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

By Takahiro S. Doi,* Toshitada Takahashi,* Osamu Taguchi,‡ Takachika Azuma,§ and Yuichi Obata*

From the *Laboratory of Immunology and the ‡Laboratory of Experimental Pathology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464; and the §Research Institute for Biological Sciences, Science University of Tokyo, Noda 278, Japan

Summary
To investigate the function of NF-κB RelA (p65), we generated mice deficient in this NF-κB 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^2/2 fetal liver transplants, similar to the relA^1/1 and 1/2 cases. T cells were found to mature to Thy-1^1/CD3^1/TCR^ab^ and CD4^1 or CD8^1, while B cells had the ability to differentiate to IgM^1/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-κB 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-κB and their activation pathways have been extensively studied, especially in terms of immune and acute phase responses. The mammalian NF-κB 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-κB proteins form multiple interchangeable heterodimers and homodimers and their activity is regulated by binding of IκB inhibitory factors which determine the localization of NF-κB dimers, either in the cytoplasm or in the nucleus. Upon activation, N F-κB dimers dissociate from IκB and translocate to the nucleus and then bind to the κB sites in promoters and enhancers of NF-κB responsive genes, consequently activating their transcription.

The RelA/p50 heterodimer was the original NF-κB identified, as a transcription factor for Igκ light chain gene (2), and has the strongest transactivating activity among NF-κB dimers as well as the most widely distributed κB binding activity (3). In the B cell lineage, RelA/p50 is the major NF-κB in pre-B cells, while c-Rel/p50 is predominant in mature B cells and RelA/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 TN Fα (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-κB proteins have faced similar problems. To overcome this drawback, mutant mice lacking RelA (11), c-Rel (12), RelB (13), p50 (14), or IκBα (15) have been derived by homologous recombination to assess specific functions of individual NF-κB 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 R elA-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 R elA-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 R elA-deficient stem cells could then differentiate to mature T and B cells. To investigate the roles of RelA further, we examined R elA-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 by 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, PM C1-neo inserted into the first exon of relA at an Ncol 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 x 10⁷ CCE embryonic stem (ES) cells (kindly provided by Dr. M. otoya 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 μg/ml; Sigma Chem. Co.). Growing colonies were isolated from a C57BL/6 (B6) mouse genomic library using a cDNA probe (codons 185-277, reference 21) 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.

Differentiation and Function of RelA-deficient Lymphocytes

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Igκ plates were coated with affinity-purified rabbit anti-mouse IgG (5 μg/ml, 100 μl/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-Phenylenediamine 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. $3 \times 10^5$ spleen cells from transplanted mice were plated in 96-well plates (200 μl per well). Con A (2 μg/ml; Boehringer Mannheim GmbH, Mannheim, Germany), anti-CD28 (1 μg/ml; Caltag Laboratories, South San Francisco, CA), LPS (20 μg/ml; Sigma), anti-IgM (60 μg/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 μg/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 ($5 \times 10^3$, 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 diUTP 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...
matings of heterozygous mice were conducted. Until embryonic day (ED) 13.5, all embryos were apparently normal and homozygous mutants (−/−) 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. 2A). On ED15.5, a portion of the embryos became necrotic and were typed homozygous mutant (−/−), while normal embryos were all wild type (+/+ or heterozygous (+/−)). 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. 2B). Positive immunostaining with anti-R elA antibody correlated with the presence of R NA transcripts, showing that R elA proteins were present in almost all tissues of ED13.5 relA+/+ embryos, while no staining of relA−/− embryos (Fig. 2C). Although a different vector construct was used in our study, the generated R elA-deficient mice showed the same phenotype as those of Beg et al. (11), indicating that R elA is essential for embryonic development of the mouse. In contrast to R elA-deficient mice, mice lacking other N F-κB proteins are known to develop normally at least until birth (12–14). The difference may simply reflect the fact that R elA is expressed ubiquitously from an early stage of development while the others are expressed in restricted tissues from a much later stage (34, 35). Identification of R elA responsive genes in developing embryos, especially in the liver, should open new avenues for elucidation of the function of N F-κB 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 R elA-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−/− but also from relA+/+ 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 +/+ 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-2Kb antigen. The fetal liver cells were from crosses between 129 and B6, both of which express H-2Kb, while the recipient SCID themselves express H-2Kd. As shown in Fig. 3, >95% of lymphocytes of mice transplanted with fetal liver cells expressed H-2Kb, 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).
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

