Ku70 Is Required for Late B Cell Development and Immunoglobulin Heavy Chain Class Switching

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Summary

Immunoglobulin (Ig) heavy chain (HC) class switch recombination (CSR) is a late B cell process that involves intrachromosomal DNA rearrangement. Ku70 and Ku80 form a DNA end-binding complex required for DNA double strand break repair and V(D)J recombination. $Ku70^{-/-}$ (K70T) mice, like recombination activating gene (RAG)-1– or RAG-2–deficient (R1T or R2T) mice, have impaired B and T cell development at an early progenitor stage, which is thought to result at least in part from defective V(D)J recombination (Gu, Y., K.J. Seidl, G.A. Rathbun, C. Zhu, J.P. Manis, N. van der Stoep, L. Davidson, H.L. Cheng, J.M. Sekiguchi, K. Frank, et al. 1997. *Immunity*. 7:653–665; Ouyang, H., A. Nussenzweig, A. Kurimasa, V.C. Soares, X. Li, C. Cordon-Cardo, W. Li, N. Cheong, M. Nussenzweig, G. Iliakis, et al. 1997. *J. Exp. Med.* 186:921–929). Therefore, to examine the potential role of Ku70 in CSR, we generated K70T mice that carry a germline Ig HC locus in which the JH region was replaced with a functionally rearranged VH(D)JH and Ig λ light chain transgene (referred to as K70T/HL mice). Previously, we have shown that B cells from R1T or R2T mice carrying these rearranged Ig genes (R1T/HL or R2T/HL mice) can undergo CSR to IgG isotypes (Lansford, R., J. Manis, E. Sonoda, K. Rajewsky, and F. Alt. 1998. *Int. Immunol.* 10:325–332). K70T/HL mice had significant numbers of peripheral surface IgM^+ B cells, which generated serum IgM levels similar to those of R2T/HL mice. However, in contrast to R2T/HL mice, K70T/HL mice had no detectable serum IgG isotypes. In vitro culture of K70T/HL B cells with agents that induce CSR in normal or R2T/HL B cells did lead to the induction of germline CH transcripts, indicating that initial signaling pathways for CSR were intact in K70T/HL cells. However, treatment with such agents did not lead to detectable CSR by K70T/HL B cells, and instead, led to cell death within 72 h. We conclude that Ku70 is required for the generation of B cells that have undergone Ig HC class switching. Potential roles for Ku70 in the CSR process are discussed.

Key words: immunoglobulin class switch recombination • Ku70 • recombination activating gene 2 • B cell development

There are two types of DNA rearrangements involved in the generation of Ig genes during B cell development. The first is the V(D)J recombination reaction in which Ig heavy chain (HC)[1] and light chain (LC) variable region genes are assembled from component germline V, D, and J DNA segments (1, 2). This process involves a site-specific recombination event initiated by the recombination activating gene (RAG)-1 and RAG-2 gene products and completed, at least in part, by a set of proteins also used in DNA double strand break repair (DSBR) in all cell types (3). The expression of an Ig μ HC followed by an Ig κ or λ LC as a result of productive VDJ recombination is required to promote differentiation of bone marrow progenitor B cells to surface (s)IgM^+ B lymphocytes. The $sIgM^+$ B cells then populate peripheral lymphoid organs, where upon appropriate activation, they can undergo further differentia-

[1]*Abbreviations used in this paper:* C, constant region gene; CSR, class switch recombination; DC, digestion–circularization; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; DSB, double strand break; DSBR, double strand break repair; HC, heavy chain; HL, heavy plus light chain transgene; K70T, $Ku70^{-/-}$; LC, light chain; RAG, recombination activating gene; R1T and R2T, RAG-1– and RAG-2–deficient; sIg, surface Ig; wt, wild-type.

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tion events, including the second type of DNA rearrangement referred to as Ig HC class switch recombination (CSR) (4).

CSR exchanges the μ constant region gene (Cμ) for one of a set of downstream CH genes which include Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, or Cα (for a review, see reference 5). CSR events usually occur within large regions of repetitive DNA sequence (S regions), which lie upstream of Cμ and other CH genes. CSR appears to be targeted by transcription through the S region of the involved CH gene before recombination (6, 7). CSR is highly regulated; switching to different downstream CH genes is modulated by the type of B cell activation in combination with different cytokines or accessory signals (for a review, see reference 8). For example, LPS treatment of splenic B cells induces CSR to either Cγ2b or Cγ3. In contrast, addition of IL-4 plus LPS inhibits switching to Cγ2b and Cγ3, and induces switching to Cγ1 or Cε. Other combinations of activators (e.g., stimulation via CD40) and cytokines induce CSR to other sets of CH genes (for a review, see reference 8).

