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## References

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# The p65 Subunit of NF- $\kappa$ B Is Redundant with p50 During B Cell Proliferative Responses, and Is Required for Germline C<sub>H</sub> Transcription and Class Switching to IgG3<sup>1</sup>

Bruce H. Horwitz,<sup>\*†</sup> Piotr Zelazowski,<sup>‡</sup> Yi Shen,<sup>‡</sup> Karen M. Wolcott,<sup>§</sup> Martin L. Scott,<sup>\*</sup> David Baltimore,<sup>2\*</sup> and Clifford M. Snapper<sup>3‡</sup>

B cells lacking individual NF- $\kappa$ B/Rel family members exhibit defects in activation programs. We generated small resting B cells lacking p65 or p50 alone, or lacking both p50 and p65, then evaluated the ability of these cells to proliferate, secrete Ig, and undergo Ig class switching. B cells lacking p65 proliferated well in response to all stimuli tested. However, these cells demonstrated an isolated defect in switching to IgG3, which was associated with a decrease in  $\gamma$ 3 germline C<sub>H</sub> gene expression. Whereas, previously reported, B cells lacking p50 alone had a severe proliferative defect in response to LPS, a moderate defect in response to CD40 ligand (CD40L), and normal proliferation to Ag receptor cross-linking using dextran-conjugated anti-IgD Abs ( $\alpha\delta$ -dex), B cells lacking both p50 and p65 exhibited severely impaired proliferation in response to LPS,  $\alpha\delta$ -dex, and CD40L. This defect could be overcome by simultaneous administration of  $\alpha\delta$ -dex and CD40L. In response to this latter combination of stimuli, B cells lacking both p50 and p65 secreted Ig and underwent isotype switching to IgG1 as efficiently as B cells lacking p50 alone. These data demonstrate a role for the p65 subunit of NF- $\kappa$ B in germline C<sub>H</sub> gene expression as well as functional redundancy between p50 and p65 during proliferative responses. *The Journal of Immunology*, 1999, 162: 1941–1946.

The NF- $\kappa$ B/Rel family of transcriptional regulators contains five members: p50 (NF $\kappa$ B1), p65 (RelA), c-Rel (Rel), RelB, and p52 (NF $\kappa$ B2) (1–3). These proteins bind to DNA in a sequence-specific manner and influence transcription either as homodimers or as heterodimers with other family members. NF- $\kappa$ B/Rel complexes representing many of the possible dimeric combinations have been identified in cell extracts; some of these complexes differ in DNA binding specificity and ability to activate transcription (4, 5). Dimers of NF- $\kappa$ B/Rel family polypeptides are present in the cytoplasm of most cells bound to inhibitory proteins of the I $\kappa$ B family (6–8). Cellular activation can lead to rapid degradation of I $\kappa$ B molecules, allowing translocation of NF- $\kappa$ B/Rel proteins to the nucleus, where they strongly enhance the transcription of a wide range of genes, including many involved in immune and inflammatory processes (4, 5).

Stimulation of purified small resting B cells represents a powerful system for studying the role of NF- $\kappa$ B/Rel proteins during activation programs. B cell activators including LPS, Ag receptor cross-linkers, and CD40 agonists induce I $\kappa$ B degradation and nuclear translocation of NF- $\kappa$ B/Rel proteins. B cells lacking the in-

dividual NF- $\kappa$ B/Rel subunits p50, RelB, c-Rel, or lacking only the transactivation domain of c-Rel demonstrate specific defects in B cell proliferative responses, Ig heavy chain constant region (C<sub>H</sub>) gene expression, and/or isotype class switching (9–13). Whereas B cells lacking p52 are essentially normal in the assays performed (14), B cells lacking p50 proliferate poorly in response to LPS, while proliferation of B cells lacking c-Rel or RelB is moderately reduced in response to LPS, Ag receptor stimulation, and CD40 ligation. Furthermore, B cells lacking p50 or the transactivation domain of c-Rel demonstrate defects in switching to IgG3, IgA, and IgE, or IgG3, IgG1, and IgE, respectively. Because C<sub>H</sub> gene expression is thought to be a prerequisite for class switching (15, 16), it is not surprising that in B cells lacking p50 there are alterations in expression of C<sub>H</sub> $\gamma$ 3 and C<sub>H</sub> $\epsilon$ , while in B cells lacking the transactivation domain of c-Rel, there are alterations in expression of C<sub>H</sub> $\gamma$ 3 and C<sub>H</sub> $\gamma$ 1. However, despite the observation that there is reduced switching to IgA and IgE in B cells lacking p50 or the transactivation domain of c-Rel, respectively, in these cases expression of the corresponding germline transcripts has appeared normal (11, 12). This raises the possibility that NF- $\kappa$ B/Rel may have other roles in the regulation of the class switching process in addition to regulation of germline C<sub>H</sub> gene expression.

Although the experiments summarized above suggest several roles for NF- $\kappa$ B/Rel proteins during the B cell activation program, important questions remain. Activation of purified small resting B cells lacking p65 has not been reported. Mice lacking RelA, the gene for p65, die during embryonic development (17, 18). However, B cells lacking p65 are produced after adoptive transfer of p65-deficient fetal liver cells into irradiated hosts (18, 19). In one study, splenocytes isolated from animals that had received p65-deficient fetal liver cells showed impaired [<sup>3</sup>H]TdR uptake in response to LPS or anti-IgM (18). However, because small resting B cells were not purified away from other splenocytes including large preactivated B cells and T cells, and the percentage of B cells present in each population was apparently not normalized, a cell

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autonomous role for p65 during B cell activation has not yet been clearly demonstrated. Furthermore, the role of p65 in class switching and  $C_H$  gene expression has not been addressed, nor has the possibility that p65 exhibits redundant functions with other NF- $\kappa$ B/Rel family members during B cell activation programs. Therefore, a number of important questions regarding the role of p65 in B cell activation remain unresolved.

In this article, we characterize the ability of purified small resting B cells lacking either p65 alone or both p50 and p65 to proliferate, secrete Ig, and undergo isotype class switching. We have found that although B cells lacking p65 alone proliferate well to all stimuli tested, they exhibit a defect in switching to IgG3 caused by decreased germline transcription of  $C_H\gamma$ 3. B cells lacking both p50 and p65 proliferate poorly in response to all individual mitogens but proliferate well to combinations of these agents. Interestingly, B cells lacking both p50 and p65 can secrete Ab and efficiently switch to IgG1 in response to these combination stimuli.

## Materials and Methods

### Reagents

LPS serotype 0111:B4, phenol extracted, was purchased from Sigma (St. Louis, MO). H8<sup>9</sup>/1 (monoclonal mouse IgG2b (b allotype) anti-mouse IgD (a allotype)) and AF3 (monoclonal mouse IgG2a (a allotype) anti-mouse IgD (b allotype)) Abs were purified from ascites. Dextran-conjugated H8<sup>9</sup>/1 and AF3 Abs ( $\alpha\delta$ -dex)<sup>4</sup> were prepared by conjugation of the respective mAbs to high m.w. dextran ( $2 \times 10^6$  m.w.), as previously described (20). The concentration of dextran-conjugated mAb that is indicated in the text reflects only the anti-Ig Ab concentration and not that of the entire conjugate. Membrane CD40 ligand (mCD40L) was prepared from Sf9 insect cells infected with a CD40 Ligand (CD40L)-containing recombinant baculovirus vector. Recombinant CD8-CD40L fusion protein (sCD40L) was constructed and expressed in a soluble form as previously described, partially purified from culture supernatants (SN) by precipitation with ammonium sulfate, and chromatographed over a CM-Sepharose column. Recombinant murine IL-4, IL-5, IFN- $\gamma$ , and human TGF- $\beta$  were kind gifts of Alan Levine (Case Western Reserve University School of Medicine, Cleveland, OH), Richard Hodes (National Institutes of Health, Bethesda, MD), Genentech (South San Francisco, CA), and Wendy Waegell (Celtrix Pharmaceuticals, Santa Clara, CA), respectively. The following FITC-labeled monoclonal Abs were used for flow cytometric analysis: Rat IgG1 anti-mouse IgG1 (Zymed Laboratories, South San Francisco, CA) and rat IgG2a anti-mouse IgG3 (PharMingen, San Diego, CA). Rat IgG1 anti-IgE mAb (R1.E4) (21) was purified from ascites and conjugated to FITC by a standard protocol. R1.E4 recognizes an epitope of the IgE molecule that is masked when IgE is bound to Fc $\epsilon$ R2; thus, R1.E4 recognizes intrinsic but not cytophilic IgE.

### Adoptive transfer of fetal liver cells

For transplantation experiments, p65<sup>-/-</sup> embryos were generated from crosses of p65<sup>+/-</sup> animals, and p50<sup>-/-</sup>;p65<sup>-/-</sup> embryos were generated from crosses of p50<sup>-/-</sup>;p65<sup>+/-</sup> animals. All fetal liver donors were on a mixed background of C57BL/6 and 129. Fetal livers were harvested and placed in 1 ml of Iscove's modified Dulbecco's medium, 2% FBS, and single-cell suspensions prepared by passage through a needle. To determine genotypes, 1/20<sup>th</sup> of the fetal liver suspension was washed in 1 ml of PBS, resuspended in 100  $\mu$ l PCR buffer containing 10 mg proteinase K/ml, 0.045% NP40, and 0.045% Tween 20, and then incubated at 55°C for 30 min. The protease was inactivated for 10 min at 100°C, and 2  $\mu$ l of this lysate was subject to genotyping by standard PCR reaction. Within 6 h of harvest, 300  $\mu$ l of medium containing  $5 \times 10^5$  fetal liver cells and  $5 \times 10^5$  wild-type bone marrow cells harvested from a C57BL/6-CD45.1 mouse was injected into the tail vein of a C57BL/6-CD45.1 female host. Host animals were irradiated using a <sup>137</sup>Cs source with doses of 800 and 400 Rads, separated by 3 h, before injection of fetal liver cells. After transplantation, host mice were maintained in autoclaved cages on autoclaved water containing trimethoprim-sulfamethoxazole.

### Culture medium

RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FBS (Sigma), 2 mM L-glutamine, 0.05 mM 2-ME, 50  $\mu$ g/ml penicillin (Life Technologies, Grand Island, NY), and 50  $\mu$ g/ml streptomycin (Life Technologies) was used for culturing cells.

### Preparation and culture of B cells

Two to six months after transplantation, single-cell suspensions were made from spleens of chimeric animals, and RBC were lysed in ammonium chloride. Cells were stained with phycoerythrin-labeled rat IgG2a anti-mouse B220 mAb (clone RA3-6B) (PharMingen) and FITC anti-mouse CD45.1 mAb (PharMingen). Small resting fetal liver-derived B cells (low forward and side scatter, B220<sup>+</sup>, CD45.1<sup>-</sup>) were obtained by electronic cell sorting on an EPICS Elite cytometer (Coulter, Hialeah, FL). Reanalysis of sorted cells immediately after isolation showed fetal liver-derived B cell purities of >99%. B cells were cultured at 37°C in a humidified incubator containing 6% CO<sub>2</sub> at a cell density of  $2 \times 10^5$  cells/ml.

### Measurement of DNA synthesis by [<sup>3</sup>H]TdR incorporation

B cells were cultured for 48 h in a final volume of 0.2 ml in RPMI 1640 medium in flat-bottom 96-well trays (Costar, Cambridge, MA). [<sup>3</sup>H]TdR (1  $\mu$ Ci; sp. act. 20 Ci/mmol; Amersham, Arlington Heights, IL) was added to the cultures for the last 8–12 h. Cultured cells were then harvested onto glass fiber filter paper with an LKB-Wallac (Turku, Finland) 1295–001 cell harvester. Specific incorporation of [<sup>3</sup>H]TdR was analyzed by scintillation spectroscopy, and results are expressed as the arithmetic mean  $\pm$  SEM of triplicate cultures.

### Quantitation of secreted Ig isotype concentrations in culture SN

Ig isotype concentrations were measured by ELISA. To determine concentrations of secreted IgM, IgG3, (IgG1, IgG2b, and IgG2a), and IgGA in culture SN, Immulon 2, 96-well flat-bottom plates (Dynatech Laboratories, Alexandria, VA) were coated with unlabeled affinity-purified polyclonal goat anti-mouse IgM, IgG3, IgG, and IgA Abs (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 goat polyclonal anti-mouse IgM, IgG3, IgG1, IgG2b, IgG2a, and IgA Abs (Southern Biotechnology Associates) as indicated, washed again, and a fluorescent product was generated by cleavage of exogenous 4-methyl-umbilliferyl phosphate (Sigma) by the plate-bound alkaline phosphatase-conjugated Abs. For determination of IgE concentrations, a similar procedure was followed except that the plates were coated with a monoclonal rat IgG1 anti-mouse IgE (R1.E4) (purified from ascites), followed by samples and standards, then monoclonal biotin-rat IgG1 anti-mouse IgE (clone R35–92) (PharMingen), then streptavidin-alkaline phosphatase (PharMingen), and then 4-methylumbilliferyl phosphate. Fluorescence was quantitated on a MicroFLUOR ELISA reader (Dynatech, Chantilly, VA), and fluorescence units were converted to Ig concentrations by interpolation from standard curves that were determined with known concentrations of purified myeloma Ig. Each assay system showed no significant cross-reactivity or interference from other Ig isotypes (IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) found in the culture SN.

### Measurement of membrane Ig-positive cells by flow cytometry

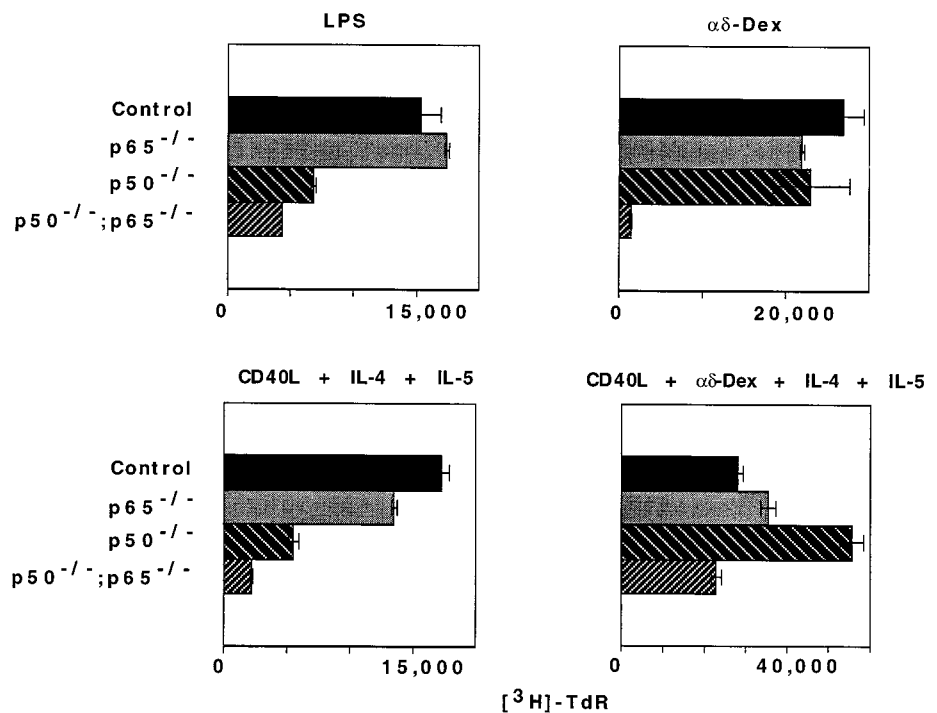
All steps were performed on ice. Cultured cells were harvested, washed twice in cold staining buffer and then resuspended in 100  $\mu$ l of the same buffer. FITC-labeled mAbs were added at a final concentration of 10  $\mu$ g/ml. Fluorescence analysis was conducted using a FACScan (Becton Dickinson, Mountain View, CA). Only viable cells, which were identified on the basis of their characteristic forward and side scatter and exclusion of propidium iodide, were analyzed.

### RT-PCR for $C_H$ germline transcripts

RNA was extracted from cultured B cells using RNA-zol (Tel-Test, Friendswood, TX), according to the manufacturer's instructions. A total of 3  $\mu$ g of RNA in 25  $\mu$ l of ddH<sub>2</sub>O were reversed transcribed using SuperScript RNase H<sup>-</sup> reverse transcriptase (Life Technologies, Gaithersburg, MD). PCR conditions and primers sequences were described elsewhere (11). Reactions were performed in the presence of 0.1  $\mu$ l of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, 370 Mbq/ml; Amersham) in 50  $\mu$ l reaction mix. The PCR products were separated on 5% PAGE, the gel was dried and placed into a PhosphorImager cassette, and the relative intensity of the bands was measured by densitometry using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

<sup>4</sup> Abbreviations used in this paper:  $\alpha\delta$ -dex, dextran-conjugated anti-IgD Abs; CD40L, CD40 ligand; mCD40L, membrane-bound CD40L; sCD40L, soluble CD40L; SN, supernatant.

**FIGURE 1.** Proliferation of B cells lacking NF- $\kappa$ B family members in response to LPS,  $\alpha\delta$ -dex, and CD40L. B cells were stimulated with indicated agents. Concentrations were LPS (20  $\mu$ g/ml),  $\alpha\delta$ -dex alone (3 ng/ml) or when with CD40L, IL-4, and IL-5 (0.3 ng/ml), mCD40L (1:1000 v/v), IL-4 (3000 U/ml), and IL-5 (150 U/ml). [ $^3$ H]TdR incorporation was measured, and data are expressed as the mean of triplicate cultures,  $\pm$  SEM. Cells cultured in medium alone had <300 cpm of [ $^3$ H]TdR incorporation.



## Results and Discussion

### Proliferation of B cells lacking NF- $\kappa$ B

We have previously demonstrated that there is a severe defect in lymphopoiesis after transplantation of p50/p65-deficient fetal liver cells into irradiated hosts. However, fetal liver-derived lymphocytes will accumulate if wild-type bone marrow cells are transplanted simultaneously with the p50/p65-deficient fetal liver cells (19). To directly compare the function of B cells lacking different NF- $\kappa$ B genes, we used this method to produce B cells lacking p50 alone, p65 alone, both p50 and p65, as well as control B cells. Fetal liver-derived small resting B cells were isolated by FACS of low forward and side scatter, and B220<sup>+</sup> and CD45.1<sup>-</sup> cells from spleens of previously transplanted animals. The absence of CD45.1 identifies these cells as ones derived from transplanted fetal liver cells, which are CD45.1<sup>-</sup>, rather than ones derived from transplanted or residual host bone marrow cells, which are CD45.1<sup>+</sup>.

To examine the ability of splenic B cells to proliferate *in vitro*, they were stimulated with B cell mitogens, and incorporation of [ $^3$ H]TdR was assessed after 2 days (Fig. 1). As previously demonstrated, B cells lacking p50 proliferated significantly less well in response to LPS than control B cells, although proliferation in response to Ag receptor cross-linking was normal. It has previously been argued that the absence of p50 has a quantitative effect on the ability of B cells to proliferate in response to CD40 activation, because p50-deficient B cells exhibited a variable defect in this response dependent on the mode of activation (11). In the experiments reported here, p50-deficient B cells exhibited a moderate defect in proliferation in response to CD40L plus IL-4 and IL-5 compared with control B cells.

B cells lacking p65 proliferated as well as control cells to all stimuli tested. This result was initially surprising because a previous report had suggested a defect in B cell proliferation in the absence of p65 (18). However, in that report proliferation was measured in populations of total splenocytes, not B cells purified away from other cell types nor fractionated based on size. This could complicate interpretation because the previously observed

defect in proliferation could be secondary to a defect in another cell type present in the population or to inclusion of large preactivated B cells. Furthermore, the proliferation data was not clearly normalized to the percentage of B cells present in each spleen sample. In the current study, small resting B cells were rigorously purified from other cell types and equivalent numbers of purified cells were assayed. Thus, our data suggest that under conditions used in this study, small resting B cells lacking p65 do not have an intrinsic defect in their ability to proliferate in response to the mitogens tested.

In contrast to p65-deficient B cells, B cells lacking both p50 and p65 exhibit severe defects in proliferation. In addition to defective proliferation in response to LPS (as observed for B cells lacking p50 alone), p50/p65-deficient B cells do not proliferate in response to Ag receptor cross-linking. Furthermore, they proliferate threefold less well than B cells lacking p50 alone in response to CD40L, IL-4, and IL-5. This additional defect was verified independently by demonstrating that 4 days after stimulation there were at least threefold more viable cells in cultures of B cells lacking p50 than in cultures of B cells lacking both p50 and p65 (data not shown). Thus, proliferation is much more severely affected in the absence of both p50 and p65 than in the absence of either subunit alone. This observation suggests that in response to Ag receptor cross-linking or CD40 activation, p50 and p65 perform at least partially redundant functions. Although the molecular nature of this redundancy is not yet clear, it is intriguing to speculate that there is redundancy between NF- $\kappa$ B complexes that contain p50 and those that contain p65 in their ability to mediate the transcriptional events necessary for proliferation. Thus, proliferation would only be defective in the absence of both p50 and p65, but not in the absence of either subunit individually. It is possible that these redundant complexes are either homodimers of each subunit or heterodimers with other Rel family members that have been shown to be necessary for proliferation, such as c-Rel or RelB (10, 13).

The observation that B cells lacking p50 and p65 proliferate poorly to all individual stimuli tested raises the possibility that B

Table I. Secretion of Ig isotypes by control and p65-deficient B cells<sup>a</sup>

Secreted Ig (ng/ml)	Control	p65 <sup>-/-</sup>
IgM		
LPS	38,700	4,500
LPS + IL-4 + IL-5	30,000	7,000
IgG3		
LPS	2,620	195
IgG1		
LPS + IL-4 + IL-5	6,870	4,125
IgG2a		
LPS + IFN- $\gamma$	5,250	4,125
IgG2b		
LPS	3,125	1,350
IgE		
LPS + IL-4 + IL-5	450	380
IgA		
CD40L + IL-4 + IL-5 + $\alpha\delta$ -dex + TGF- $\beta$	2,500	2,300

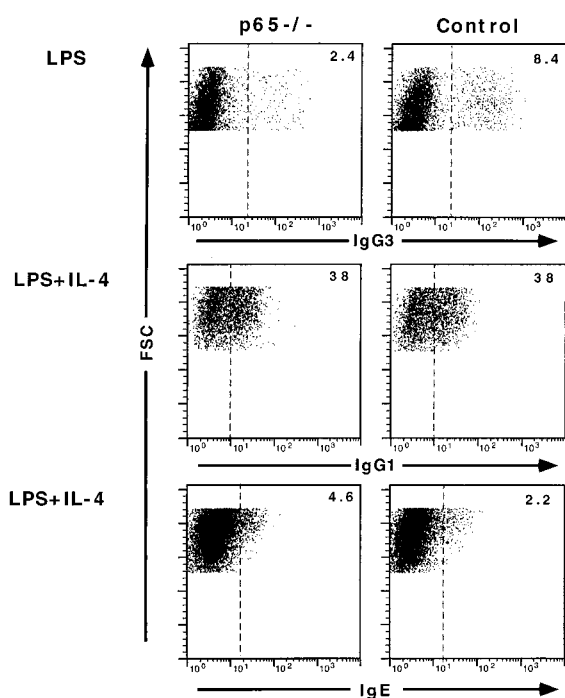
<sup>a</sup> Control and p65<sup>-/-</sup> B cells were stimulated with LPS (20  $\mu$ g/ml) alone, LPS with IL-4 (3000 U/ml) and IL-5 (150 U/ml), LPS with IFN- $\gamma$  (10 U/ml), or with mCD40L (1:1000 v:v), IL-4, IL-5,  $\alpha\delta$ -dex (0.3 ng/ml), and TGF- $\beta$  (1 ng/ml). Concentrations of secreted IgM, IgG3, IgG1, and IgE were measured in culture SN by ELISA 6 days after initiation of culture. The values represent the mean of triplicate cultures.

cell proliferation absolutely requires the presence of either p50 or p65. This does not appear to be the case because p50/p65-deficient B cells will proliferate essentially as well as control B cells in response to the combined stimuli of CD40L,  $\alpha\delta$ -dex, IL-4, and IL-5 (Fig. 1). Interestingly, this combined treatment will also rescue activation defects observed in B cells lacking p50 (11) or the transactivation domain of c-Rel (12). Thus, it is possible that the combined stimuli activate signaling pathways that bypass the requirements for NF- $\kappa$ B/Rel family members during B cell activation programs. Alternatively, the combined treatment might be a much more potent activator of NF- $\kappa$ B nuclear translocation than any of these agents individually, allowing functional compensation between family members that does not occur when activating agents are used individually.

#### Ab secretion and class switching in B cells lacking p65

To evaluate the ability of B cells lacking p65 to undergo Ig isotype switching, the amount of Ig isotypes in supernatants of stimulated cultures was measured by ELISA after 6 days. In response to both LPS and LPS, IL-4, and IL-5, less IgM was detected in cultures of p65-deficient B cells than in cultures of control B cells (Table I). In addition, we detected a marked decrease in the amount of IgG3 produced by p65-deficient B cells compared with control B cells, although levels of other switched isotypes examined were comparable (Table I). The observation that cultures of B cells lacking p65 proliferate as well as cultures of wild-type cells but that there is less IgM detected in SN of p65-deficient cells raises the possibility that the absence of p65 may lead to a defect in secretion of IgM. A defect in maturation to Ab secretion has been reported in B cells lacking p50 (11). However, the apparent ability of p65-deficient B cells to secrete most other isotypes at normal levels argues against p65 playing a global role in B cell maturation.

The observation that IgG3 production is reduced in the absence of p65 suggests that isotype switching to IgG3 is p65 dependent. To examine switching directly, the presence of switched isotypes on the surface of stimulated B cells was measured by flow cytometry. After stimulation with LPS, there is a three- to fourfold reduction in the percentage of p65-deficient B cells that express surface IgG3 compared with control B cells (Fig. 2). In contrast, a

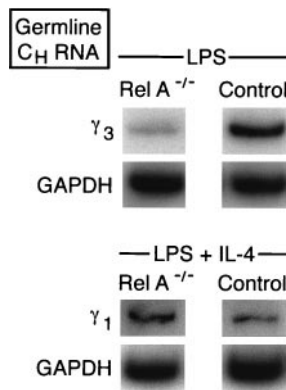


**FIGURE 2.** p65-deficient B cells are defective for class switching to IgG3. Control and p65-deficient B cells were stimulated with LPS (20  $\mu$ g/ml) for switching to IgG3 or LPS and IL-4 (3000 U/ml) for switching to IgG1 and IgE. Flow cytometry was performed 4 days after initiation of culture, and data is expressed as the log of fluorescent intensity of staining with the indicated Ab on the x-axis and a linear representation of forward scatter on the y-axis. Numbers in the right upper corner of each plot refer to the percentage of cells expressing the indicated isotype on the cell surface. Data is representative of three similar experiments.

similar percentage of control and p65-deficient B cells express surface IgG1 and IgE after stimulation with LPS and IL-4 (Fig. 2). Comparable percentages of control and p65-deficient B cells expressing surface IgA were also observed after stimulation with CD40L, IL-4, IL-5,  $\alpha\delta$ -dex, and TGF- $\beta$  (data not shown). These results demonstrate that p65 is required for switching to IgG3 but not to other measured isotypes.

It has previously been suggested that NF- $\kappa$ B/Rel may regulate class switching by influencing transcription of germline C<sub>H</sub> genes (11, 12, 22–25). However, in some situations NF- $\kappa$ B/Rel must play an alternative role in the switching process, because B cells deficient in p50 or the transactivation domain of c-Rel exhibit certain defects in switching that cannot be explained by defects in germline C<sub>H</sub> gene expression (11, 12, 26). To determine whether the defects in switching to IgG3 noted in the absence of p65 could be explained by a defect in germline C<sub>H</sub> gene expression, we used a previously described semiquantitative RT-PCR assay to measure germline transcripts in stimulated B cells (11). After stimulation with LPS, there was a marked reduction in germline C<sub>H</sub> $\gamma$ 3 transcripts in cells lacking p65 compared with control cells, although there were similar amounts of GAPDH transcript present (Fig. 3). The amount of germline C<sub>H</sub> $\gamma$ 1 transcript present after stimulation with LPS and IL-4 was similar in p65-deficient and control B cells (Fig. 3). These results strongly suggest that the defect in switching to IgG3 observed in the absence of p65 is caused by a defect in transcription of the germline C<sub>H</sub> $\gamma$ 3 gene, and, furthermore, that p65 is required only for transcription of the C<sub>H</sub> $\gamma$ 3 gene, but not other C<sub>H</sub> genes.

Defects in  $\gamma$ 3 gene expression have previously been noted in cells lacking p50 and the transactivation domain of c-Rel, but in



**FIGURE 3.** p65-deficient B cells show defective expression of germline C<sub>H</sub>γ<sub>3</sub> RNA. Control or p65-deficient B cells were stimulated as indicated. Concentration of reagents are as in Fig. 2. RNA was purified 48 h after the initiation of culture, and levels of germline transcripts and GAPDH were determined by RT-PCR.

both these cases transcription of other constant region genes was also effected (11, 12). Because there are functional NF-κB binding sites throughout the Ig heavy chain locus, it is unclear whether the effects on germline transcription are mediated by interactions with κB binding sites located within I region promoter sequences (22–24) or whether NF-κB could be influencing transcription by binding to remote sites such as those present in the 3′α-enhancers (25). Within the Iγ<sub>3</sub> promoter, an NF-κB/Rel binding site has been identified that is necessary for NF-κB-dependent transcription of a reporter gene (22). It is possible that dimers of NF-κB/Rel family members influence transcription by binding to this site. Because presumably a single κB site can only be bound by a single dimeric complex at any one time, it is interesting that mutation of three different family members p50, p65, and c-Rel strongly influence transcription of this gene. These observations suggest that either sequential binding of these factors is required or that at least one of these proteins influences transcription by binding to a different κB site. Finally, it has not been clearly demonstrated that all NF-κB family members are influencing transcription by binding to *cis*-acting NF-κB sites. NF-κB subunits could influence germline constant region transcription in an indirect fashion, perhaps by altering expression of other genes involved in the switching process. It has previously been suggested that nuclear NF-κB activity may regulate expression of c-Rel (27, 28). Therefore, it is possible that there is reduced c-Rel expression after LPS stimulation of p65-deficient B cells and that the absence of complexes containing c-Rel causes a direct defect in γ<sub>3</sub> germline transcription.

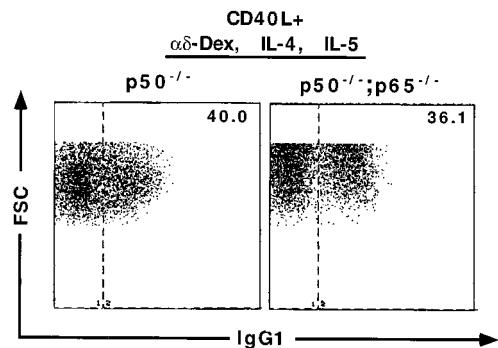
#### *Ab secretion and class switching of B cells lacking both p50 and p65*

The observation that p50/p65-deficient B cells will proliferate in response to CD40L, αδ-dex, IL-4, and IL-5 allowed us to evaluate the ability of these stimulated cells to secrete Ig and undergo iso-

**Table II.** Secretion of Ig isotypes by p50-deficient and p50/p65-deficient B cells after stimulation with mCD40L, αδ-dex, IL-4, and IL-5<sup>a</sup>

	p50 <sup>-/-</sup>	p50 <sup>-/-</sup> ;p65 <sup>-/-</sup>
IgM (ng/ml)	10,000	28,700
IgG1 (ng/ml)	4,620	10,500

<sup>a</sup> p50<sup>-/-</sup> and p50<sup>-/-</sup>;p65<sup>-/-</sup> B cells were stimulated with mCD40L (1:1000 v:v), αδ-dex (0.3 ng/ml), IL-4 (3000 U/ml), and IL-5 (150 U/ml). