B Cells Lacking RelB Are Defective in Proliferative Responses, but Undergo Normal B Cell Maturation to Ig Secretion and Ig Class Switching

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Summary

A number of distinct functional abnormalities have been observed in B cells derived from p50/NF-κB or c-rel knockout mice. RelB, another member of the NF-κB/Rel family of transcription factors, is expressed during the latter stages of B cell maturation and can bind to regulatory sites within the Ig heavy chain locus. Therefore, we tested the ability of B cells from relB knockout mice (relB-/-) to proliferate, undergo maturation to IgM secretion, and switch to the expression of downstream Ig isotypes in response to distinct activators including LPS, anti-CD40 mAb or CD40 ligand, and/or dextran anti-IgD antibodies in combination with various cytokines, including IL-4, IL-5, IFN-γ, and TGF-β. B cells lacking RelB showed up to 4-fold reductions in DNA synthesis in response to LPS, CD40, and membrane Ig-dependent activation relative to controls. However, relB-/- B cells were comparable to control B cells in their ability to undergo maturation to IgM secretion and switch to the expression of IgG3, IgG1, IgG2b, IgG2a, IgE, and/or IgA under all activation conditions tested. Thus, RelB, like c-Rel and p50/NF-κB, plays a role in B cell proliferation. However, in contrast to c-Rel and p50/NF-κB, it is not critically involved in maturation to Ig secretion or expression of Ig isotypes.

The NF-κB/Rel family of transcription factors, which includes p50 (and its precursor p105), p52 (and its precursor p100), RelA, RelB, and c-Rel, has been implicated in the rapid regulation of a wide array of genes involved in inflammation and immunity (1–3). These transcription factors are expressed in most cell types in an inactive cytoplasmic form and can be induced by a wide range of stimuli leading to degradation of the inhibitors, collectively termed IkB (4, 5). The NF-κB/Rel members form either homodimers or various combinations of heterodimers, possessing distinct binding affinities for particular promoters and enhancers. After cellular activation, IkB is rapidly degraded allowing NF-κB/Rel dimers to enter the nucleus and bind to κB consensus sequences, which are located in the promoters and enhancers of multiple genes. In this manner NF-κB/Rel family members can participate in the transcriptional regulation of that gene. Activation of normal murine B cells through membrane (m)Ig crosslinking, CD40 activation, or with LPS leads to rapid induction of NF-κB (6–8). Further, normal B cells and B cell tumors demonstrate a patterned expression of NF-κB/Rel family members during ontogeny and upon activation (9). Thus, pre-B cells express mainly p50 and p65, mature B cells p50 and c-Rel, and plasmacytoma lines or LPS-activated B cells express p52 and RelB along with the others. Indeed, the late expression of RelB has led to the suggestion that it may play a role in B cell maturation to Ig secretion.

Although the precise role of the NF-κB/Rel family members in normal B cell function is largely unknown, multiple NF-κB/Rel binding sites have been identified in the Ig heavy chain locus. Thus, p50 binding sites have been identified in the germine promoters (17 regions) of the constant heavy (CH) genes encoding γ (10), ε (11), γ1, and α (Lin, S.C., and J. Stavnezer, manuscript submitted for publication). In addition, multiple p50 binding sites have been identified in the switch regions for γ3, γ1, and γ2b, particularly at sites of switch recombination (12–14). A recent study has also identified functionally relevant κB binding sites in a pair of Ig heavy chain enhancers located 3' to the constant heavy gene for α (i.e., 3'αE [hs1,2] and 3'αE-hs4). Specifically, 3'αE (hs1,2) was found to bind RelB, c-Rel, and p50, whereas 3'αE-hs4 contained specific binding sites for RelB, p50, and p52 (15).

Several recent studies have demonstrated striking functional deficits in B cells from knockout mouse deficient in either c-Rel or p50. Thus, B cells from c-Rel-deficient mice were defective in DNA synthesis in response to LPS, CD40 ligand, or mlg cross-linking, relative to control B...
cells (16). A different functional phenotype was observed for B cells derived from p50 knockout mice. In this case, p50<sup>-/-</sup> B cells were markedly defective in their proliferative response to LPS, but showed comparable responses to mlg crosslinking or CD40 activation, compared to control B cells (17, 18). p50<sup>-/-</sup> B cells also demonstrated selective defects in B cell maturation to IgM secretion, germline C<sub>H</sub> transcription, and Ig class switching (18).