CSR fuses two participating intrachromosomal S regions to juxtapose the VDJ exons to a downstream CH exon (for a review, see reference 5). A common by-product of this process is an excised circle that results from the fusion of the reciprocal S region sequences associated with the intervening DNA (9–11). To date, there is no evidence that CSR utilizes the type of site-specific cleavage mechanism used to initiate V(D)J recombination. Thus, S regions share no clear-cut consensus sequences, and the CSR events are distributed throughout the relatively large (1–10 kb) S regions (for a review, see reference 12). However, the process does appear to require both transcription and cell division/DNA replication (for a review, see reference 13). Most recently, DNA double strand breaks (DSBs) were found within Sγ3 regions in B cells induced to switch to Cγ3, potentially implicating a CSR mechanism that uses DSBs as an intermediate (14).

The DNA DSBs generated by agents such as ionizing radiation and by the RAG proteins during V(D)J recombination are repaired by a common set of proteins (15, 16). Three of these proteins are components of the enzyme referred to as the DNA-dependent protein kinase (DNA-PK). The Ku70 and Ku80 proteins associate to form a DNA end-binding activity that has been shown in vitro to activate the large DNA-PK catalytic subunit (DNA-PKcs) (17). The murine *scid* mutation is a point mutation in the DNA-PKcs gene (18, 19); mice homozygous for this mutation (SCID mice) lack mature B and T cells due to defective V(D)J recombination (20–24). The lack of CSR in SCID pro-B cell lines treated with anti-CD40 and IL-4 has implicated the DNA-PKcs gene product as necessary for CSR (25). However, SCID mice have a leaky phenotype, and older SCID animals can generate some peripheral B cells as well as low levels of serum IgG isotypes (26). Therefore, it remains unclear whether DNA-PKcs is directly or absolutely required for CSR in mature B cells.

Targeted mutations of the Ku70 and Ku80 genes have been generated and show similar phenotypes (27–31). Ku70- and Ku80-deficient cells display ionizing radiation sensitivity, early senescence, and apparent doubling time defects (27, 31). In both types of mutant cells, DNA damage-sensing machinery appears intact, and the growth defects appear linked to loss of cells from cycle as a result of the repair defects. In correspondence with the cellular defects, both Ku70- and Ku80-deficient mice have a SCID phenotype due to an impairment in V(D)J recombination, as well as several other defects, including growth retardation (27, 30–32). Given the potential linkage of DSBs and DNA-PKcs to switching, the Ku70 and Ku80 proteins are also strong candidates for a role in this process. However, it was not possible to assess potential roles for Ku in CSR in Ku-deficient mice because of the lack of mature B cells in these animals.

Recently, we have described a method to test the role of gene products required for V(D)J recombination in downstream processes such as CSR (33). In this context, we generated RAG-1–deficient (R1T) and RAG-2–deficient (R2T) mice which contained a rearranged Ig HC V(D)J gene inserted in place of the endogenous JH locus, plus a rearranged λ1 Ig LC transgene (referred to as R1T/HL and R2T/HL mice, respectively). The transgenic HC and λ LC proteins form a complete Ig molecule, the expression of which led to significant reconstitution of the peripheral B cell compartments of R1T/HL and R2T/HL mice (33). Moreover, analyses of serum isotype levels and in vitro–stimulated B cells from these mice indicated the ability to undergo relatively normal levels of CSR in the absence of the RAG gene products (33). We have now used this same strategy to test the ability of B cells of Ku70-deficient mice to undergo Ig HC class switching.

Materials and Methods

Generation of the Ku70$^{-/-}$/HL mice. The Ku70$^{-/-}$(K70T)/HL mice were made by a three-way cross of mice containing a rearranged B1–8 V(D)J gene segment that has been inserted into the endogenous JH locus (34), with mice containing a λ1 LC transgene (35), and with K70T mice (27). The mice were identified by Southern blotting and PCR analyses of tail-derived genomic DNA as described previously (27, 33, 35). The R2T/K70T/HL mice were generated by a cross of K70T/HL with R2T mice (36). R2T/HL mice were obtained as described previously (33).

Spleen Cell Cultures. Single-cell suspensions of spleen cells were cultured at 5×10^5 cells/ml in RPMI medium supplemented with 10% FCS and 25 ng/ml LPS with or without 50 ng/ml of mouse recombinant IL-4 (R & D Systems, Inc., Minneapolis, MN) as described previously (37). Cells were harvested for flow cytometry analyses, and culture supernatants were assayed on day 5. Stimulations with anti-CD40 (PharMingen, San Diego, CA) were performed using 500 ng/ml of the antibody, placed directly into culture media.

