

NF- κ B RelA-deficient Lymphocytes: Normal Development of T Cells and B Cells, Impaired Production of IgA and IgG1 and Reduced Proliferative Responses

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Summary

To investigate the function of NF- κ B RelA (p65), we generated mice deficient in this NF- κ B family member by homologous recombination. Mice lacking RelA showed liver degeneration and died around embryonic day 14.5. To elucidate the role of RelA in lymphocyte development and function, we transplanted fetal liver cells of 13.5-day embryos from heterozygote matings into irradiated SCID mice. Within 4 weeks, both T and B cells had developed in the SCID mice receiving *relA*^{-/-} fetal liver transplants, similar to the *relA*^{+/+} and *relA*^{+/-} cases. T cells were found to mature to Thy-1⁺/TCR $\alpha\beta$ ⁺/CD3⁺/CD4⁺ or CD8⁺, while B cells had the ability to differentiate to IgM⁺/B220⁺ and to secrete immunoglobulins. However, the secretion of IgG1 and IgA was reduced in RelA-deficient B cells. Furthermore, both T and B cells lacking RelA showed marked reduction in proliferative responses to stimulation with Con A, anti-CD3, anti-CD3+anti-CD28, LPS, anti-IgM, and PMA+calcium ionophore. The results indicate that RelA plays a critical role in production of specific Ig isotypes and also in signal transduction pathways for lymphocyte proliferation.

The NF- κ B family of transcription factors are conserved from flies to humans and regulate the expression of a wide variety of cellular and viral genes (reviewed in reference 1). Biochemical and molecular characteristics of NF- κ B and their activation pathways have been extensively studied, especially in terms of immune and acute phase responses. The mammalian NF- κ B proteins, RelA (p65), c-Rel, RelB, p50/p105, and p52/p100, share the “rel” homology domain which facilitates dimerization, nuclear translocation and DNA binding. Transactivation domains are also present at the COOH termini of RelA, c-Rel, and RelB. These NF- κ B proteins form multiple interchangeable heterodimers and homodimers and their activity is regulated by binding of I κ B inhibitory factors which determine the localization of NF- κ B dimers, either in the cytoplasm or in the nucleus. Upon activation, NF- κ B dimers dissociate from I κ B and translocate to the nucleus and then bind to the κ B sites in promoters and enhancers of NF- κ B responsive genes, consequently activating their transcription.

The RelA/p50 heterodimer was the original NF- κ B identified, as a transcription factor for Ig κ light chain gene (2), and has the strongest transactivating capacity among NF- κ B dimers as well as the most widely distributed κ B

binding activity (3). In the B cell lineage, RelA/p50 is the major NF- κ B in pre-B cells, while c-Rel/p50 is predominant in mature B cells and RelB/p52 in plasmacytomas and LPS-activated B cells (4). This suggests that RelA/p50 plays an important role in certain steps of B cell development, although genes regulated by RelA/p50 have yet to be identified. In the T cell lineage, RelA/p50 has been reported to be critical for antigen activation (5) and cytokine production (3). Studies *in vitro* have suggested that RelA/p50 regulates expression of the T cell receptor β chain gene (6), cytokine genes such as IL-2 (7), IL-6 (8), and TNF α (9), and the IL-2 receptor α chain gene (10). However, because of the presence of several related proteins and their pleiotropic effects, the specific roles of RelA *in vivo* remain to be elucidated. Studies on the functions of other NF- κ B proteins have faced similar problems. To overcome this drawback, mutant mice lacking RelA (11), c-Rel (12), RelB (13), p50 (14), or I κ B α (15) have been derived by homologous recombination to assess specific functions of individual NF- κ B proteins. All except RelA-deficient mice demonstrate normal birth and development but with certain abnormalities in immune responses. In the case of RelA deficiency, however, embryonic mortality occurs, concomi-

tant with liver degeneration (11), so that clarification of the function of this family member in the immune system has faced difficulties.

In the present study, we generated RelA-deficient mice with a targeting vector expected to yield a null mutation, different from the vector used in the previous study which would be expected to produce a truncated form of RelA (11). Our RelA-deficient mice also died during embryogenesis but in an attempt to explore the role of RelA in lymphoid development, we transplanted the fetal liver cells from *relA*^{-/-} embryos into SCID mice and found that the RelA-deficient stem cells could then differentiate to mature T and B cells. To investigate the roles of RelA further, we examined RelA-deficient T and B cells for their functions and their proliferative responses to various stimuli.

Materials and Methods

Construction of the Targeting Vector. The mouse *relA* gene was isolated from a C57BL/6 (B6)¹ mouse genomic library using a mouse *relA* cDNA probe (codons 185-277, reference 16). The targeting vector was constructed in pBluescript as shown in Fig. 1. It contained 7 kb of the mouse *relA* gene including exons 1 to 6, *PMCI-neo* inserted into the first exon of *relA* at an NcoI site 3 bp downstream of the translation initiation codon, and the herpes simplex virus-thymidine kinase gene (*HSV-tk*) flanking at the 3' end of the *relA* sequence. We expected that this targeting vector would generate a null mutant allele by homologous recombination.

Derivation of *relA*-deficient Mice. 20 µg DNA of the targeting vector was transfected into 5 × 10⁷ CCE embryonic stem (ES) cells (kindly provided by Dr. Motoya Katsuki, Institute of Medical Science, University of Tokyo, Tokyo, Japan [17]) by electroporation (T-300; Biotechnologies & Experimental Research Inc., San Diego, CA). Transfected cells were cultured for 10 d in positive-negative selection medium (18) containing G418 (400 µg/ml; Sigma Chem. Co., St. Louis, MO) and Gancyclovir (5 µM, Demosine; F. Hoffmann-La Roche Ltd., Palo Alto, CA, provided by Tanabe Seiyaku Co. Ltd., Osaka, Japan). Growing colonies were tested for homologous recombination by DNA blot analysis using 5' and 3' flanking region probes. Eight clones with the targeted allele were obtained and injected into B6 blastocysts. The blastocysts were then transferred into the uterus of pseudopregnant Jcl: MCH(ICR) (MCH) mice. Three clones produced chimeric mice which transmitted a mutant allele to offspring by mating with B6 mice. Three *relA* mutant mouse strains, RKO-1, -2, and -3, were maintained by brother-sister mating of heterozygous mice in our animal facility. All three strains showed identical phenotypes and RKO-1 mice were mainly used in this study. B6 and MCH mice were purchased from Japan SLC Inc. (Hamamatsu, Japan) and Clea Japan Inc. (Tokyo, Japan), respectively.

DNA and RNA Blot Analyses. DNA and total RNA were isolated using the proteinase K/SDS and the guanidium thiocyanate/CsCl procedures, respectively (19, 20). DNA was digested by restriction enzymes and separated by agarose gel electrophoresis

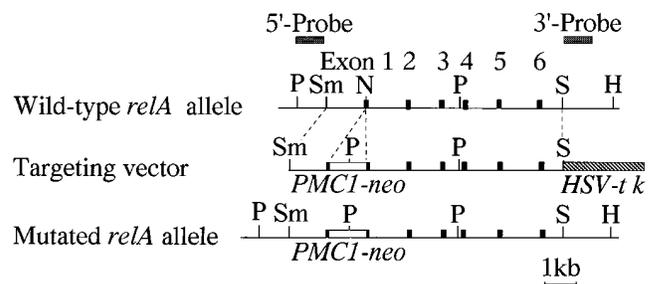


Figure 1. Structure of the *relA* targeting vector. The wild-type mouse *relA* allele is shown at the top. The targeting vector is in the middle and the predicted mutant allele is at the bottom. The area predicted to undergo homologous recombination is indicated by the dotted lines. Exons are indicated by closed boxes. The probes used for diagnostic DNA blot analysis are also indicated at the top. Restriction enzyme sites: H, HindIII; N, NcoI; P, PstI; Sm, SmaI; S, SphI.

and then transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). Total RNA was separated by 2.2 M formaldehyde agarose gel electrophoresis and transferred to nitrocellulose filters. RNA blots were analyzed by cDNA probes for *relA*, *c-rel* (codons 144-277, reference 21), *p50* (codons 391-518, reference 22) and *relB* cDNA (codons 458-580, reference 23). DNA blots were analyzed with 5' and 3' flanking region probes (see Fig. 1).

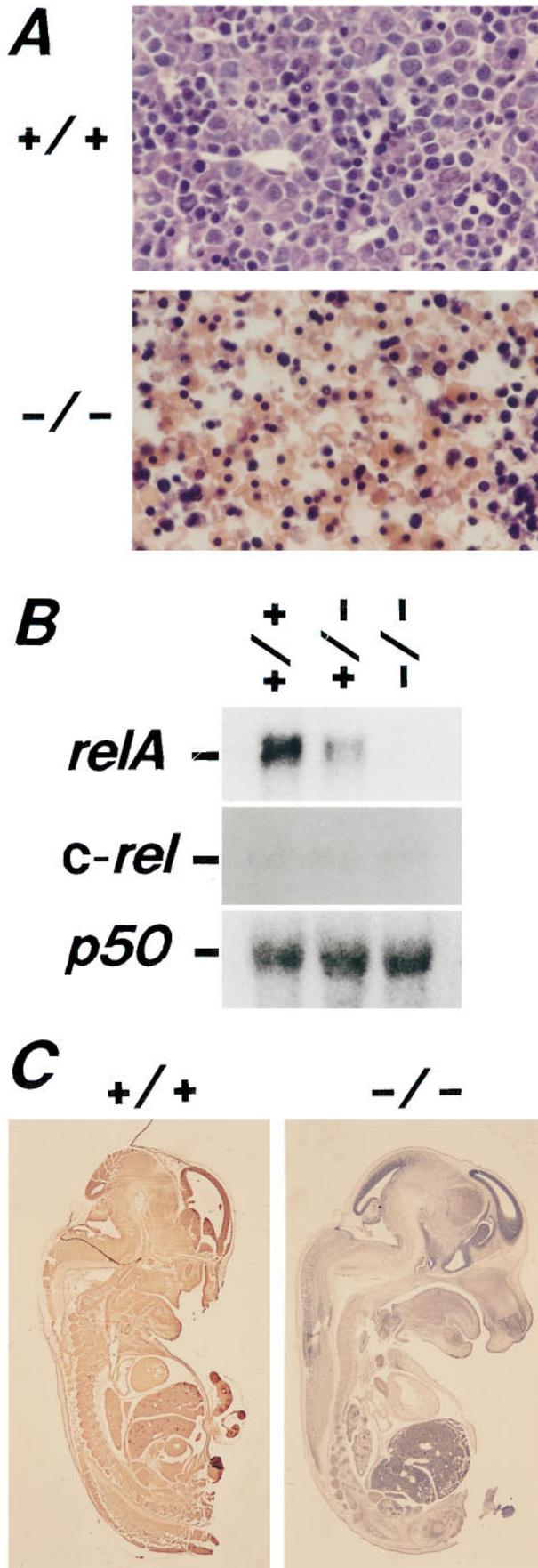
Immunohistochemistry and Histology. Whole embryos were fixed in buffered formalin and embedded in paraffin. Sagittal sections (5 µm) were reacted with rabbit anti-RelA specific antibody (no. sc-372; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and binding sites were visualized by an avidin-biotin enzyme complex (ABC) method (Vectastain *Elite* ABC kit; Vector Laboratories Inc., Burlingame, CA), and then counterstained in hematoxylin. For histological examination, the sections were stained also with hematoxylin-eosin (HE).