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

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
and IgG1 and IgG2a in the latter. Thus, each NF-κB (14) and c-Rel (12): IgG1, IgA, and IgE in the former and certain Ig classes has been also reported in mice deficient in which ultimately control Ig class switching and production. Ig isotypes, presumably by regulating the transcription of Ig chain gene, surprisingly the absence of RelA has no effect on the levels of IgGk 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 1. Levels of IL-2 Production by Spleen Cells from SCID Mice Transplanted with Fetal Liver Cells in Response to Various Stimuli

| relA genotype of fetal liver donor* | Stimuli† | IL-2 levels U/ml§ |
|-----------------------------------|----------|-------------------|
|                                   | Con A    | Anti-CD3          | Anti-CD28 | Anti-CD3 + Anti-CD28 | PMA + calcium ionophore | N one |
| +/+                               | 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  |
| N one                             | 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 x 10⁶ fetal liver cells from relA +/+, +/−, or −/− embryos were transplanted into irradiated SCID mice.
†3 x 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.
§The levels of IL-2 were measured by bioassay using NRB cells (see the Materials and Methods).

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

| relA genotype of fetal liver donor* | Stimuli* | [3H] Thymidine uptake qpm |
|-----------------------------------|----------|--------------------------|
|                                   | Con A    | Anti-CD3                | Anti-CD28 | Anti-CD3 + Anti-CD28 | LPS | Anti-IgM | PMA + calcium ionophore | N one |
| +/+                               | 19,328 ± 3,771† | 46,465 ± 4,898 | 94,775 ± 2,612 | 18,336 ± 2,924 | 15,571 ± 2,003 | 73,837 ± 2,261 | 305 ± 73 |
|                                   | (n = 6)‡ | (n = 4)                | (n = 3)   | (n = 6)       | (n = 4)          | (n = 2)       | (n = 6)        |
| +/−                               | 23,031 ± 3,491† | 49,169 ± 6,557 | 92,642 ± 5,692 | 20,076 ± 7,856 | 18,560 ± 4,059 | 85,702 ± 6,130 | 505 ± 146 |
|                                   | (n = 9)‡ | (n = 7)                | (n = 6)   | (n = 9)       | (n = 7)          | (n = 5)       | (n = 9)        |
| −/−                               | 3,621 ± 960† | 18,343 ± 3,030 | 18,865 ± 4,575 | 4,717 ± 1,026 | 5,879 ± 1,076 | 20,863 ± 6,150 | 403 ± 168 |
|                                   | (n = 9)‡ | (n = 7)                | (n = 6)   | (n = 9)       | (n = 7)          | (n = 5)       | (n = 9)        |
| N one                             | 381 ± 145 | 911 ± 352 | 860 ± 215 | 807 ± 285 | 689 ± 285 | 1,707 ± 734 | 298 ± 75 |
|                                   | (n = 7)‡ | (n = 5)                | (n = 4)   | (n = 7)       | (n = 5)          | (n = 3)       | (n = 7)        |

*See Table 1.
†Number of mice analyzed.
‡Mean ± SD.
Thus, it was strongly suggested that the main component of the NF-κB 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-2Rα expression with stimulation by various agents (40). Before stimulation, the levels of IL-2Rα 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-2Rα 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-2Rα (41). These observations suggest the following two possibilities: (a) neither RelA nor c-Rel is required for the expression of IL-2Rα, or (b) both can participate in the control of IL-2Rα expression and one works in the absence of the other. Identification of binding subunits to the κB motif of IL-2Rα 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-2Rα 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−/− − 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

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**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.
reA /− 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 reA in proliferative responses is thus wider. Furthermore, reA /− embryonic fibroblasts also showed reduced proliferation after PMA stimulation, down to 30% of the levels of their reA +/+ or +/− counterparts (data not shown). As expression of reA 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 reA in the immune system, despite the fact that the reA /− genotype is lethal for embryos. Further study with in vitro and in vivo immunization by T-dependent and -independent antigens should facilitate clarification of reA roles. In addition, since lymphocytes can be rescued from dying embryos by transplantation of fetal liver cells as described here, mice lacking multiple NF-κB proteins, such as reA and c-Rel, or reA and reB, should now be testable for their actions exerted in concert.

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Address correspondence to Yuiichi Ohita, Laboratory of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464, Japan.

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