Flow Cytometry Analysis. Single-cell suspensions from bone marrow, spleen, lymph node, peripheral blood, peritoneum, and thymus were prepared as described previously from 4–8-wk-old mice (38). Cells from day 3 cultures were washed twice in PBS/3% FCS and stained with various antibodies conjugated with fluorescein (IgM, IgD, and IgG1), phycoerythrin (IgM, IgG3, and Igλ), and Cy-Chrome (B220; PharMingen). The cells were analyzed using a FACSCalibur® and interpreted using Cellquest

(Becton Dickinson, Mountain View, CA) and Flo-Jo (Tree Star, Inc., San Carlos, CA) software. The FACS® profiles representing 5,000–20,000 events were gated for live, lymphoid cells determined by forward scatter versus side scatter. The surface phenotypes presented are representative of data collected from littermate mice comprising three wild-type (wt; which contained at least one normal K70 allele and one normal R2 allele), three K70T, four K70T/HL, two K70T/R2T/HL, and three R2T/HL animals.

ELISAs. ELISAs to detect serum and supernatant isotype secretion were carried out as reported previously (33). For all analyses except IgE, we used isotype-specific antibodies purchased from either Southern Biotechnology Associates, Inc. (Birmingham, AL) or Zymed Laboratories, Inc. (South San Francisco, CA). IgE-specific antibodies were obtained from PharMingen and used at a 1:500 dilution. Immulon 1 (Dynatech Laboratories, Inc., Chantilly, VA) 96-well plates were used to detect serum isotypes, and Immulon 2 (Dynatech Laboratories, Inc.) 96-well plates were used to detect supernatant isotypes. Cultures were established in duplicate for each assay. Mice ranged in age from 4 to 8 wk, and culture supernatants were assayed at 5 d of stimulation.

Preparation of RNA and Germline Transcript Amplification. RNA was prepared using the TRIZOL reagent (GIBCO BRL, Gaithersburg, MD) as per the instructions of the manufacturer. cDNA was generated using Superscript (GIBCO BRL), again as instructed by the manufacturer. 1/10 the total cDNA was amplified using primers for Iγ-1, Cγ-1, and mb-1 as described elsewhere (39).

Digestion–Circularization PCR. Genomic DNA was prepared from cells obtained from day 3 splenic cultures, using the QIAamp kit (QIAGEN Inc., Chatsworth, CA). 1 μg of genomic DNA from each sample was digested with EcoR1 in a total volume of 100 μl. To favor circular ligation products, 250 ng of the digested DNA was placed in a T4 DNA ligase reaction volume of 50 μl. Primers and PCR reaction conditions are described elsewhere (40), and were followed as described. The PCR products were separated on a 10% polyacrylamide gel and stained with ethidium bromide. The semiquantitative nature of each digestion–circularization (DC)-PCR was established using two- to fivefold dilutions of sample DNA along with murine 129 genomic DNA to produce a final total amount of 100 ng DNA per reaction.

Results

Rescue of the Peripheral B Cells in K70T Mice with Ig HC and LC Transgenes. Prior studies have demonstrated the absence of peripheral $sIgM^+$ B cells in K70T mice in association with impaired V(D)J recombination (27, 29). To circumvent this block, we generated, by backcrossing, K70T mice which contained an Ig HC locus that harbors a rearranged HC variable region gene in place of the endogenous JH plus DQ52 locus (34) along with a λ1 LC transgene (35). We have demonstrated previously that the products expressed from these rearranged Ig HC and LC transgenes can generate a substantial number of IgM^+ B cells in an R1T or R2T background (33). In the current study, K70T, R2T, or K70T/R2T double mutant mice harboring these two productively rearranged Ig loci are referred to as K70T/HL, R2T/HL, and K70T/R2T/HL mice, respectively. Wt or K70T mice harboring the rearranged HC and LC genes are referred to as wt and wt/HL mice, respectively. We observed no significant differences in wt versus wt/HL mice other than predominant λ LC expression from the transgene in the latter.

Flow cytometric analyses of the lymphocyte populations of spleens and lymph nodes of 4–6-wk-old K70T/HL mice revealed an expected lack of T lymphocytes (not shown) but a significant reconstitution of $B220^+/IgM^+$ peripheral B cells that were also $sIg\lambda^+$ (Fig. 1). However, the numbers of sIg^+ cells in the K70T/HL mice were ~10–15% of those of wt/HL and ~25% of those of R2T/HL mice (see Fig. 1 and its legend). As expected, the K70T mice lacked both $sIgM^+$ B cells and T cells in their spleens or lymph nodes (Fig. 1, and data not shown). The splenic B cells of K70T/HL mice, like those of R2T/HL mice, appeared phenotypically normal—staining positive for surface IgM and dimly for IgD (data not shown). These findings indicate that expression of the HC and LC transgenes was suffi-

