Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression

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The c-rel proto-oncogene, which is expressed predominantly in hemopoietic cells encodes a subunit of the NF-xB-like family of transcription factors. In mice with an inactivated c-rel gene, whereas development of cells from all hemopoietic lineages appeared normal, humoral immunity was impaired and mature B and T cells were found to be unresponsive to most mitogenic stimuli. Phorbol ester and calcium ionophore costimulation, in contrast to certain membrane receptor-mediated signals, overcame the T cell-proliferative defect, demonstrating that T cell proliferation occurs by Rel-dependent and independent mechanisms. The ability of exogenous interleukin-2 to restore T cell, but not B cell, proliferation indicates that Rel regulates the expression of different genes in B and T cells that are crucial for cell division and immune function.

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c-rel was originally identified as the cellular homolog of v-rel, the oncogene of Rev-T, an avian retrovirus that causes acute lymphoid leukemia in galliform birds and immortalizes B and T cells in vitro [Gilmore 1991]. c-rel was subsequently shown to be highly homologous to the NF-xB1 (p50) and relA (p65) subunits of the ubiquitous transcription factor NF-xB. NF-xB binds to decameric motifs [kB elements] found in promotors and enhancers of viral and cellular genes, particularly genes that encode proteins involved in immune, acute phase, and inflammatory responses [Baeuerle 1991; Blank et al. 1992; Nolan and Baltimore 1992; Grillum et al. 1993; Baeuerle and Henkel 1994]. In vertebrates, dimeric Rel/NF-xB proteins are encoded by a small multigene family. These genes include nfkbl (p50 and p105), nfkbl2 (p52 and p100), rela (p65), relb (RelB), c-rel (Rel), and v-rel. Two Rel-related homologs, dorsal and dfj have been identified in Drosophila. Dorsal is a transcriptional regulator that determines the developmental fate of cells along the dorsoventral axis during embryogenesis [Steward 1987], whereas dfj is required for immune function [Ip et al. 1993]. Rel-related proteins share a conserved 300-amino-acid amino-terminal domain [Rel homology domain (RHD)] that encompasses sequences required for DNA binding, protein dimerization, and nuclear localization [Grillum et al. 1993]. Rel, p65, and RelB all contain carboxy-terminal transcriptional trans-activation domains [Nolan and Baltimore 1992; Grilli et al. 1993]. The nontrans-activating subunits p50 and p52 are derived from the amino terminus of the precursor proteins p105 and p100, respectively, by proteolytic cleavage [Ghosh et al. 1990; Kieran et al. 1990; Schmid et al. 1991; Mercurio et al. 1992]. In most cells prior to stimulation, a large proportion of Rel/NF-xB factors reside in the cytoplasm as inactive complexes through association with inhibitory proteins collectively termed IKB. A wide range of stimuli promote NF-xB nuclear translocation by a mechanism that involves IKB phosphorylation [Ghosh and Baltimore 1990], which appears to target IKB for degradation [Brown et al. 1993; Henkel et al. 1993; Sun et al. 1993].

Precisely what physiological role Rel serves remains unclear. In the chick embryo, Rel is expressed after embryonic day 3 (E3) at low levels in most tissues with high level expression confined to cells undergoing programmed cell death [Abbadie et al. 1993]. A role for Rel in programmed cell death is supported by the finding that enforced c-rel expression in chicken bone marrow cells induces apoptosis [Abbadie et al. 1993; Huguet et al. 1994]. In mouse embryos, Rel is first detected at E17, with expression confined to the medullary region of the thymus [Weih et al. 1994]. Whereas the pattern of Rel expression during murine and avian embryonic development is different, in adult vertebrates, Rel is largely restricted to hemopoietic organs [Brownell et al. 1987; Moore and Bose 1989; Grumont and Gerondakis 1990a],

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Figure 1. The generation of mice lacking Rel. (A) The c-rel locus and targeting vector. The restriction map of that region of the murine c-rel gene encoding exons 4–9 is shown with the coding and noncoding exons depicted as solid and open boxes, respectively. The targeting vector, pC-REL, comprises the PGK-neomycin resistance gene. The structure of the targeted allele is indicated, with c-rel exons 4–9 replaced by PGKNeo. The size of the diagnostic restriction fragments detected by Southern blot analysis are indicated. Probe a is a 0.6-kb HindIII-BglII fragment from the c-rel 3′-flanking sequence, probe b is a 0.5-kb SstII-EcoRI fragment located between exons 3 and 4, and probe c a 0.6-kb EcoRI-BglII fragment from the bacterial neomycin resistance gene. Abbreviations for the restriction enzyme sites are: [H] HindIII, [RI] EcoRI, and [Bg] BglII. (B) Southern blot analysis of the targeted c-rel gene. Genomic tail DNAs from the offspring of heterozygote c-rel mutant (+/–) intercrosses were subjected to Southern blotting. Lanes 1–3 BglII-digested DNAs hybridized with probe a. The 10.5- and 6-kb fragments correspond to the wild-type and targeted c-rel alleles, respectively. HindIII-digested genomic DNAs hybridized with probe b (lanes 4–6) or probe c (lanes 7,8) detect 20- and 14-kb fragments corresponding to the normal and mutant c-rel alleles, respectively. All Southern blots were exposed to autoradiography for 48 hr. (C) Rel is not detectable in splenocytes from c-rel+/– mutant mice. Splenocytes (2 × 10⁶) from normal (+/+) and homozygous c-rel mutant (–/–) mice were boiled in 0.5% SDS, electrophoresed on an 10% SDS-polyacrylamide gel, and subjected to Western blot analysis with antibodies specific for Rel. Filters were exposed for 48 hr autoradiography at −70°C.

with the highest levels in B and T lymphoid cells (Brownell et al. 1987; Grumont and Gerondakis 1990a). In the B cell lineage, Rel levels change in a developmental stage-specific manner (Grumont and Gerondakis 1990a). A 5- to 10-fold increase in Rel that accompanies the transition from pre-B to B cell is linked to a qualitative change in NF-κB subunit usage. Whereas the major NF-κB complex in pre-B cells is p50/p65, in B cells this complex is largely replaced by p50/Rel (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994). In turn, the finding that p50/Rel has a 20-fold higher affinity than p50/p65 for the κB site in the Cκ intronic enhancer (Miyamoto et al. 1994) lends support to a model that implicates Rel in the induction of Cκ transcription in B cells (Miyamoto et al. 1994). In contrast, no clear association between c-rel expression and the differentiation of T lymphoid cells has emerged. During activation of mature T cells by mitogenic stimuli, the rapid induction of Rel expression is regulated by a combination of transcriptional and post-translational mechanisms (Grumont and Gerondakis 1990a,b). This induction of Rel appears to be important for interleukin-2 receptor α (IL-2Rα) chain gene transcription (Tan et al. 1992) and links Rel to the regulation of autocrine-mediated T cell proliferation. A role for Rel in lymphocyte proliferation is supported indirectly by the ability of v-rel to transform both B and T lineage cells (Gilmore 1991).

To determine the function of the Rel subunit of the NF-κB family of transcription factors in mammalian cells, mice were generated that lack a functional c-rel gene. While Rel-deficient mice develop normally and contain normal numbers of hemopoietic cells, B and T lymphocytes in these animals display proliferative and immune effector defects that appear in part to be attributable to an absence of cytokine production.

Results

The generation of c-rel null mice

The strategy used to disrupt the mouse c-rel locus in embryonal stem (ES) cells involved replacing c-rel exons encoding amino acid residues 145–588 with a PGKNeo cassette (Fig. 1A). This disruption should truncate the c-rel protein, removing sequences required for DNA binding, Rel/NF-κB dimerization, nuclear transport, and transcriptional trans-activation. Homologous recombination between the electropo-
rated targeting vector and endogeneous c-rel gene in the W9 ES cell line occurred at a frequency of \(-1\) in 150 G418-resistant colonies. In targeted ES cells, Southern analysis detected a 10.5-kb BglII (Fig. 1B, lane 2, probe a) and a 20-kb HindIII (Fig. 1B, lane 5, probe b) fragment that corresponded to the normal copy of c-rel (lanes 1,4), plus novel 6-kb BglII (probe a, lane 2) and 14-kb HindIII fragments (probe b, lane 5) from the targeted allele. Consistent with the predicted structure of the targeted locus, the neo gene (probe c, lane 8) hybridized to the 14-kb HindIII fragment. Mice homozygous for the c-rel mutation (Fig. 1B, lanes 3,6) were derived by intercrossing heterozygous (Rel +/-) mice. The absence of Rel in Western blots of total splenic extracts from mice homozygous for the targeted c-rel gene (Fig. 1C, lane 2) confirmed that this was a null mutation. Two transgenic lines carrying the c-rel mutation, each established from independent ES clones, were found to display the same phenotype. Consequently, all results presented here are from line 64.

**Lymphocyte development and hemopoiesis is normal in Rel\(^{-/-}\) mice**

Genotypic analysis of 243 offspring from Rel\(^{-/-}\) intercrosses showed that 25.5% were homozygous for the disrupted c-rel allele. This frequency of Rel\(^{-/-}\) progeny clearly establishes that Rel is not essential for mouse fetal or neonatal development.

The morphology and behavior of Rel\(^{-/-}\) mice up to 9 months of age were indistinguishable from heterozygous and wild-type littermates. These mice exhibited normal organ architecture, and no histopathology was evident. Although murine Rel expression is restricted to hemopoietic cells (Grumont and Gerondakis 1990a), no difference in hemopoietic lineage cellularity was observed in the Rel\(^{-/-}\) mice. In addition, no significant differences were observed among Rel\(^{++/+}\), Rel\(^{-/-}\), and Rel\(^{-/-}\) mice in the frequencies of progenitor cells in the bone marrow for granulocytic, macrophage, eosinophil, or megakaryocytic lineage cells [Table 1]. A survey of cell surface marker expression on Rel\(^{-/-}\) bone marrow, splenic, mesenteric lymph node, and thymic cell populations, using immunofluorescence staining and flow cytometry, confirmed that the number and distribution of hemopoietic cells was normal. A representative sample of these results is shown Figure 2. In particular, normal expression of IgK on splenic B cells [Fig. 2B] and the IL-2Ra chain (CD25) on thymic T cells [Fig. 2C] establishes that Rel is not essential for the transcription of the genes encoding these proteins. In summary, although Rel is expressed in B and T lymphocytes, macrophages, and neutrophils, these findings show it is not essential for the development of mature cells from hemopoietic precursors.

### Table 1. Frequency of progenitor cells of various lineages in the bone marrow

| Mouse | Stimuli   | No. of colonies | G  | GM | M  | Eo | Meg | Blast |
|-------|-----------|-----------------|----|----|----|----|-----|-------|
| +/-   | GM-CSF    | 26 ± 6          | 46 | 14 | 22 | 18 | 0   | 0     |
|       | G-CSF     | 10 ± 2          | 91 | 0  | 9  | 0  | 0   | 0     |
|       | M-CSF     | 27 ± 0          | 14 | 14 | 72 | 0  | 0   | 0     |
|       | multi-CSF | 40 ± 0          | 47 | 11 | 9  | 0  | 21  | 12    |
|       | IL-6      | 5 ± 0           | 89 | 11 | 0  | 0  | 0   | 0     |
|       | SCF       | 7 ± 0           | 73 | 0  | 0  | 0  | 0   | 27    |
|       | SCF + G-CSF | 16 ± 2      | 69 | 8  | 8  | 0  | 0   | 15    |
| +/-   | GM-CSF    | 35 ± 8          | 46 | 13 | 31 | 10 | 0   | 0     |
|       | G-CSF     | 10 ± 3          | 91 | 0  | 9  | 0  | 0   | 0     |
|       | M-CSF     | 32 ± 4          | 4  | 12 | 84 | 0  | 0   | 0     |
|       | multi-CSF | 50 ± 0          | 36 | 21 | 17 | 6  | 11  | 9     |
|       | IL-6      | 10 ± 2          | 67 | 27 | 6  | 0  | 0   | 0     |
|       | SCF       | 20 ± 2          | 77 | 7  | 6  | 0  | 0   | 0     |
|       | SCF + G-CSF | 26 ± 2     | 70 | 3  | 7  | 0  | 0   | 20    |
| +/-   | GM-CSF    | 33 ± 1          | 61 | 18 | 14 | 7  | 0   | 0     |
|       | G-CSF     | 7 ± 6           | 100| 0  | 0  | 0  | 0   | 0     |
|       | M-CSF     | 23 ± 2          | 21 | 13 | 67 | 0  | 0   | 0     |
|       | multi-CSF | 51 ± 12         | 41 | 21 | 6  | 6  | 18  | 8     |
|       | IL-6      | 7 ± 1           | 100| 0  | 0  | 0  | 0   | 0     |
|       | SCF       | 16 ± 5          | 78 | 4  | 9  | 0  | 0   | 9     |
|       | SCF + G-CSF | 18 ± 0     | 88 | 0  | 0  | 0  | 0   | 12    |

Frequency of progenitor (colony-forming) cells in culture of 25,000 bone marrow cells stimulated by 10 ng/ml of GM-CSF, G-CSF, M-CSF, or multi-CSF (IL-3), 500 ng/ml or IL-6 or 100 ng/ml of stem cell factor (SCF). Cultures were scored after 7 days of incubation and colonies were enumerated and typed from stained cultures.

\(\text{G}\) Granulocyte; \(\text{GM}\) granulocyte-macrophage; \(\text{M}\) macrophage; \(\text{Eo}\) eosinophil; \(\text{Meg}\) megakaryocyte; \(\text{Blast}\) blast cell. Total colony counts are mean values ± s.d. from quadruplicate cultures.
B and T cells from Rel−/− mice exhibit proliferation defects

Although the maturation of Rel−/− hemopoietic cells was normal, the association between Rel expression and lymphocyte activation prompted an analysis of lymphocyte proliferative responses. Splenic B and T cells from mice of the three c-rel genotypes were stimulated with a number of agents over a 72-hr period and proliferation monitored by [3H]thymidine incorporation. These results are summarized in Figure 3. In Rel−/− B cell cultures, stimulation with optimal concentrations of lipopolysaccharide (LPS), CD40 ligand, mitogenic antibodies specific for the IgM receptor or the novel B cell surface protein RP (Miyake et al. 1994) failed to induce proliferation (Fig. 3A). Whereas thymidine incorporation by Rel−/− B cells was 0.5% (anti-RP) to 2.5% (CD40 ligand) of normal levels, proliferation in the Rel−/− B cell cultures ranged between 14% (anti-RP) and 20% (anti-IgM) of the normal response. This indicates that Rel−/− B cells are partially defective for proliferation. Clonal B cell growth was monitored by seeding splenocytes into agar cultures containing LPS. These results, shown in Table 2, demonstrate that the number of Rel−/− B lymphocyte colonies was 100-fold lower when compared with wild-type cells. Consistent with proliferation in liquid cultures, Rel−/− B cell colony formation was 10-fold lower than that of normal splenocytes.

The T cell proliferative responses of Rel−/− and Rel+/− cells were also compromised and are outlined in Figure 3B. Whereas engagement of the CD3 chain of the T cell receptor complex normally induces proliferation (Crabtree and Clipstone 1994) that can be augmented by CD28 costimulation (Bryan et al. 1994), Rel−/− T cells failed to proliferate in response to anti-CD3 activation (3% of normal T cell [3H]thymidine incorporation after 72 hr), and this defect was not overcome by CD28 costimulation. Concanavalin A (Con A) also failed to promote normal proliferation of Rel−/− T cells (20% of a normal T cell response, at 72 hr). In contrast, proliferation in Rel−/− T cell cultures stimulated with PMA and ionomycin was ~70% of a normal response. These results indicate that although Rel is required for surface receptor-mediated B and T cell proliferation, in T cells, mitogenic signals that act in the cytoplasm can bypass the defect imposed by the loss of Rel.

IL-2 complements the proliferative defect in T but not B cells from Rel-deficient mice

Up-regulation of IL-2 and IL-2Ra expression during T cell activation establishes an autocrine signaling loop (Crabtree and Clipstone 1994). Because IL-2Ra chain is up-regulated in activated Rel−/− T cells (A. Strasser, unpubl.) aberrant IL-2 production remained a possible explanation for the proliferative block. Whereas significant levels of IL-2 were produced by normal T cells stimulated with concanavalin A, anti-CD3 or anti-CD3/anti-CD28 (Table 3), IL-2 levels in the corresponding Rel−/− T cell stimulations were ~50-fold lower. In contrast,
Figure 3. B and T cells from Rel-deficient mice exhibit proliferative defects in vitro. (A) B cell proliferation. Resting splenic B cells isolated from normal (●), Rel +/+ (■), or Rel -/- (▲) mice were stimulated for a period of 3 days with anti-IgM, LPS, CD40 ligand, and anti-RP. (B) T cell proliferation. Resting splenic T cells isolated from normal, Rel +/+ and Rel -/- mice were stimulated with anti-CD3, anti-CD3 plus anti-CD28, Con A, or phorbol ester plus ionomycin over a period of 3 days. (C) Exogenous IL-2 overcomes the proliferative block in Rel-deficient T cells. T cells (Con A or anti-CD3/anti-CD28) and B cells (LPS or anti-IgM) from normal mice (○) or Rel-deficient mice stimulated in the absence (solid circles) or presence of exogenous IL-2 (hatched circles) for a 3-day period. Lymphocyte proliferation in all experiments was measured by [3H]thymidine incorporation, and these results are the mean of four experiments.

Table 2. The frequency of B-lymphocyte colony-forming cells is diminished in Rel -/- mice

| Mouse | Spleen | Mesenteric lymph node |
|-------|--------|-----------------------|
|       | LPS    | saline                |
| +/+   | 156 ± 7| 2 ± 0                 |
| +/-   | 23 ± 10| 0                     |
| -/-   | 1 ± 0  | 0                     |

Quadricuplet cultures containing 25,000 cells/ml in agar medium with or without 10 μg of lipopolysaccharide. Colony formation was scored after 7 days of incubation. Total colony counts are mean values ± S.D.

much higher levels of IL-2 were present in the culture supernatant of Rel -/- T cells treated with PMA and ionomycin, although the amount of IL-2 was severalfold lower than in normal cells. To determine whether the markedly reduced levels of IL-2 in stimulated Rel -/- T cell cultures was associated with the proliferative defect, proliferation of stimulated Rel -/- T cells was compared in the presence and absence of exogenous IL-2. These experiments shown in Figure 3C demonstrate that in the presence of excess murine IL-2, Rel -/- T cells stimulated with Con A or anti-CD3/anti-CD28 proliferate as effectively as normal T cells. Because IL-2 has been shown to augment B cell growth in vitro (Kishimoto and Hirano 1988) it was also tested for the ability to rescue the Rel -/- B cell-proliferative defect. In contrast to Rel -/- T cells, IL-2 did not enhance proliferation of Rel -/- B cells stimulated with anti-IgM or LPS (Fig. 3C), CD40 ligand, and anti-RP (results not shown). Cultures supplemented with other B cell growth factors such as IL-4, IL-5, and IL-6 also failed to promote Rel -/- B cell proliferation (A. Strasser, unpubl.). This demonstrates that the lymphocyte proliferative defect in Rel -/- mice is different for B and T cells.

Rel-deficient mice display impaired antibody production in response to antigenic challenge

To determine if the in vitro block in Rel -/- B cell proliferation was reflected in the humoral immunity of Rel-
deficient mice, the responsiveness of mature B cells to antigenic challenge was assessed by analyzing serum immunoglobulins in naive and immunized animals. Serum isotype levels from naive mice of each genotype are shown in Figure 4A. Whereas the levels of IgM (3-fold), IgG2b (6-fold), and IgG3 (4-fold) in Rel−/− mice were somewhat reduced compared with Rel+/− and wild-type littermates, IgG1 levels in Rel−/− animals were 100-fold less and IgG2a was undetectable. Rel−/− mice also appear to be partially defective for IgG1 production because serum IgG1, but not other isotypes, was 10-fold lower than in normal littermates.

The humoral immune responses to antigenic challenge are shown in Figure 4, B and C. IgG3, a prominent isotype produced during a normal response to the T cell-independent antigen NP-LPS, was marginally reduced 7 days postimmunization in the Rel−/− mice compared with normal and Rel+/− littermates (Fig. 4B) and continued to decrease over the subsequent 14 days of the immune response. Whereas the T-dependent antigen NP-KLH normally elicits a strong IgG1 response (Lalor et al. 1992), NP-specific IgG1 levels in Rel−/− animals were 50- to 100-fold lower over the course of antigenic challenge compared with wild-type controls (Fig. 4C). Although normal and Rel+/− mice immunized with NP-LPS had equivalent levels of NP-specific serum IgG3, anti-NP-specific IgG1 levels in NP-KLH immunized Rel−/− mice were intermediate between those found in normal and Rel−/− animals. In summary, these findings establish that Rel is required for the synthesis of IgG1 and IgG2a and appears to be important for T cell-dependent humoral responses.

The expression of other Rel family proteins is normal in Rel−/− lymphocytes

The transcription of nfkbl (Ten et al. 1992), c-rel (Hannink and Temin 1990; Grumont et al. 1993), and Ikba (LeBail et al. 1993; Chiao et al. 1994) is regulated by Rel/NF-KB complexes that bind to kB sites within the promoters of these genes. To determine whether the absence of Rel alters the expression of other Rel family proteins, equivalent amounts of protein from whole cell extracts of purified resting splenic B and T lymphocytes were analyzed by Western blotting using antibodies specific for p50, p52, p65, RelB, and Rel (Fig. 5). The levels of p65, p50, p52, and RelB were equivalent in B and T cells isolated from mice of the three genotypes. Whereas Rel, as expected, was undetectable in Rel−/− cells, the level of Rel in B cells from heterozygous mice was only 25% of that in normal B cells. This is in contrast to Rel−/− T cells, where the amount of Rel is 50% of that in normal resting cells. RNase protection analysis confirmed that c-rel mRNA in Rel−/− B cells is only 25% of normal levels [R. Grumont, unpubl.]. These results show that up-regulation of other Rel family members, equivalent amounts of protein from whole cell extracts of purified resting splenic B and T lymphocytes were analyzed by Western blotting using antibodies specific for p50, p52, p65, RelB, and Rel (Fig. 5). The levels of p65, p50, p52, and RelB were equivalent in B and T cells isolated from mice of the three genotypes. Whereas Rel, as expected, was undetectable in Rel−/− cells, the level of Rel in B cells from heterozygous mice was only 25% of that in normal B cells. This is in contrast to Rel−/− T cells, where the amount of Rel is 50% of that in normal resting cells. RNase protection analysis confirmed that c-rel mRNA in Rel−/− B cells is only 25% of normal levels [R. Grumont, unpubl.]. These results show that up-regulation of other Rel family members does not occur in resting B and T cells to compensate for the loss of Rel and that positive autoregulation of c-rel transcription appears to occur in B but not T cells.

KB-binding complexes in Rel−/− B cells resemble those found in pre-B cells

Electrophoretic mobility shift assays (EMSA) were used to analyze nuclear Rel/NF-κB complexes in resting and stimulated B and T cells (Fig. 6). Consistent with previous reports (Rooney et al. 1991; Grumont and Geron-
Lymphocyte activation defects in Rel-deficient mice

The subunit composition of nuclear Rel/NF-κB complexes in B and T cells was determined by shift-Western blotting (Demczuk et al. 1993). DNA–protein complexes from EMSA gels were electrophoretically transferred to stacked nitrocellulose and anionic membranes. The radiolabeled DNA probe, which only binds to the anionic membrane, was detected by autoradiography, whereas nitrocellulose-bound proteins were probed with antibodies specific for the Rel family members. Because the composition of κB-binding complexes in resting and LPS-stimulated B cells was found to be the same, only the data from resting cells are presented in Figure 7. In normal splenic B cells, p50/Rel and a lesser amount of p50/p65 are the major complexes in Ca (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994).

Figure 5. Expression of Rel/NF-κB proteins in Rel-deficient B and T cells. Approximately 1 x 10⁶ untreated splenic B or T cells isolated from mice of each genotype were boiled in 0.5% SDS, electrophoresed on 10% SDS–polyacrylamide gels, and subjected to Western blot analysis with antibodies specific for Rel, p50, p65, RelB, and p52 as described in Materials and methods. Filters were exposed for 48 hr autoradiography at −70°C.

dakis 1994; Liou et al. 1994), two major nuclear complexes, here denoted Ca and Cβ, are detected in normal resting B cells (Fig. 6, lane 1). Although Rel is a major component of Ca in normal B lymphocytes (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994), a complex of similar mobility and abundance was still present in Rel−/− B cell nuclear extracts (Fig. 6, lane 7). Both complexes were up-regulated in all LPS-stimulated B cells (Fig. 6, lanes 2,5,8) and DNA binding was competed with an excess of wild-type (Fig. 6, lanes 3,6,9) but not mutant κB probe (results not shown). In resting T cells, two nuclear complexes, here denoted C1 and C2, bound to κB3 probe (Fig. 6, lanes 1,4,7). Whereas both complexes were markedly up-regulated in normal Con A-stimulated T cells (Fig. 6, lanes 2, 5, 8), C1 and C2 levels in Con A–treated Rel+/− T cells were only marginally higher than in unstimulated cells (Fig. 6, lane 5) and complex C2 was down-regulated in stimulated Rel−/− cells (Fig. 6, lane 8). Competition analysis (Fig. 6, lanes 3,6,9) confirmed the binding specificity of these complexes.

Figure 6. Analysis of NF-κB complexes in resting and stimulated B and T cells. Splenic B and T cells were isolated from litter-matched Rel+/+, Rel+−, and Rel−− mice. Nuclear extracts (1.5–3 μg) from resting B and T cells, B cells stimulated with LPS for 4 hr, or T cells treated with Con A for 24 hr were incubated with 1 μg of poly[d(I-C)] and 32P-labeled κB3 probe. The resulting NF-κB complexes were resolved on 5% nondenaturing polyacrylamide gels and exposed to autoradiography for 6 hr at −70°C. (Lanes 1,4,7) Nuclear extracts from unstimulated cells, (lanes 2,5,8) LPS-stimulated B cells and Con A–treated T cells, (lanes 3,6,9) competition analysis with a 50-fold molar excess of unlabeled κB3 oligonucleotide. Ca and Cβ correspond to the upper and lower complexes from B cell nuclear extracts; C1 and C2 are slow and fast mobility complexes in T cell nuclear extracts.
The Cs complex is comprised of p50 homodimers (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994). In B cells of each genotype, p65 (Fig. 7, lane 10–12) and a small amount of RelB (Fig. 7, lane 13–15) were detected in Cα. Consistent with the Western blot analysis of whole cell extracts (Fig. 5), the levels of Rel in Cα from Rel−/− cells (Fig. 7, lane 5) were reduced compared with normal B cells (Fig. 7, lane 4). Conversely, levels of p65 in Cα from Rel+/− and Rel−/− cells were severalfold higher than in normal B lymphocytes (Fig. 7, lanes 10–12) despite cellular p65 levels being the same in normal and Rel-deficient B cells (Fig. 5). This inverse relationship indicates that decreasing Rel levels in Cα are compensated for by a corresponding increase in p65. whereas p50 was present in Cs (Fig. 7, lanes 7–9), it was not detected in Cα by shift-Western blotting (Fig. 7, lanes 7–9), even though equivalent amounts of p50–DNA adduct eluted from SDS–polyacrylamide gels was immunoprecipitated from all Cα complexes with the same antisera (R. Grumont, unpubl.). The reason for this is unclear, but may reflect masking of the epitope in p50/Rel and p50/65 heterodimers, but not p50 homodimers.

In normal resting splenic T cells (Fig. 7), C1 contains mainly Rel (Fig. 7, lane 4), p65 (Fig. 7, lane 10), and p50, whereas only p50 was detected in C2 (Fig. 7, lane 11). The presence of p50 in C1 was confirmed by immunoprecipitation of the cross-linked complex (R. Grumont, unpubl.). In contrast to B cells, no increase in the level of other Rel family proteins in the kb-binding complexes compensated for the loss of Rel in T cells (Fig. 7, lanes 9, 12, 15, 18). Although the amount of p50, p65, and Rel in C1 and C2 increased in normal Con A-stimulated T cells, a finding consistent with the up-regulation of these complexes, a decrease in constitutive nuclear C1 levels in stimulated Rel−/− T cells was consistent with the down-regulation of p50 and p65 levels within this complex (R. Grumont, unpubl.). Work currently in progress aims at learning why C1 and C2 up-regulation during T-cell stimulation is blocked in the absence of Rel.

Discussion

The Rel/NF-κB family of transcription factors have been implicated in the regulation of many genes involved in a range of physiological processes (Baeuerle et al. 1991; Grilli et al. 1993). Here we describe the phenotype of mice lacking the Rel subunit of NF-κB. The normal development of Rel-deficient mice, coupled with the expected frequency of Rel−/− offspring from heterozygous intercrosses, indicates that c-rel is not crucial for mammalian embryogenesis. Although Rel has been implicated in the induction of programmed cell death in avian embryonic and hematopoietic cells (Abbadie et al. 1993), the absence of Rel does not influence the apoptotic process in mouse lymphocytes (A. Strasser, unpubl.). Although normal numbers of mature hematopoietic cells in adult Rel-deficient mice demonstrate that this subunit of NF-κB is not essential for the generation or maturation of hematopoietic precursors, these animals exhibit impaired immunity attributable to defects in the activation of mature B and T cells. This indicates that different intracellular signal transduction pathways critical for lymphocyte proliferation appear to be used during the antigen-independent expansion of lymphoid precursors.
and the antigen-driven proliferation of mature lymphocytes.

Rel regulation of lymphocyte activation and immune function

A number of transcription factors have been shown to be important for B cell proliferation. For example, Oct-2-deficient B lymphocytes are refractory to LPS and anti-IgM stimulation but respond normally to CD40 ligand (Corcoran and Karvalas 1994), whereas p50\(^{-/-}\) B cells fail to respond to LPS, but anti-IgM induces proliferation, albeit less efficiently than for normal B cells (Sha et al. 1995). In contrast, the inability of Rel-deficient B cells to respond to LPS, anti-IgM, CD40 ligand, and engagement of the RP receptor, demonstrates that Rel is a crucial focal point for different B cell mitogenic signals. Whereas a large proportion of Rel in B cells is associated with p50 (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994), which may account for the lack of LPS responsiveness by both Rel- and p50-deficient cells, differences in the proliferative response of Rel\(^{-/-}\) and p50\(^{-/-}\) B cells to a range of stimuli indicates that p65/Rel or Rel homodimers may be more important than p50/Rel for other types of B cell activation.

A crucial role for Rel/NF-κB complexes in T cell proliferation was originally inferred from studies demonstrating that the antioxidant PDTC, an inhibitor of NF-κB function, prevented T-cell proliferation and cytokine production (Costello et al. 1993). Whereas the in vitro proliferative response induced by T cell receptor and CD28 costimulation is impaired in Rel-deficient T cells, most likely attributable to a block in IL-2 synthesis, phorbol ester and calcium ionophore promote proliferation and IL-2 secretion in the absence of Rel. This suggests that in T cells, either Rel is upstream of the point at which phorbol ester and calcium ionophore engage a common intracellular signal required for IL-2 transcription or that these agents operate through a distinct pathway. It remains to be determined which T cell immune responses are defective. Although T-cell independent humoral immunity is only marginally reduced in Rel\(^{-/-}\) mice, the T cell-dependent IgG1 immune response is reduced 100-fold and serum IgG2a is undetectable. This deficiency in antibody production is not likely to be attributable to a T cell proliferative defect that arises from a lack of IL-2 production because IL-2-deficient mice, although exhibiting T cell proliferative defects in vitro (Schorle et al. 1991), mount relatively normal T and B cell immune responses (Kündig et al. 1993). Instead, it may reflect deficiencies in the production of certain cytokines by helper T cells that are required for switching to specific isotypes.

Genes regulated by Rel

Rel appears to be an important component of gene regulatory programs associated with the activation of mature lymphocytes. Here we identify the IL-2 gene as a target regulated by Rel. Whereas IL-2 gene transcription is controlled by an enhancer that encompasses binding sites for constitutive and inducible factors (for review, see Crabtree and Clipstone 1994), including a κB-binding site for p50 homodimers (Hoyos et al. 1989) and a CD28 response element that binds Rel-containing complexes (Ghosh et al. 1993; Bryan et al. 1994), it remains to be determined to what extent direct binding of Rel/NF-κB proteins to the enhancer element contribute to IL-2 gene expression. The genes regulated by Rel that are critical for B cell proliferation are yet to be identified. Although all known B cell growth factors fail to rescue the proliferative defect (A. Strasser, results not shown), it remains a possibility that a novel autocrine B cell growth factor is absent in Rel-deficient mice. Alternatively, Rel may regulate the expression of other transcription factors that are required for B cell proliferation. One candidate is c-myc, the transcription of which is controlled in B cells by NF-κB complexes that contain Rel (Lee et al. 1995).

The involvement of Rel in the regulation of genes important for autocrine cell growth has implications for the mechanism by which v-Rel transforms lymphocytes. Although the v-rel oncprotein was initially thought to act as a repressor of κB-dependent transcription (Ballard et al. 1990; Inoue et al. 1991; Richardson and Gilmore 1991), recent studies have shown that v-Rel can specifically up-regulate the expression of a number of genes, including IL-2Rα in transformed cells (Hrdlickova et al. 1994). If v-Rel, like Rel, also induces IL-2 expression, autocrine growth could be important in v-rel-mediated transformation.

Regulation of Rel/NF-κB proteins

Although an ordered pattern of Rel/NF-κB gene expression occurs during B cell differentiation (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994), no change was observed in the expression of other Rel-related proteins in resting Rel\(^{-/-}\) B or T cells [Fig. 5]. This indicates that other Rel family members are not up-regulated to compensate for the loss of Rel, nor is Rel essential for basal transcription of the genes encoding these proteins. In contrast, lower than expected levels of Rel in resting Rel\(^{+/+}\) B lymphocytes is consistent with constitutive c-rel transcription in these cells controlled by positive autoregulation, a conclusion supported by murine c-rel promoter mapping studies (Grumont et al. 1993). The partial mutant phenotype displayed by Rel\(^{-/-}\) lymphocytes, particularly T cells, which exhibit a proliferative defect despite only a 50% reduction in Rel levels, emphasizes that the regulation of Rel levels must be tightly controlled to maintain normal cellular functions.

In Rel-deficient B cells, there is an elevation in p50/p65 levels compared with normal B cells. This finding indicates that the switch from p65 to Rel that normally accompanies pre-B to B cell differentiation (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994) is attributable to subunit competition arising from increased c-rel expression rather than a post-translational modification that specifically decreases p50–p65
dimerization. The rapid induction of p50/p65 and p50 homodimers in the nucleus of mitogen-activated Rel-deficient B lymphocytes also demonstrates that the proliferative defect in these cells does not result from a failure to induce NF-κB proteins, such as has been described for LPS-stimulated p50−/− B cells (Sha et al. 1995). Rather, p50/p65, an efficient trans-activator of κB regulated genes, cannot functionally substitute for Rel-containing complexes in B cells.