Transplantation of Fetal Liver Cells. SCID mice between 5-8 wk age were irradiated (2.5 Gy; Hitachi MBR-1520R; Hitachi, Tokyo) and each injected intravenously with 3 × 10⁶ fetal liver cells from ED13.5 *relA*^{+/+}, *relA*^{+/-}, or *relA*^{-/-} embryos. 4 wk after the transplantation, these mice were used for experiments. SCID mice contain very few lymphocytes in the thymus, spleen and lymph nodes (24), due to a defect in the gene coding for DNA-dependent protein kinase p350 (25). SCID mice were maintained in our breeding colony.

Serological Analysis. Two-color analysis of cell surface antigens was performed with a FACScan[®] (Becton Dickinson and Co., Mountain View, CA). Thymocytes and spleen cells were stained with antibodies to H-2K^b (E121.46; Seikagaku Kogyo, Tokyo, Japan), Thy-1.2 (30-H12; Becton Dickinson and Co.), TCRαβ (H57-597; provided by Dr. R. T. Kubo, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO [26]), CD3 (145-2C11; provided by Dr. J. A. Bluestone, The University of Chicago, IL [27]), CD4 (GK1.5; obtained from Dr. N. Shinohara, Mitsubishi Kasei Institute for Life Science, Machida, Japan, [28]), CD8 (53-6.7; obtained from Dr. N. Shinohara [29]), CD25 (IL-2Rα, 7D4; reference 30), CD44 (Pgp-1, NU5-50; Seikagaku Kogyo), B220 (RA3-6B2; Pharmingen, San Diego, CA), and IgM (DAKO, Glostrup, Denmark).

ELISA for Measurement of Levels of Serum Ig. For measurement of IgM, IgG1, IgG2a, IgG2b, IgA, and IgE, 96-well flat-bottom plates (Immuno Plate 430341; Nunc, Roskilde, Denmark) were pre-coated with affinity-purified goat anti-mouse Igκ (5 µg/ml, 100 µl/well; Bethyl Laboratories Inc., Montgomery, TX). For

¹Abbreviations used in this paper: ABC, avidin-biotin enzyme complex; B6, C57BL/6; ED, embryonic day; ES, embryonic stem; HE, hematoxylin-eosin; *HSV-tk*, herpes simplex virus-thymidine kinase; MCH, Jcl: MCH(ICR); TUNEL, TdT-mediated dUTP nick end labeling.



Ig κ , plates were coated with affinity-purified rabbit anti-mouse IgG (5 μ g/ml, 100 μ l/well; Southern Biotechnology Associates, Inc., Birmingham, AL). Diluted serum samples and standard Ig were added and bound Ig were detected with horseradish peroxidase-labeled affinity-purified goat anti-mouse Ig isotype-specific antibodies or an anti- κ light chain-specific antibody (Southern Biotechnology). *o*-Phenyldiamine solution (0.04%; Sigma) was added to each well as a substrate. The optical density at 490 nm was measured with a microplate reader (model 3550; Bio-Rad, Hercules, CA).

IL-2 Bioassay. 3×10^5 spleen cells from transplanted mice were plated in 96-well plates (200 μ l per well). Con A (2 μ g/ml; Boehringer Mannheim GmbH, Mannheim, Germany), anti-CD28 (1 μ g/ml; Caltag Laboratories, South San Francisco, CA), LPS (20 μ g/ml; Sigma), anti-IgM (60 μ g/ml; Capel Research Products, Durham, NC), and PMA (10 ng/ml; Sigma) plus calcium ionophore (100 ng/ml; Sigma) were added to the medium. For anti-CD3 antibody stimulation, plates were pre-coated with the antibody (10 μ g/ml). After 18 h of culture, the supernatants were collected for the assay. To measure the levels of IL-2, serially diluted culture supernatants were added to IL-2-dependent NRB cells (5×10^3 , reference 31) in 96-well plates. NRB cells were cultured for 44 h and their proliferation was quantitated using a Cell-Titer 96TM Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and a microplate reader (model 3550; Bio-Rad). Recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was used as a standard.

Proliferation Assay. Conditions for cell culture and stimulation were the same as for the IL-2 bioassay. After 48 h of culture, the proliferation response was measured by uptake of [³H]thymidine (New England Nuclear, Boston, MA) over a 16-h pulse. To determine the percentage of cells in apoptosis during the course of the proliferation assays, cells at 0, 24, 48, and 64 h after stimulation were stained by the TdT-mediated dUPT nick labeling (TUNEL) technique (32, 33), using an In Situ Cell Death Detection Kit (Boehringer Mannheim GmbH) and the results assessed by FACScan[®].