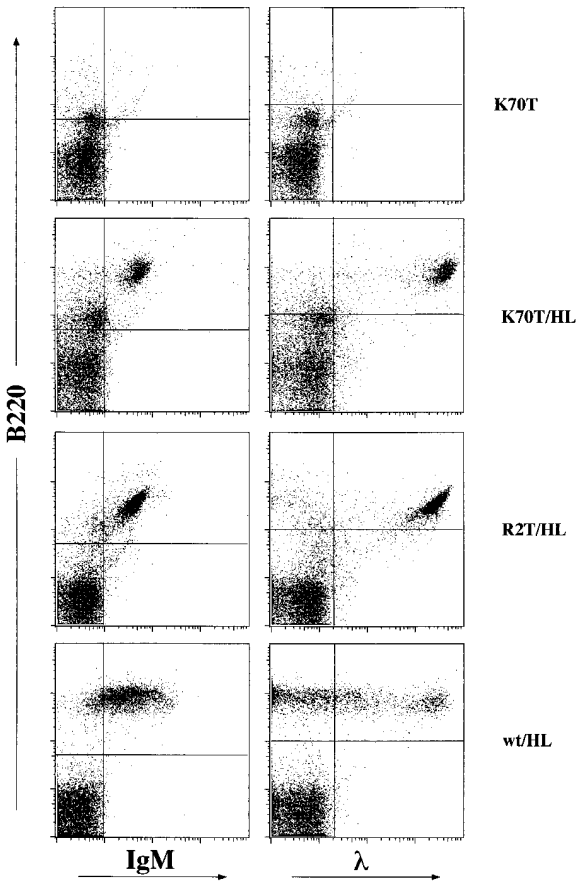

Figure 1. Reconstitution of peripheral B cells in the spleen of K70T mice. Flow cytometric analysis of splenocytes from K70T/HL mice, stained for surface IgM and Igλ, and compared with K70T, R2T/HL, and wt/HL age-matched controls. The results are representative of experiments with at least three mice of each genotype. Live $B220^+/IgM^+$ B cell counts are as follows: $1.95 \pm 0.78 \times 10^7$ in K70±T/HL, $0.73 \pm 0.44 \times 10^7$ in R2T/HL, and $0.18 \pm 0.12 \times 10^7$ in K70T/HL mice. Two R2T/K70T/HL mice were analyzed and found to have a slightly higher but not significantly different total B cell count ($0.30 \pm 0.11 \times 10^7$) compared with K70T/HL mice.

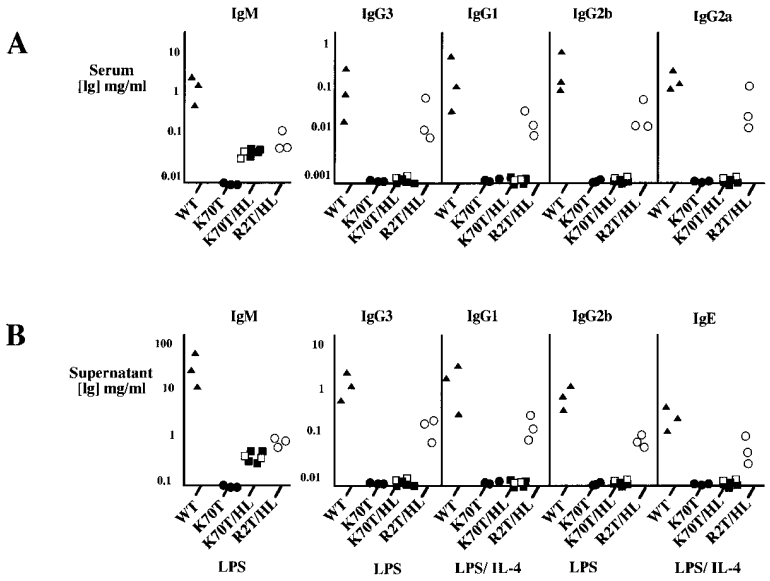

Figure 2. Detection of isotype antibody levels in the serum or in splenic culture supernatants. (*A*) Serum concentrations of three wt (*filled triangles*), three K70T (*filled circles*), four K70T/HL (*filled squares*), two R2T/K70T/HL (*open squares*), and three R2T/HL (*open circles*) mice were obtained by ELISA. All mice were between 4 and 8 wk of age. (*B*) Supernatants from splenic cultures stimulated with LPS or LPS plus IL-4 for 5 d were obtained and analyzed for the depicted Ig isotypes by ELISA.

cient to generate significant numbers of peripheral B cells in the K70T mice, although the reconstitution was not as efficient as that found in R2T/HL mice.

Reconstitution of peripheral B cells of SCID mice by expressed HC and LC transgenes is often low and variable compared with that obtained with transgenic HC and LC reconstitution of R1T or R2T mice (35, 41, 42). Some studies have suggested that the relative inefficiency and variability in transgenic reconstitution of SCID mice may result from the inability of the HC or LC transgenes to completely shut down endogenous V(D)J recombination, and as a result, that developing B cells are still lost due to unrepaired RAG-initiated DSBs (41). It is clear that the λ LC transgene used in our study does not result in a complete shutdown of endogenous κ gene rearrangement, as a substantial proportion of the splenic B cells in wt/HL mice express IgM in association with κ chains (Fig. 1, and data not shown). However, two R2T/K70/HL double mutant, Ig-transgenic mice were analyzed and found to have low numbers of peripheral B cells similar to those of the K70T/HL mice (see legend to Fig. 1). This finding suggests that factors associated with Ku70 deficiency other than inability to repair V(D)J-initiated breaks may affect B cell reconstitution in the K70T background.