RelB-deficient mice exhibit a number of abnormalities in their hematopoietic system including a reduction in the number of thymic epithelial and dendritic cells and myeloid hyperplasia (19, 20). These abnormalities are associated with a mixed inflammatory cell infiltration of several organs and splenomegaly due to extramedullary hematopoiesis. However, although T and B cell development in relB<sup>-/-</sup> mice appears grossly normal, the functional capacity of B cells derived from relB knockout mice have not been studied. In light of RelB binding to regulatory sites in the Ig heavy chain locus and the induction of RelB expression during late phases of B cell maturation we tested the capacity of highly purified relB<sup>-/-</sup> B cells to proliferate, undergo maturation to Ig secretion, and switch to the expression of downstream Ig isotypes in response to multiple distinct stimuli.

**Materials and Methods**

**Mice.** Mice with a targeted disruption of the relb gene were originally established from relb<sup>-/-</sup> mice of (I29/Sv × C57BL6/J) background and were subsequently maintained by heterozygote intercrosses (19). Nur77/N10-transgenic mice lack mature T cells due to the expression of a Nur77/N10 cDNA under the control of the T cell-specific mouse proximal lck promoter and the human CD2 gene locus control region (21). RelB-deficient mice that also express the Nur77/N10 transgene are described elsewhere (Weih, F., S.K. Durham, D.S. Barton, W.C. Sha, D. Baltimore, and R. Bravo, manuscript submitted for publication). The first set of experiments were performed using relB<sup>-/-</sup> and relB<sup>-/-</sup> mice. The second and third set of experiments were performed with relB<sup>-/-</sup> Nur77/N10 and relB<sup>-/-</sup> Nur77/N10 mice. The latter mice were used in addition to the former since they demonstrated markedly reduced pathology due to the relB<sup>-/-</sup> phenotype in the absence of mature T cells. Results from both sets of experiments were found to be essentially equivalent. To minimize potential contributions from non-B cells due to differences in background genes between the different strains, small resting relB<sup>-/-</sup> and relB<sup>-/-</sup> B cells were purified by electronic cell sorting from splenocytes pooled from multiple littermates. All animals were housed and bred within the same room in the Veterinary Sciences Department of the Bristol-Myers Squibb Pharmaceutical Research Institute. The absence of pathogens was assessed by extensive periodic, comprehensive serology and histopathology of sentinel animals housed within the same room.

**Reagents.** H<sub>B</sub>/1 (monoclonal mouse IgG2b [b allotype] anti-mouse IgD [a allotype]) and AF3 (monoclonal mouse IgG2a [a allotype] anti-mouse IgD [b allotype]) antibodies were purified from ascites. Dextran-conjugated H<sub>B</sub>/1 and AF3 antibodies (a-dex) were prepared by conjugation of the respective mAbs, to high molecular weight dextran (2 × 10<sup>6</sup> M.W.) as previously described (22). The concentration of dextran-conjugated antibodies that is noted in the text reflects only the anti-Ig antibody concentrations and not that of the entire dextran conjugate. Membrane-bound (m) CD40L was prepared from SF9 insect cells infected with a CD40L-containing recombinant baculovirus vector (23). Anti-CD40 mAb (3/23, rat IgG2a anti-mouse CD40 mAb) (24) was a kind gift from G.G. Klaus (National Institute for Medical Research, London, England) and was purified from ascites. *Escherichia coli* LPS serotype 0111:B4, phenol-extracted was purchased from Sigma (St. Louis, MO). Recombinant murine IL-4, IL-5, IFN-γ, and human TGF-β2 were kind gifts from Alan Levine (Case Western Reserve University School of Medicine, Cleveland, OH), Richard Hodes (National Institutes of Health, Bethesda, MD), Genentech, Inc. (South San Francisco, CA), and Wendy Waegell (Celtrix Pharmaceuticals, Santa Clara, CA), respectively.