Results and Discussion

Requirement of RelA for Embryonic Development of the Mouse. Mice heterozygous for a disrupted *relA* were normal and fertile, but no homozygous *relA*-deficient mice were born from heterozygote mating. Sequential DNA blot analysis and histological examination of embryos from timed

Figure 2. Liver degeneration and the absence of *relA* transcripts and RelA protein in *relA*^{-/-} embryos. (A) Histological features of livers of ED14.5 *relA*^{+/+} and *relA*^{-/-} embryos. In the liver of a *relA*^{+/+} embryo, hepatocytes with large cell size and light nuclear staining are mixed with the hematopoietic cells with dark nuclear staining. In the liver of *relA*^{-/-} embryos, hepatocytes are disintegrated, while the hematopoietic cells are apparently normal. Magnification is 240-fold. (B) RNA blot analysis of ED13.5 *relA*^{+/+}, *relA*^{+/-}, and *relA*^{-/-} embryos. 10 μ g of total RNA were loaded per lane and analyzed with the *relA*, *c-rel*, *relB*, and *p50* cDNA probes. *relA* transcripts were present in the *relA*^{+/+} and *relA*^{+/-} embryos, but not in the *relA*^{-/-} embryos. The *relA*^{+/+}, *relA*^{+/-} and *relA*^{-/-} embryos expressed similar amounts of *c-rel* and *p50* transcripts. No *relB* transcripts were detected in ED13.5 embryos (data not shown). (C) RelA protein in ED13.5 *relA*^{+/+} and *relA*^{-/-} embryos. With rabbit anti-RelA antibody and the ABC method, RelA protein is ubiquitously detected in the *relA*^{+/+} embryo, but is completely absent in the *relA*^{-/-} embryo. Magnification is eightfold.

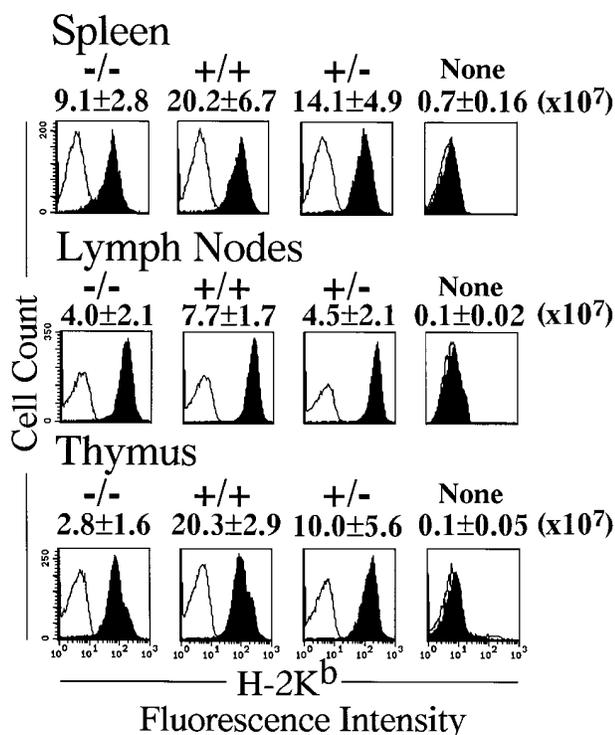


Figure 3. The fetal liver origin of lymphocytes in transplanted SCID mice. By transplantation of the fetal liver from *relA*^{+/+}, *relA*^{+/-}, or *relA*^{-/-} ED 13.5 embryos into irradiated SCID mice, the number of lymphocytes increased. Average numbers of the cells in the spleen, lymph nodes, and thymus in groups of three mice receiving transplants are indicated above the distribution plots. Staining in the presence and absence of mAb to H-2K^b is indicated by solid and open curves, respectively. Flow cytometric analysis of spleen cells, lymph node cells, and thymocytes showed the expression of H-2K^b, indicating the fetal liver origin.

matings of heterozygous mice were conducted. Until embryonic day (ED) 13.5, all embryos were apparently normal and homozygous mutants (*relA*^{-/-}) were present in an expected ratio. On ED14.5, the *relA*^{-/-} embryos were still present but some showed signs of abnormalities in their liver (Fig. 2 A). On ED15.5, a portion of the embryos became necrotic and were typed homozygous mutant (*relA*^{-/-}), while normal embryos were all wild type (*relA*^{+/+}) or heterozygous (*relA*^{+/-}). RNA blot analysis of *relA*^{+/+} embryos from ED8.5 until birth as well as CCE ES cells showed the presence of *relA* transcripts, while no *relA* transcripts could be detected in *relA*^{-/-} embryos at any stage (Fig. 2 B). Positive immunostaining with anti-RelA antibody correlated with the presence of RNA transcripts, showing that RelA proteins were present in almost all tissues of ED13.5 *relA*^{+/+} embryos, while no staining of *relA*^{-/-} embryos (Fig. 2 C). Although a different vector construct was used in our study, the generated RelA-deficient mice showed the same phenotype as those of Beg et al. (11), indicating that RelA is essential for embryonic development of the mouse. In contrast to RelA-deficient mice, mice lacking other NF- κ B proteins are known to develop normally at least until birth (12–14). The difference may simply reflect the fact that RelA is expressed ubiquitously from an early