Lack of Serum IgG Isotypes in K70T/HL Mice. The Ig HC locus harbored by the K70T/HL mice contains an Ig HC transgene in place of the endogenous JH locus, which has been shown previously to allow for CSR to downstream HC CH genes (33, 34). To assay for CSR in vivo, we assayed for serum Ig levels in the various lines of mice harboring the inserted HC rearrangement and the LC transgene. As observed previously, R2T/HL mice had low but significant levels of IgM; the low level production of IgM by B cells of these mice both in vivo and in vitro has been documented previously (33). However, the R2T/HL mice had substantial levels of the different IgG isotypes, indicative of class switching (Fig. 2 *A*). In contrast, the K70T/HL mice had similar levels of serum IgM as R2T/HL mice but no detectable serum IgG isotypes (Fig. 2 *A*). These data indicate that Ku70 is necessary for the accumulation of serum IgG isotypes.

Lack of Class Switching in K70T/HL Splenocytes In Vitro. To test if K70T/HL B cells could be induced to undergo CSR in vitro, splenocytes from the various mouse lines were cultured in the presence of either LPS or LPS plus IL-4. Treatment with LPS alone induces proliferation of cultured B cells accompanied by switching to IgG2b and IgG3, whereas treatment with LPS plus IL-4 induces proliferation and switching to IgG1 and IgE. Daily live cell counts revealed that K70T/HL B cells initially accumulated after the various treatments, but at a rate ~30% that of wt/HL cells (data not shown). All cultures were assayed on day 3 of the various stimulations for expression of sIgG3 (Fig. 3 *A*) or sIgG1 (Fig. 3 *B*). Cultures from wt/HL and R2T/HL mice had significant numbers of $sIgG3^+$ B cells after LPS treatment and significant numbers of $sIgG1^+$ cells after LPS plus IL-4 treatment (Fig. 3, *A* and *B*). However, there were no $sIgG3^+$ or $sIgG1^+$ cells in K70T/HL cell cultures (Fig. 3, *A* and *B*); in fact, the vast majority of the K70T/HL cells and similarly cultured K70T spleen cells were dead by day 3 of these culture conditions, in contrast to wt/HL or R2T/HL cells, which were primarily viable even in day 5 cultures (data not shown).

To further assay for class switching, supernatants from day 5 cultures were assayed for secreted Ig isotypes by ELISA. Supernatants from all of the cultures of K70T/HL and R2T/HL spleen cells contained low (compared with wt/HL cultures) but significant levels of IgM (Fig. 2 *B*). As observed previously, the LPS-cultured R2T/HL spleen cells produced substantial levels of IgG2b and IgG3, whereas LPS plus IL-4–treated R2T/HL cultures produced substantial levels of IgG1 and IgE (Fig. 2 *B*). In striking contrast, there was no detectable secretion of either type of IgG isotype or of IgE by K70T/HL spleen cells cultured under any

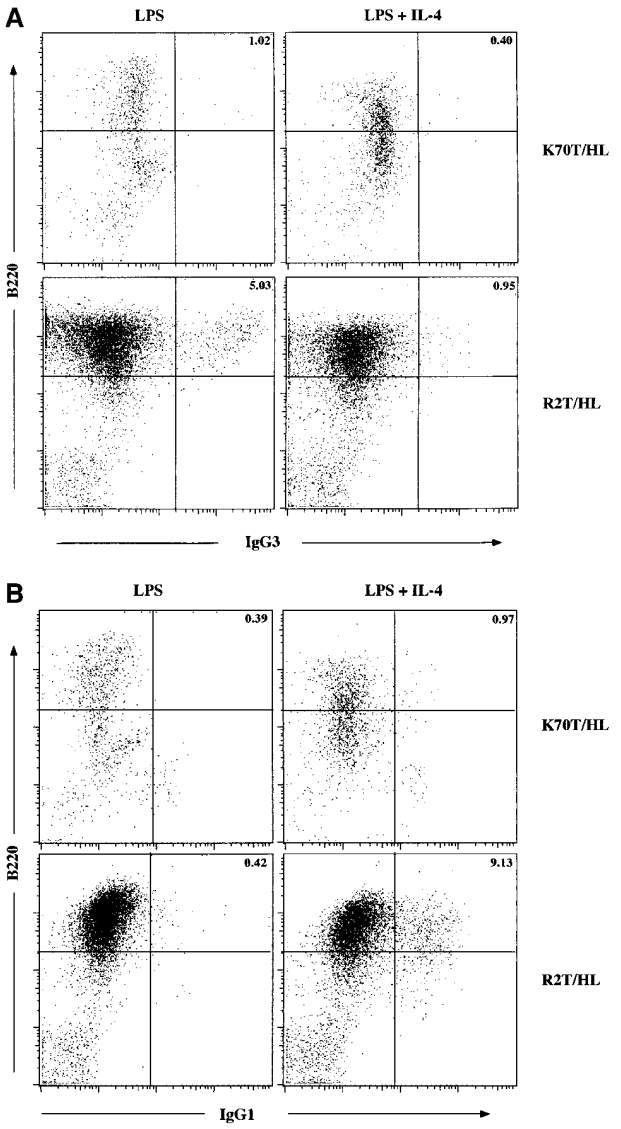

Figure 3. Surface isotype staining of stimulated day 3 splenic B cells. Splenic cultures were stimulated for 72 h with LPS or LPS plus IL-4, and stained with B220 plus IgG3 (*A*) or IgG1 (*B*). Numbers in the upper right hand corner of the panels represent percentage of live gated cells.

conditions (Fig. 2 *B*). Furthermore, supernatants from cultured R2T/K70T/HL spleen cells had IgM levels similar to those of K70T/HL cells, but also lacked expression of any IgG isotypes or IgE, showing that the lack of switching in these cultures was not do to any potential adverse effects of RAG reinduction (Fig. 2 *B*).

To test the potential relationship between proliferation, CSR, and cell death in the activated cultures of K70T/HL B cells, we tested their response to stimulation with anti-CD40 or anti-CD40 plus IL-4. Although anti-CD40 plus IL-4 induces normal B cells or R2T/HL B cells to proliferate and switch to IgG1 and IgE, treatment with anti-CD40 alone induces proliferation but does not induce class switching (43). Notably, significant numbers of live $B220^+/IgM^+$ cells were found in the anti-CD40–treated K70T/HL cultures but not in the anti-CD40 plus IL-4–treated cultures at day 3 (Fig. 4), suggesting the possibility that the observed cell death may be associated at least in part with induction of CSR as opposed to general proliferation.

Taken together, these results show that K70T/HL B cells are prone to rapid cell death and fail to undergo detectable Ig HC class switching after culture with agents that induce proliferation and CSR in normal cells.

Lack of DNA Products of CSR in Stimulated K70T/HL Spleen Cells. To assay for evidence of CSR not detectable by surface expression or Ig secretion, we used a DC-PCR assay to search for DNA products of CSR. The DC-PCR assay detects switched chromosomal DNA products of CSR in which the intervening DNA sequence has been deleted (39, 40). For this purpose, DNA isolated from day 3 splenocytes of the various cell types stimulated with either LPS or LPS plus IL-4 was digested with EcoR1, ligated under conditions that favor circularization, and assayed by PCR amplification for products of Cμ-Cγ1 CSR. There were readily detectable products of CSR to γ1 in DNA from both wt/HL and R2T/HL mice, but not in that from K70T/HL mice, even when the assay used 5–50 times more genomic DNA than was required to detect products in DNA from stimulated wt/HL cells (Fig. 5).

K70T/HL Splenic B Cells Can Be Induced to Produce HC Germline Transcripts. After appropriate activation, B cells can be induced to generate germline transcripts from the CH genes targeted for CSR; these transcripts are induced within 24 h in wt B cells and are a prerequisite for CSR (7). To test if the K70T/HL B cells responded to activation signals for CSR, we assayed the various types of spleen cells for ability to generate germline Cγ1 transcripts after LPS or LPS plus IL-4 treatment. For this purpose, total RNA prepared from day 2 splenic cultures was assayed by reverse transcription PCR amplification to estimate relative expression levels of germline γ1 and, as a control for B cell

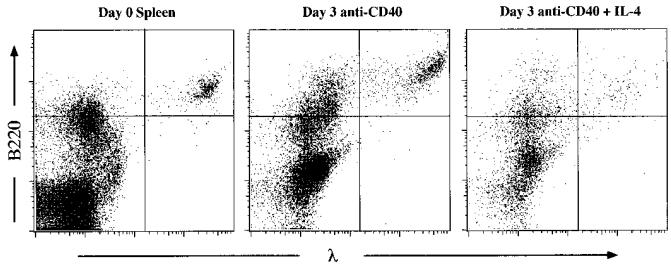

Figure 4. Day 3 in vitro–stimulated K70T/HL splenic B cells. Splenic cultures were analyzed by FACS® after a 72-h stimulation with anti-CD40 or anti-CD40 plus IL-4, for the presence of cells staining with antibodies to B220 and Igλ. Live cell gates were used in all analyses. The population of $B220^{lo}/IgM^-$ cells seen in these cultures has not been further characterized, but may correspond to cells at the pro- or pre-B cell stage (e.g., due to loss of expression of one of the transgenes), as these are also seen in K70T or R2T splenocyte populations.

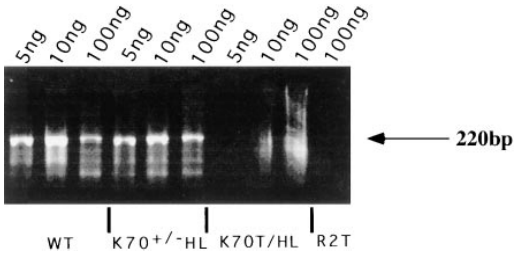

Figure 5. DC-PCR assay for Sμ to Sγ1 recombination. A semiquantitative DC-PCR assay to detect evidence of recombination to Sγ1 was used on splenic cultures after 72 h of stimulation with LPS plus IL-4. B cell DNA in the amounts noted above each lane were diluted into murine genomic DNA to keep the total DNA amount constant at 100 ng.

contribution, mb-1 transcripts (Fig. 6). At this 2-d stimulation point, levels of mb-1 transcripts from equivalent amounts of R2T or K70T RNA were <5% of those in RNA from K70T/HL or control cultures, consistent with the lack of peripheral B lineage cells in the former cultures (Fig. 6). Iγ1 germline transcripts were readily detectable in RNA from cultures of wt/HL and R2T/HL spleen cells, but as expected, not in RNA from cultures of K70T cells. Strikingly, however, the level of Iγ1 transcripts in RNA from K70T/HL cultures was comparable to those in RNA from wt/HL and R2T/HL cultures (Fig. 6). This finding indicates that K70T/HL B cells are able to respond to signals which induce CSR and generate germline transcripts, yet they fail to generate detectable evidence of CSR or its products.

Discussion

Ku70 Is Required for Normal Peripheral B Cell Functions. The use of knockout strategies for analysis of the potential function of genes required for VDJ recombination in late B cell processes such as CSR or somatic mutation has been hindered by the fact that such genes are necessary for the development of B cells beyond the pro-B cell stage where VDJ recombination occurs. In the approach used in this study, we have bypassed the required V(D)J recombination steps by providing functionally assembled Ig HC and LC genes in the form of the VDJ knock-in HC locus and an Igλ LC transgene. We have already documented the applicability of this system by using these transgenes to reconstitute peripheral B cells in R1T and R2T backgrounds (33). Similar to R1T and R2T mice, K70T mice have a SCID phenotype (27, 29). The SCID phenotype of K70T mice has been considered to result from the inability of progenitor lymphocytes to complete the V(D)J recombination reaction (27, 29). In agreement with this notion, substantial numbers of peripheral B cells were reconstituted in the K70T background as a result of expression of the transgenic HC and LC loci. This finding proves that a major portion of the SCID phenotype of K70T mice results from their inability to form functional Ig genes and is not due to other potential defects.

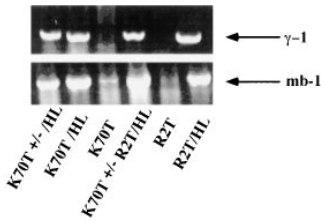

Figure 6. Cγ1 germline transcripts are detected after LPS plus IL-4 stimulation of K70T/HL B cells. Splenic cultures were assayed at 48 h for the presence of germline transcripts arising from Iγ1-Cγ1. Previously described methods (43) were used with mb-1–derived transcripts to control for the total number of B cells. PCR products were separated on a 10% polyacrylamide gel and stained with ethidium bromide.

However, it is notable that the numbers of B cells in the spleens of the K70T/HL mice were significantly lower than those of wt/HL mice, and on average, three to five times less than those of R2T/HL mice. The latter finding implies that factors in addition to the VDJ recombination defects and the lack of T cells (which are likewise absent from R2T/HL mice) also contribute to impaired B cell development in these mice. In this context, one possibility is that RAG-induced DNA breaks still occur at significant levels in differentiating K70T/HL mice but are not repaired, and lead to cell death (27, 35, 41). In this regard, it is clear that the λ LC transgene used does not fully exclude endogenous κ gene rearrangement, as evidenced by the substantial number of κ^+ cells in the spleens of wt/HL mice (Fig. 1, and data not shown). In addition, several recent reports have shown that RAG-1 and RAG-2 are reactivated in germinal center B cells and in splenic B cells stimulated in vitro with activators and cytokines (44–47). Thus, unrepaired DSBs generated by reactivation of RAG might also contribute to the loss of mature B lineage cells both in vivo and in vitro. However, we have observed similarly decreased numbers of peripheral B cells in R2T/K70T/HL double mutant mice and similar death of in vitro–stimulated R2T/K70T/HL B cells (Fig. 1, and data not shown), suggesting that major aspects of the defects in peripheral B cell numbers and activation are not secondary to effects of DSBs generated by RAG proteins. In this regard, additional defects, as exemplified by the growth defects and early senescence phenotypes of K70T fibroblasts (27, 29), may also contribute to impaired reconstitution due to affects on processes not directly related to Ig gene rearrangement or switching.

Ku70 Deficiency Impairs Class Switching by B Lymphocytes. Because the Ig HC locus we used to rescue B cell development in the K70T background was a knock-in locus in which the chromosomal JH region was replaced with a functionally rearranged V(D)J segment, we have been able to test the requirement for Ku70 with respect to CSR. Moreover, we have already established the efficacy of this approach in monoclonal B cell populations in the absence of T cells, by showing that R1T or R2T B cells can undergo relatively normal CSR on this chromosome (33). Thus, our current studies have clearly shown that K70T B cells have a major impairment in ability to undergo Ig HC class switching both in vivo and in vitro, demonstrating that Ku70 is required, either directly or indirectly, for ef-

fecting the CSR process. Parallel studies of Ku80-deficient mice that used the same HC knock-in transgene as in our studies have led to nearly identical findings and conclusions regarding the requirement for Ku80 in class switching (48). Together, these studies imply that defects found in the context of Ku70 or Ku80 deficiency likely stem from the function of these proteins in the Ku70/Ku80 complex.

Although we cannot unequivocally rule out the possibility that Ku70 deficiency negatively impacts on class switching via pathways not directly related to the CSR process, several findings argue against most conceivable indirect mechanisms, and thereby support a direct role. Thus, the possibility that Ku70 deficiency impairs initial signaling events needed for CSR has been ruled out by our demonstration that appropriately activated splenic B cells from the K70T/HL mice were able to induce germline CH transcripts. Another possibility by which Ku deficiency affects CSR in an indirect way is that K70T/HL B cells die or arrest (as seen in K70T or K80T fibroblasts [27, 29, 31]) before they are able to undergo CSR. In this regard, activated B cells usually undergo at least three cell cycles before downstream isotypes appear on their surface (49). However, this notion is countered by our observation that K70T/HL B cells activated with anti-CD40 alone, which does not stimulate CSR, exhibited significant proliferative capacity, and many were viable even after a 3-d stimulation (Fig. 4). Together, these findings are most consistent with a role for Ku70, in the context of the Ku70/80 complex, in the actual CSR process itself. Such a role would also be consistent with the finding that Ku70 and Ku80 are induced in B cells undergoing CSR (50).

Implications for the CSR Mechanism. In exploring potential roles for Ku in CSR, one must consider both its known requirement for end-joining and its potential function in the context of the DNA-PK enzyme (3). However, it is not clear how Ku functions in end-joining reactions such as those used in coding end or recognition sequence signal end-joining in V(D)J recombination. In such reactions, it has been speculated that the Ku complex might serve an end-protective function or via its intrinsic helicase activities, might be involved in end-processing (16–18, 31, 51–55). Another potential function is end-alignment or synapse formation (30, 51, 53, 55); recent studies have shown that Ku can bring DNA ends together in vitro (56). Ku also has been speculated to function in end-joining by activating the DNA-PKcs, which then could function to promote additional relevant processes (17, 19). A DNA-PK–related function (either direct or indirect) for Ku in CSR would go along with a role for DNA-PKcs in this process, as indicated by finding that CSR could not be induced in SCID pro-B cell lines (25). In the context of the potential leakiness of B cell development and switching in older SCID mice (26), it is possible that we might observe leaky switching in K70T/HL mice if older animals were examined.

The mechanisms and genes involved in CSR remain unknown. Based on the observation that S regions share long stretches of repetitive sequences, it was proposed that some type of homology-mediated recombination process, potentially based on short or imprecise homologies, may play a role in CSR (for a review, see reference 4). Consistent with this notion, RAD51, a member of the RAD52 DNA repair pathway involved in homologous recombination, was found to be specifically expressed and localized to the nucleus of B cells carrying out CSR (57). However, mice lacking RAD54, another member of the RAD52 repair pathway which mediates DNA pairing and strand exchange, appeared to have normal class switching (58). Therefore, the role of homology-mediated joining in CSR remains to be further elucidated. In this regard, Ku has been linked to end-joining reactions in both yeast and mammalian cells (59–61), but has not been found to be required for the homologous recombination reactions also used to repair DNA breaks in these organisms (62, 63). Based on previous studies of Ku-deficient lymphocytes and fibroblasts (27, 29–31), our favored scenario to explain the effects of Ku deficiency on class switching is that stimulated Ku70-deficient B lymphocytes attempt to undergo CSR but die or exit the cell cycle due to inability to complete this reaction in the absence of Ku. Given what is known about functions of Ku, this model would imply the use of an end-joining reaction in some phase of this process. Such a CSR intermediate has been suggested from the finding of DSBs in S regions of B cells induced to undergo class switching (14).

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