DSB assay

Splenocytes were activated in culture as previously described (Wuerffel *et al*., 1997). Genomic DNA was prepared using the Cell Culture DNA Kit (Qiagen) or the Puregene DNA Isolation Kit (Gentra). The DSB assay for Sγ3 DNA (Wuerffel *et al*., 1997) was performed with modifications. The partially double-stranded linker was ligated directly to 1.5 μg of unmodified genomic DNA for 18 h at 16°C. PCR amplification was done in two rounds of 15 cycles each (1 min at 95°C/ 2 min at 67°C/3 min at 76°C). Primers Sγ3 AP and L.1 (Wuerffel *et al*., 1997) were used in the first round. Ten μl of product was taken to program the second round of amplification using primers Sγ3 AP and XL1, a nested primer (5′-GTGACCCGGGAGATCTGAATTCCCC-3′) specific for a subset of ligated broken fragments. Reaction products were radioactively labeled and analyzed by denaturing polyacrylamide gel electrophoresis. Broken fragments amplified by this method are three nucleotides shorter than the corresponding fragments of the ladder which are amplified using AP and L1 only.

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