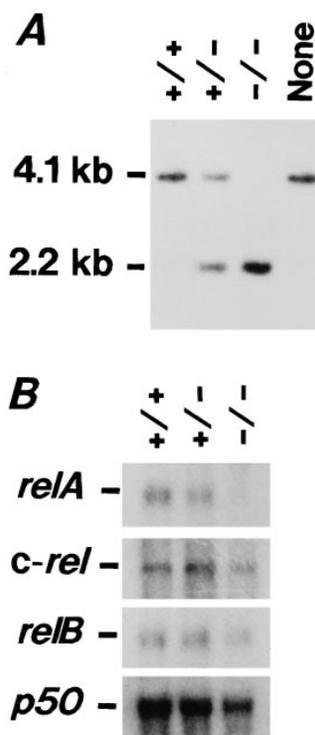


Figure 4. DNA and RNA blot analyses of the spleen cells of the transplanted SCID mice. (A) DNA blot analysis. DNA digested by PstI was analyzed with a 5' flanking probe (see Fig. 1); the wild-type *relA* allele yielding a 4.1-kb fragment, and the mutant allele a 2.2-kb fragment. (B) RNA blot analysis. Note the lack of *relA* transcripts in the spleen cells of mice transplanted with *relA*^{-/-} fetal liver cells. The *relA*^{-/-}, *relA*^{+/+}, and *relA*^{+/-} spleen cells express similar amounts of *c-rel*, *relB*, and *p50* transcripts.

stage of development while the others are expressed in restricted tissues from a much later stage (34, 35). Identification of RelA responsive genes in developing embryos, especially in the liver, should open new avenues for elucidation of the function of NF- κ B in embryonic development.

Transplantation of *relA*^{-/-} Fetal Liver Cells. During normal embryonic development, hematopoietic stem cells emerge in the fetal liver on ED9.5 (36). To test whether the RelA-deficient hematopoietic stem cells can develop in the fetal liver and also whether they can differentiate to mature lymphocytes, fetal liver cells of ED13.5 embryos were transplanted into irradiated SCID mice. Transplantation of fetal liver cells not only from *relA*^{+/+} and *relA*^{+/-} but also from *relA*^{-/-} embryos greatly increased the number of cells in the thymus, spleen and lymph nodes of SCID mice. The numbers of cells in spleen and lymph nodes of the mice transplanted with *relA*^{-/-} fetal liver cells were similar to those receiving either *relA*^{+/+} or *relA*^{+/-} fetal liver cells. The thymus from mice transplanted with *relA*^{-/-} fetal liver cells contained fewer cells than those with wild-type or heterozygous fetal liver cells. The origin of the lymphocytes in transplanted mice was determined by testing the expression of H-2K^b antigen. The fetal liver cells were from crosses between 129 and B6, both of which express H-2K^bD^b, while the recipient SCID themselves express H-2K^bD^d. As shown in Fig. 3, >95% of lymphocytes of mice transplanted with fetal liver cells expressed H-2K^b, indicating that they were definitely of fetal liver origin. The donor origin of the lymphocytes in transplanted mice with fetal liver cells was further confirmed by the presence of disrupted *relA* genes and the absence of *relA* transcripts (Fig. 4).

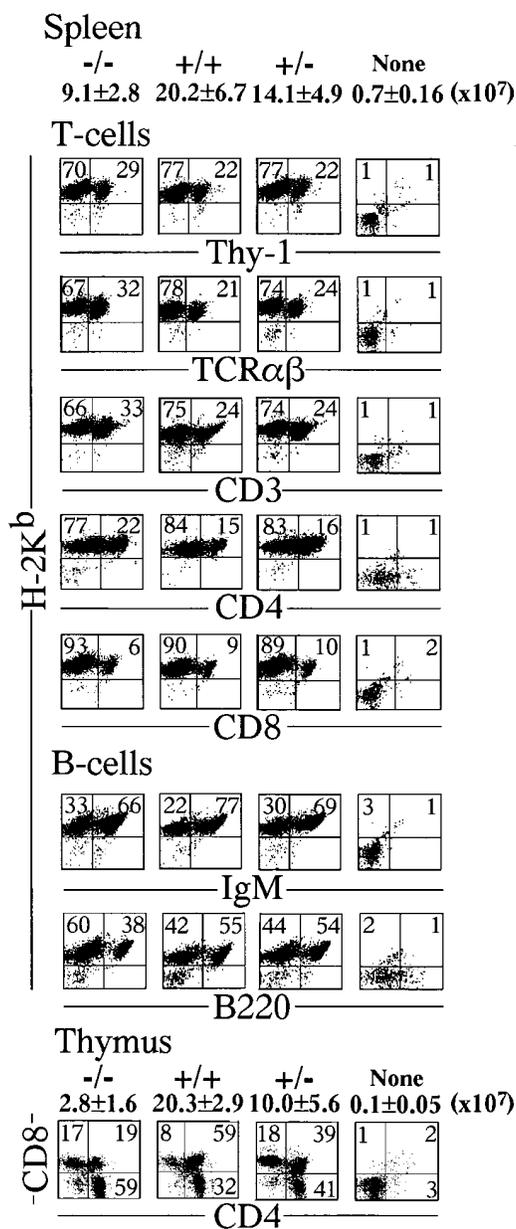


Figure 5. Surface antigen profiles of spleen cells and thymocytes of SCID mice transplanted with ED13.5 fetal liver cells. Cells were examined by two-color staining using various combinations of antibodies. The expression of H-2K^b by spleen cells confirms their fetal liver origin. As for T cell markers, Thy-1, TCRαβ, CD3, CD4, and CD8 were examined and as for B cell makers, IgM and B220 were examined. Even *relA*^{-/-} fetal liver cells develop normally to mature T and B cells in the transplanted SCID mice.

Thus, these results indicated that RelA-deficient hematopoietic stem cells can indeed develop in the fetal liver and also proliferate in SCID mice.

Development of T and B Cells in the Absence of RelA. To test whether RelA-deficient stem cells can differentiate into T and B cells, the cell surface markers on lymphocytes of transplanted mice were examined. As shown in Fig. 5, the

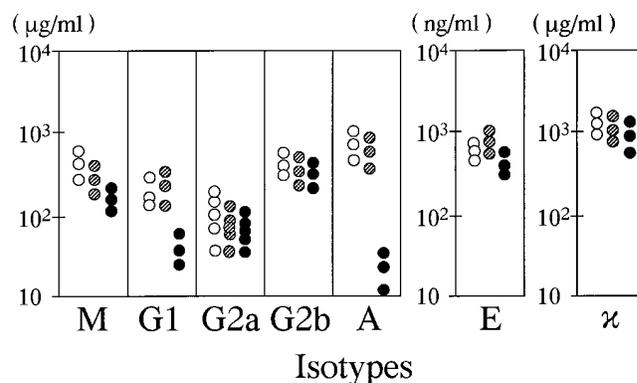


Figure 6. The serum Ig levels in SCID mice transplanted with fetal liver cells. 4 wk after the transplantation, mice with *relA*^{-/-} fetal livers were able to secrete Ig in their sera, with the total amount being comparable to those with *relA*^{+/+} or *relA*^{+/-} cells, but the IgA and IgG1 levels were found to be significantly lower. SCID mice secrete hardly detectable levels of any Ig isotypes. Open, striped, and closed circles correspond to mice transplanted with *relA*^{+/+}, *relA*^{+/-}, and *relA*^{-/-} fetal liver cells, respectively.

relA^{-/-} hematopoietic stem cells differentiated to Thy-1⁺/TCRαβ⁺/CD3⁺/CD4⁺ or CD8⁺ T cells and IgM⁺/B220⁺ B cells in the periphery, similarly to the *relA*^{+/+} and *relA*^{+/-} stem cells. In addition, testing of the expression of activation markers of T cells, IL-2Rα (CD25) and CD44 (Pgp-1), revealed 6.6 and 2.5% of *relA*^{-/-} spleen cells to be positive, respectively, with no significant differences from the *relA*^{+/+} or *relA*^{+/-} cases (data not shown). The results with cells in lymph nodes were essentially identical to those of spleens (data not shown). The *relA*^{-/-} thymuses with much fewer cells than those of *relA*^{+/+} and *relA*^{+/-}, showed reduction of the CD4⁺/CD8⁺ immature population, which may have been caused by the absence of RelA. Altogether, these results suggested that RelA is not necessary for the maturation of lymphocytes or that the loss of RelA function can be compensated for by other members of the NF-κB family. In this regard, it was interesting to note that none of the mice deficient in any NF-κB subunit, whether RelA, c-Rel, RelB, or p50, showed abnormality in the development of T cells and B cells. There is a vast amount of evidence indicating the importance of NF-κB in lymphocyte development (for review see references 1, 3, 37). Thus, it is most likely that the development of T and B cells proceeds with certain combinations of NF-κB subunits and may not require the presence of specific NF-κB dimers.

Reduced Production of IgG1 and IgA by *relA*^{-/-} B Cells. As the RelA-deficient B and T cells matured normally, the role of RelA in lymphocyte function was examined. To assess B cell function, the levels of serum Ig isotypes in SCID mice transplanted with fetal liver cells were measured (Fig. 6). The results showed that *relA*^{-/-} B cells were capable of secreting Ig as well as switching classes of Ig isotypes. The levels of total Ig and individual classes of IgM, IgG2a, IgG2b, IgE, and Igκ produced by *relA*^{-/-} B cells were

Table 1. Levels of IL-2 Production by Spleen Cells from SCID Mice Transplanted with Fetal Liver Cells in Response to Various Stimuli

<i>relA</i> genotype of fetal liver donor*	Stimuli [†]					
	Con A	Anti-CD3	Anti-CD28	Anti-CD3 + Anti-CD28	PMA + calcium ionophore	None
	IL-2 levels U/ml [§]					
+/+	167 ± 16.2	63.7 ± 10.3	5.7 ± 0.84	446 ± 19.8	361 ± 69.8	4.5 ± 1.42
+/-	146 ± 18.5	42.8 ± 5.27	5.2 ± 0.82	448 ± 10.9	312 ± 26.1	2.0 ± 0.05
-/-	118 ± 16.8	60.3 ± 8.11	6.5 ± 1.08	402 ± 88.0	276 ± 42.3	2.4 ± 0.32
None	1.5 ± 0.66	3.4 ± 1.03	2.3 ± 0.62	5.2 ± 1.84	6.5 ± 1.32	1.4 ± 0.32

*3 × 10⁶ fetal liver cells from *relA* +/+, +/-, or -/- embryos were transplanted into irradiated SCID mice.

[†]3 × 10⁵ spleen cells were stimulated as described in the Materials and Methods.

[§]The levels of IL-2 were measured by bioassay using NRB cells (see the Materials and Methods).

^{||}Mean ± SD. The results obtained from three mice were averaged.

comparable to those with *relA*+/+ or +/- B cells. Although RelA was originally identified, together with p50, as an enhancer binding protein for an Igκ chain gene, surprisingly the absence of RelA had no effect on the levels of Igκ production. The RelA-deficient B cells, however, produced 10-fold and 100-fold less IgG1 and IgA, respectively, than the control B cells. Reduced production of certain Ig classes has been also reported in mice deficient in p50 (14) and c-Rel (12): IgG1, IgA, and IgE in the former and IgG1 and IgG2a in the latter. Thus, each NF-κB member is critically involved in the production of certain Ig isotypes, presumably by regulating the transcription of Ig genes directly and/or acting on various cytokine genes which ultimately control Ig class switching and production.

In this regard, it is interesting to note that IgA reduction has also been reported in IL-6-deficient mice (38) and that the expression of IL-6 is controlled by RelA/p50 heterodimers (8).

IL-2 and IL-2Rα in relA-/- T Cells. In T cells, the RelA/p50 heterodimer has been reported to be a potent transcription factor for the IL-2 gene after stimulation by various agents (39). The *relA*-/- spleen cells, however, produced similar levels of IL-2 to *relA*+/+ or +/- spleen cells after stimulation with Con A, anti-CD3, anti-CD3+anti-CD28, or PMA+calcium ionophore (Table 1). The results were in contrast to those for c-Rel-deficient mice which showed ~50-fold reduction in IL-2 production after stimulation by anti-CD3, and anti-CD3+anti-CD28

Table 2. In Vitro Proliferation of Spleen Cells from SCID Mice Transplanted with Fetal Liver Cells in Response to Various Mitogenic Stimuli

<i>relA</i> genotype of fetal liver donor*	Stimuli*						
	Con A	Anti-CD3	Anti-CD3 + Anti-CD28	LPS	Anti-IgM	PMA + calcium ionophore	None
	[³ H]Thymidine uptake cpm						
+/+	19,328 ± 3,771 [§] (n = 6) [‡]	46,465 ± 4,898 (n = 4)	94,775 ± 2,612 (n = 3)	18,336 ± 2,924 (n = 6)	15,571 ± 2,003 (n = 4)	73,837 ± 2,261 (n = 2)	305 ± 73 (n = 6)
+/-	23,031 ± 3,491 (n = 9)	49,169 ± 6,557 (n = 7)	92,642 ± 5,692 (n = 6)	20,076 ± 7,856 (n = 9)	18,560 ± 4,059 (n = 7)	85,702 ± 6,130 (n = 5)	505 ± 146 (n = 9)
-/-	3,621 ± 960 (n = 9)	18,343 ± 3,030 (n = 7)	18,865 ± 4,575 (n = 6)	4,717 ± 1,026 (n = 9)	5,879 ± 1,076 (n = 7)	20,863 ± 6,150 (n = 5)	403 ± 168 (n = 9)
None	381 ± 145 (n = 7)	911 ± 352 (n = 5)	860 ± 215 (n = 4)	807 ± 285 (n = 7)	689 ± 285 (n = 5)	1,707 ± 734 (n = 3)	298 ± 75 (n = 7)

*See Table 1.

[‡]Number of mice analyzed.

[§]Mean ± SD.

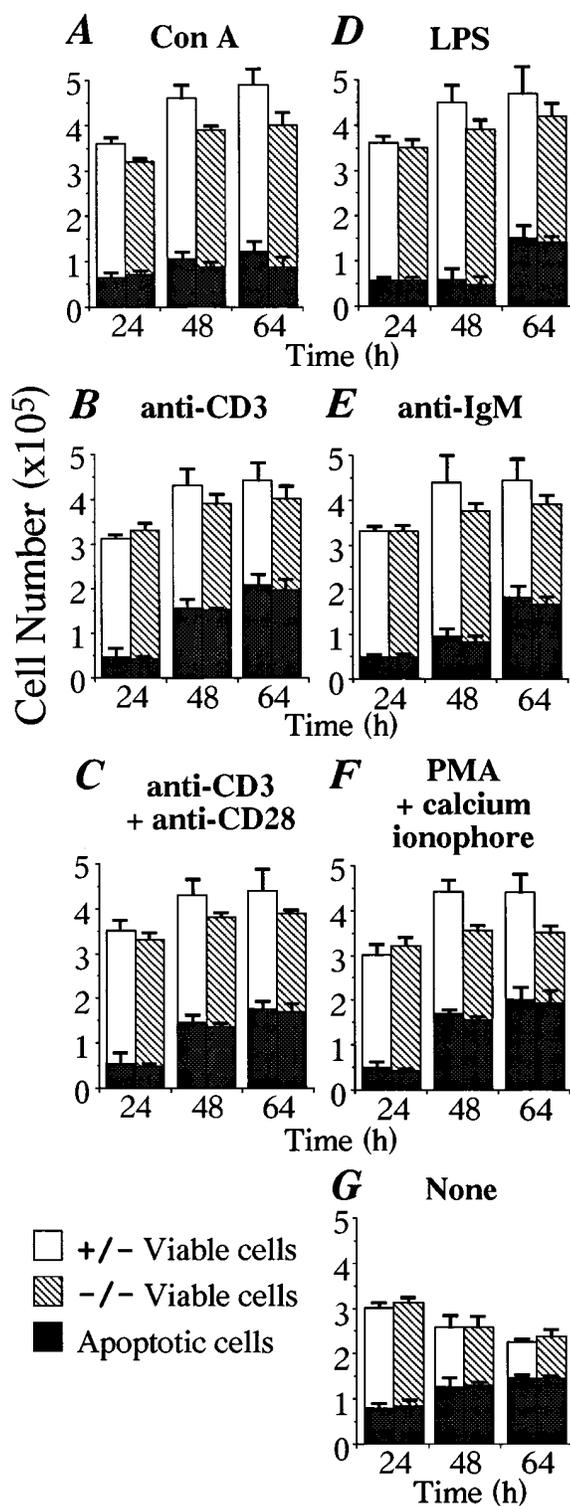


Figure 7. Cells in apoptosis during the course of proliferation assays. Spleen cells from mice transplanted with *relA*^{+/-} or *-/-* fetal liver cells were stimulated with various mitogens as in Table 2. The percentages of apoptotic cells were determined using the TUNEL technique with a FACScan® (32, 33). The numbers of viable cells were determined by the trypan blue exclusion test. At the beginning of stimulation, no cells were in apoptosis. The averages and SD values from three independent experiments are shown in the figure.

and threefold reduction by the PMA+calcium ionophore. Thus, it was strongly suggested that the main component of the NF- κ B transcription factor for the IL-2 gene in vivo is c-Rel rather than RelA.

RelA has been reported to be involved also in the upregulation of IL-2R α expression with stimulation by various agents (40). Before stimulation, the levels of IL-2R α on *relA*^{-/-}, *+/+*, or *+/-* lymphocytes were similar to one another as mentioned above. With stimulation by PMA+calcium ionophore or Con A, the levels of IL-2R α expression on *relA*^{-/-} T cells increased and did not significantly differ from *relA*^{+/+} or *relA*^{+/-} T cells (data not shown). The c-Rel-deficient T cells also showed no reduction in the basal or induced expression of IL-2R α (41). These observations suggest the following two possibilities: (a) neither RelA nor c-Rel is required for the expression of IL-2R α , or (b) both can participate in the control of IL-2R α expression and one works in the absence of the other. Identification of binding subunits to the κ B motif of IL-2R α in the absence of RelA or c-Rel and derivation of mice lacking both RelA and c-Rel should sort out these possibilities.

Impaired Proliferative Response of *relA*^{-/-} Lymphocytes. To further analyze the role of RelA in lymphocyte activation, spleen cells from mice transplanted with *relA*^{-/-} fetal liver cells were stimulated with various agents. With both T cell specific stimuli, Con A, anti-CD3 and anti-CD3+anti-CD28, and B cell-specific stimuli, LPS and anti-IgM, *relA*^{-/-} spleen cells showed a much lower [³H]thymidine uptake than *relA*^{+/+} or *+/-* spleen cells (Table 2). To test whether this low response of *relA*^{-/-} spleen cells is due to reduced cellular proliferation or to increased apoptotic cell death, the percentages of cells in apoptosis during the course of proliferation assays were determined. As shown in Fig. 7, the percentages and the actual numbers of apoptotic cells with *relA*^{-/-} were not significantly different from the *relA*^{+/-} case. Although the number of viable cells may not be as indicative as [³H]thymidine uptake because only a small component of the spleen cells can proliferate in response to certain mitogenic stimuli, *relA*^{-/-} yielded constantly fewer viable cells than *relA*^{+/-}. These results indicate that RelA-deficient lymphocytes indeed have an impaired proliferative response to various mitogens. As the production of IL-2 and the expression of IL-2R α were normal in RelA-deficient T cells, the results suggested that RelA is also involved in yet unidentified critical steps of proliferative responses. Furthermore, RelA-deficient lymphocytes exhibited impaired responses to various stimuli whose signals are transduced by distinctive pathways (42, 43). Thus, RelA may be involved in each single pathway or in a critical merging step downstream of these different pathways. Identification of RelA responsive genes involved in proliferation should reveal the role of RelA in these responses. T and B cells of c-Rel-deficient mice have also been found to demonstrate a defective proliferation response to various stimuli, generally with severe reduction (12). These results indicate that RelA and c-Rel are essential for certain steps of proliferation and that they cannot compensate for each other. It is interesting to note that

relA^{-/-} lymphocytes showed an impaired proliferative response to PMA+calcium ionophore in this study while *c-rel*^{-/-} lymphocytes respond normally to this agent (12). Presumably, the involvement of RelA in proliferative responses is thus wider. Furthermore, *relA*^{-/-} embryonic fibroblasts also showed reduced proliferation after PMA+calcium ionophore stimulation, down to 30% of the levels of their *relA*^{+/+} or *relA*^{+/-} counterparts (data not shown). As expression of *relA* is not restricted to lymphocytes, in contrast to that of *c-rel* (44), this also suggests a role in a wider range of biological processes.

In conclusion, transplantation of fetal liver cells into

SCID mice in the present investigation allowed light to be cast on a number of the functions of RelA in the immune system, despite the fact that the *relA*^{-/-} genotype is lethal for embryos. Further study with in vitro and in vivo immunization by T-dependent and -independent antigens should facilitate clarification of RelA roles. In addition, since lymphocytes can be rescued from dying embryos by transplantation of fetal liver cells as described here, mice lacking multiple NF- κ B proteins, such as RelA and c-Rel, or RelA and RelB, should now be testable for their actions exerted in concert.

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