Rel-related genes play distinct roles in hemopoietic cells

Whereas Rel-related proteins share common properties, different spatial and temporal patterns of Rel/NF-κB gene expression (Grilli et al. 1993; Weih et al. 1994) point to each family member serving a distinct biological function. This has been confirmed with the characterization of Rel−/−, p50−/− (Sha et al. 1995), and RelB (Weih et al. 1995) deficient mice. p50−/− mice, like Rel-deficient animals, undergo normal hemopoiesis but exhibit defects in B cell activation and humoral immunity. Although Rel and p50 mutant mice share some common lesions, a finding consistent with Rel being a major partner of p50 in B cells (Grumont and Gerondakis 1994; Liou et al. 1994; Miyamoto et al. 1994), differences in B cell responsive-ness to particular stimuli and proliferative defects in Rel−/− T cells serve to emphasize the unique properties of Rel. In contrast, RelB-deficient mice develop hemopoietic lesions characterized by multifocal inflammatory infiltrates, myeloid hyperplasia, and splenomegaly several weeks after birth (Weih et al. 1995). The differences in the phenotype of mice bearing null mutations of Rel, p50, and RelB suggest that in hemopoietic cells, specific Rel/NF-κB proteins either regulate genes involved in developmental and housekeeping functions or are required for the rapid induction of genes induced in response to stimuli. RelB appears to belong to that class of Rel/NF-κB factors that are important for the regulation of genes associated with constitutive functions in hemopoietic cells. This is consistent with RelB hetero-dimers being major components of the constitutive nuclear κB-binding activity in murine thymus and spleen (Lernbecher et al. 1994; Weih et al. 1994). In contrast, Rel and p50 appear to be involved in the regulation of genes that are induced during immune responses. Because Rel is necessary for lymphocyte activation and IL-2 synthesis, defects in the expression of other cytokines and proteins involved in immune and inflammatory responses for which NF-κB factors have been implicated are likely to emerge from the study of Rel-deficient mice.

Materials and methods

Targeting vector

Phage clones encoding the murine c-rel gene were isolated from a 129/Sv liver genomic library (Stratagene, San Diego, CA). A 2.5-kb EcoRI restriction fragment flanking the 5′ end of exon 4 and a 4.5-kb HindIII fragment encompassing the c-rel 3′-untranslated region and flanking sequence of c-rel were cloned into a PGKNeo cassette (McBurney et al. 1991) to generate the targeting vector pC-REL.

ES cell culturing and generation of mutant mice

Thirty micrograms of linearized pC-REL was electroporated into W9 ES cells and G418+ colonies were selected and cultured as described (Köntgen and Stewart 1993). BglII-digested DNAs isolated from 300 colonies were screened by Southern blotting using a 0.6-kb HindIII–BglII fragment (Fig. 1A, probe a) external to the 4.5-kb HindIII fragment in c-rel. Two ES clones with a single targeted copy of c-rel, identified by the presence of a novel 6-kb BglII restriction fragment, were microinjected into C57BL6 blastocysts. Chimeric offspring with agouti-colored coats were backcrossed to C57BL6 mice and c-rel−/− progeny identified by Southern blotting c-rel−/− mice were subsequently derived from c-rel−/− intercrosses.

Immunofluorescence staining and flow cytometry

Dispersed cells from thymus, bone marrow, spleen, and lymph nodes were surface stained as described previously (Strasser et al. 1991), either with directly fluoresceinated monoclonal antibodies or with biotinylated monoclonal antibodies followed by R-phycocerythin-streptavidin (Caltag, San Francisco, CA). Viable cells [5000–10,000] not stained by propidium iodide were then analyzed in a FACScan flow cytometer (Becton Dickinson). The monoclonal antibodies used were 5.1 anti-μ heavy chain, 187 anti-κ light chain, RA3-6B2 anti-CD45R-B220, B3B4 anti-CD23, T24.31.2 anti-Thy-1, 30H12 anti-Thy1.2, GK1.5 anti-CD4, 53.6.7 anti-CD8a, 145-2C11 anti-CD3ε, KT3 anti-CD3ε, H57-597 anti-TCRβ, 37.51 anti-CD28, PC61 anti-IL-2 receptor α chain, TER119 anti-erythrocyte cell surface marker, RB6-8C5 anti-Gr-1, and M1/70 anti-Mac-1. Small resting B and T lymphocytes purified from the spleens of 4- to 5-week-old mice were stained either with a cocktail of fluoresceinated monoclonal antibodies specific for T cells, erythroid cells, granulocytes, and macrophages (anti-Thy-1, anti-CD3, anti-TCRβ, anti-CD4, anti-CD8, TER119, anti-Gr-1, and anti-Mac-1) or with a cocktail of monoclonal antibodies specific for B cells, erythroid cells, granulocytes, and macrophages (anti-μ heavy chain, anti-CD45R-B220, TER119, anti-Gr-1, and anti-Mac-1). Unstained viable cells (not stained with propidium iodide) with low forward and side light-scattering properties were sorted in a FACStar+ or a FACS II (Becton Dickinson).

Bone marrow cultures and B cell colony assays

Dispersed bone marrow cell suspensions were cultured in 35-mm petri dishes using 25,000 cells in 1 ml volumes of Dulbecco’s modified Eagles medium (DMEM) containing a final concentration of 20% newborn calf serum and 0.3% agar. Cultures were stimulated by addition of purified recombinant murine growth factors at the following final concentrations: GM-CSF, G-CSF, M-CSF, multi-CSF (IL-3) 10 ng/ml, IL-6, 500 ng/ml, stem cell factor (SCF) 100 ng/ml. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO2 in air for 7 days. The cultures were then fixed for 4 hr by the addition of 1 ml of 2.5% glutaraldehyde and floated intact onto glass slides. After drying, the cultures were stained for acetylcholinesterase and then with Luxol Fast Blue and hematoxylin. All colonies in magnifications of 400×. B lymphocyte colony assays were performed essentially as described (Metcalf 1976).
IL-2 bioassays

The supernatants of T cells stimulated in culture for 72 hr were screened for IL-2 using the IL-2-dependent mouse HT2 T cell line essentially as described [Mossman et al. 1986].

Preparation of purified splenic B and T cells

Splenic cells from 6-week-old mice were first depleted of erythrocytes followed either by anti-Thy-1 [T cell removal] or anti-B220 [B cell removal] complement mediated lysis. Viable lymphocytes were then enriched on Ficoll gradients. Cells were subsequently stained and analyzed by immunofluorescence with the B and T cell populations shown to be >95% pure.

Lymphocyte activation in tissue culture

All cell lines and primary cells from mice were cultured in the high glucose version of DMEM supplemented with 13 mM folic acid, 250 mM L-asparagine, 50 mM 2-mercaptoethanol, and 10% fetal bovine serum. Spleen cells were cultured at an initial concentration of 1 × 10^6 leukocytes/ml. FACs-purified B and T cells were cultured at an initial concentration of 3 × 10^6 cells/ml. B lymphocytes were stimulated with LPS (Difco) at a concentration of 20 μg/ml, recombinant chimeric mouse CD40 ligand–CD8a fusion protein (Lane et al. 1993) at 2 μg/ml, or affinity purified goat anti-mouse IgM [Fab'1]; fragments [Cappel] at 20 μg/ml in the presence or absence of recombinant mouse IL-2, IL-4, IL-5 at 100 U/ml. T lymphocytes were stimulated in the presence or absence of recombinant mouse IL-2 at 1000 U/ml with Con A (Pharmacia) at 2 μg/ml, PMA (Sigma) at 2 ng/ml, ionomycin (Sigma) at 1 μg/ml, or by culturing in plates coated with monoclonal hamster antibodies specific for mouse CD3 (clone 145-2C11; Leo et al. 1987) and mouse CD28 (clone 37.51; Harding et al. 1992). Cell proliferation was measured as [3H]thymidine incorporation after incubating 100 μl of cultures in 96-well microtiter plates for 6 hr with 0.5 mCi [3H]thymidine [Amersham] per well.

Immunization and ELISA assays

Mice were immunized by intraperitoneal injection of either 100 μg of NP coupled to KLH precipitated in alun (T-cell dependent) or 10 μg of NP coupled to LPS (T-cell independent). Conjugates were prepared as described [Lalor et al. 1992] at conjugate ratios of 17:1 (NP/KLH) and ~10:1 (NP/LPS). Serum samples were collected from the mice prior to immunization and at 7-day intervals after immunization for a period of 3 weeks. The level of NP-specific immunoglobulin in each sample was determined by ELISA using NP1γ-BSA as a capture agent and goat anti-mouse isotype-specific sera directly conjugated to horseradish peroxidase [Southern Biotechnology Associates, Birmingham, AL] as revealing agents [Lalor et al. 1992]. The relative levels of each antigen-specific isotype were determined at the different time points by comparing each serum sample to a hyperimmune serum standard. Absolute levels of immunoglobulin isotypes in preimmune sera were determined by ELISA as described [Cocoran and Karvalas 1994] using purified myeloma protein standards [Sigma, St. Louis, MO].

Western blotting

Equivalent amounts of whole cell lysates from total splenic cells or purified splenic B or T cells were fractionated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose filters as described [Harlow and Lane, 1988]. Filters were first in-cubated with antibodies specific for murine p50, Rel, p65, RelB, and p52 followed by incubation with 125I-labeled protein A. Filters were exposed for autoradiography at ~70°C. Antibodies specific for Rel and p65 were described previously [Grumont and Gerondakis 1994], whereas antibodies specific for RelB, p50, and p52 were obtained from Santa Cruz Biotechnology.

Nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared from B and T cells essentially as described [Schrieber et al. 1989]. Briefly, ~10^7 cells were washed in mouse tonicity phosphate buffered saline [MTPBS], resuspended in 500 μl of buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiobitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), 4 μg/ml leupeptin], then pelleted. Cells were resuspended gently in 400 μl of buffer A and allowed to swell on ice for 15 min. After the addition of 25 μl of 10% NP-40, samples were vortexed for 10 sec and centrifuged at 12,000g for 30 sec. The resultant supernatant containing the cytoplasmic fraction was frozen at ~70°C. Fifty microliters of buffer C [420 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (pH 7.9), 1.5 mM MgCl_2, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 4 μg/ml leupeptin] was added to the pellet and placed on ice; then the tube was agitated periodically for 20 min. The extract was centrifuged at 10,000g and the supernatant containing the nuclear fraction was frozen at ~70°C.

EMSA and shift-Western blotting

The κB3 probe was prepared for EMSA by end-labeling the κB3 binding site [5'-GGCGGAAAATCCCCC-3'] from the murine c-rel promoter [Grumont et al. 1993] as described [Grumont and Gerondakis 1994]. EMSA reactions contained 5000 cpm of 32p-labeled probe, 0.1–1 μg of poly [dI-dC], 1.5–3 μg of protein extract, 10 μg of bovine serum albumin and reaction buffer [10 mM Tris-Cl at pH 7.5, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% NP-40] in a total volume of 15 μl. For competition analysis, a 50-fold excess of unlabeled κB3 competitor DNA was added to the reaction at room temperature 15 min prior to the addition of radiolabeled probe. EMSA reactions were then incubated for 20 min at room temperature, 2 μl of Ficoll dye was added, and the reactions fractionated on 5% non-denaturing acrylamide gels. The shift-Western blotting method was performed as described [Demczuk et al. 1993]. Briefly, protein–DNA complexes electrophoresed as for standard EMSA reactions were transferred onto stacked nitrocellulose and anion-exchange membranes. The radiolabeled probe that bound to the anionic filter was detected directly by autoradiography, whereas the Rel/NF-κB proteins in the complexes that bound to the nitrocellulose filter were detected with rabbit antibodies specific for p50, p65, Rel, RelB, and p52 as described for Western blotting.

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