**Preparation and Culture of B Cells.** Single cell suspensions were made from spleen and RBCs were lysed with ACK lysing buffer. Cells were stained with polyclonal goat anti-mouse IgM-PE (Southern Biotechnology Associates, Birmingham, AL) and monoclonal FITC-rat anti-mouse CD3e (PharMingen, San Diego, CA). Small, resting B cells (IgM<sup>CD23</sup><sup>-</sup>, low forward and side scatter profile) were obtained by electronic cell sorting on an EPICS Elite cytometer (Coultier Corp, Hialeah, FL). Reanalysis of sorted cells immediately after isolation showed B cell purities of 97–99%. Sorted cells were cultured immediately after their isolation.

**Measurement of DNA Synthesis by [3H]Tdr Incorporation.** B cells were cultured for 72 h in a final volume of 0.2 ml in complete RPMI in flat bottom 96-well trays (Costar, Cambridge, MA). [3H]Tdr (1 μCi) (Amershams Corp., Arlington Heights, IL) with a specific activity of 20 Ci/mmol was added to the cultures for an additional 18 h. Cultured cells were then harvested onto glass fiber filter paper with an LKB Wallac (Turku, Finland) 1295-001 cell harvester. Specific incorporation of [3H]Tdr was analyzed by scintillation spectroscopy and results are expressed as the arithmetic mean ± SEM of triplicate cultures.

**Quantitation of Secreted Ig Isotype Concentrations in Culture SN.** Ig isotype concentrations were measured by an ELISA assay. For determination of concentrations of secreted IgM, IgG3, IgG1, IgG2c, IgG2a, and IgA in culture SN, Immulon 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unlabelled affinity-purified polyclonal goat anti-mouse IgM, IgG3, IgG, and IgA antibodies (10 μg/ml) (Southern Biotechnology Associates, Birmingham, AL), respectively. Plates were then washed, blocked with FBS-containing buffer, and incubated with various dilutions of culture SN and standards. After washing, plates were incubated with alkaline phosphatase-conjugated affinity-purified, polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2c, IgG2a, and IgA antibodies (100 ng/ml) (Southern Biotechnology Associates) as indicated, washed again, and a fluorescent product was generated by cleavage of exogenous 4-methyl umbiliferyl phosphate (MUP) (Sigma) by the plate-bound alkaline phosphatase-conjugated antibodies. For determination of IgG concentrations, a similar procedure was followed except that plates were coated with monoclonal rat IgG2a anti-mouse IgE (clone EM95) (10 μg/ml) (PharMingen), then streptavidin-alkaline phosphatase (PharMingen) (100 ng/ml), then MUP. Fluorescence was quantitated on a 3 M FluorOFAST 96 fluorometer (Mountain View, CA) and fluorescence units were converted to Ig concentrations by interpolations...
Results and Discussion

B Cells Lacking RelB Exhibit Defects in DNA Synthesis in Response to LPS, mlg or CD40 Signaling. Activation of normal murine B cells with LPS or through engagement of CD40 or membrane (m)Ig all induce B cell mitogenesis, with further augmentation in response to IL-4 + IL-5, although the signal transduction pathways mediating this event are distinct. Further, B cells activated through these modalities show distinctive patterns of Ig secretion and Ig isotype switching in response to specific patterns of stimuli (25). In this context, and in light of distinctive roles for p50 and c-Rel in murine B cell proliferation, we determined whether any of these mitogenic pathways were dependent upon the expression of RelB. Highly purified resting B cells from relB−/− and control mice, were obtained by electronic cell sorting and stimulated for 72 h with various concentrations of LPS, αδ-dex or anti-CD40 mAb plus IL-4 + IL-5. Comparable results were obtained in three independent experiments which demonstrated up to 4-fold reductions in DNA synthesis in B cells lacking RelB compared to controls in response to any of the 3 activation modalities (Fig. 1). These results differ in several ways from those obtained using B cells from c-rel−/− or p50−/− mice. c-rel−/− B cells show a more profound defect in proliferation in response to mIg- or CD40-mediated signaling, although, like relB−/− B cells, exhibited only a partial proliferative response to LPS, relative to control B cells (16). By contrast, p50−/− B cells showed a more profound proliferative defect in response to LPS, a partial reduction upon CD40 activation, and normal DNA synthesis after mIg crosslinking relative to control B cells (17, 18). Thus, although relB−/−, c-rel−/−, and p50−/− B cells all demonstrate defects in DNA synthesis they appear to differ (i) in the severity of the defect and (ii) in their capacity to respond to different mitogenic signals.

B Cells Lacking RelB Show Normal Maturation of Ig Secretion and Switching to Downstream Ig Isotypes. A previous study indicated that RelB is expressed late during B cell maturation (9). This suggested that RelB could regulate B cell maturation to Ig secretion. Further, RelB binding sites have been identified in both 3′c~E (hsl,2) and 3′α-hs4 (15). B cells from mice made genetically deficient in 3′c~E (hsl,2) showed selective defects in germline CH transcription and consequent Ig isotype switching (26), implicating 3′c~E (hsl,2) in transcriptional regulation of CH genes in germline. Indeed, the 3′αE (hs1,2) also contains p50 binding sites (15), and B cells from p50−/− mice demonstrate a pattern of germline CH expression similar to B cells from mice lacking 3′αE (hs1,2) (15, 21). The ability of LPS and anti-Ig to regulate 3′αE (hs1,2) activity in normal B cells (27) further indicates that this enhancer is active during B cell activation. Thus, we tested the relative ability of B cells lacking RelB to undergo B cell maturation to IgM secretion and switch to the expression of downstream Ig isotypes in response to various combinations of B cell activators and cytokines.

IL-4, IFN-γ, and TGF-β each induce class switching in appropriately activated mouse B cells; IL-4 to IgG1 and IgE; IFN-γ to IgG3 and IgG2a; and TGF-β to IgG2b and IgA (for review see reference 28). This is accomplished, at least in large part, by their apparent ability to selectively
stimulate transcriptional activation of germline C\(\mu\) genes, thus targeting them for switch recombination. Combinations of stimuli optimal for induction of class switching to different sets of isotypes were used to assess switch recombination in RelB\(^{-/-}\) B cells. Maturation to IgM secretion was essentially comparable between B cells lacking RelB and similarly-activated control B cells, in response to a variety of distinct stimuli (Fig. 2, A-E). This finding contrasted sharply with observations made using p50\(^{-/-}\) B cells in which IgM secretion was markedly defective in response to LPS, or in the presence of cytokines to either \(\alpha\delta\)-dex or CD40 activation (18). Restoration of cytokine-dependent IgM secretion in p50\(^{-/-}\) B cells was accomplished only with the combined actions of CD40L and \(\alpha\delta\)-dex. Thus, despite its appearance late during B cell maturation, RelB does not appear to be critical for maturation of B cells into IgM-secretors.

Likewise, induction of all 6 non-IgM, non-IgD isotypes in B cells lacking RelB was comparable to that observed for control B cells. The essentially normal levels of IgG1 and IgG3 secretion by B cells lacking RelB correlated with comparable percentages of membrane (m)IgG1\(^+\) and mIgG3\(^+\) cells by flow cytometry, relative to similarly activated control B cells (data not shown). This contrasted sharply with p50\(^{-/-}\) B cells in which class switching to IgG3, IgE, and IgA, was markedly reduced compared to control B cells, although induction of IgG1 was significant (18). This was associated with normal steady-state levels of germline C\(\gamma\)1 and C\(\gamma\)\(\alpha\) RNA, but markedly reduced germline C\(\gamma\)3 and C\(\gamma\)\(\epsilon\) RNA in p50\(^{-/-}\) B cells relative to controls. Studies on IgM secretion and class switching by B cells from c-relnoc knockout mice have not been reported (16). However, preliminary data from our laboratories indicates selective defects in germline C\(\gamma\)1 RNA expression and Ig class switching in c-rel\(^{-/-}\) B cells, distinct from that observed in B cells from p50\(^{-/-}\) mice (P. Zelazowski et al., in preparation). Thus, although RelB binding to the 3'\(\alpha\)-E (hs 1,2) as well as 3'\(\alpha\)-hs4 has been shown to be functionally relevant in a B cell tumor line (15), and 3'\(\alpha\)-E (hs 1,2) has been implicated in regulating germline C\(\mu\) RNA transcription (26), RelB by itself does not appear to play a critical role in expression of any of the Ig isotypes.

A more comprehensive understanding of the role of the NF-\(\kappa\)B/Rel family members in normal B cell function, will require additional analyses of B cells from p52 and RelA knockout mice plus further analyses of B cells from double, and perhaps triple knockout mice. Through multi-parameter functional analyses of these model systems, a clear view of the role of the NF-\(\kappa\)B/Rel family in the B cell functional program should emerge.
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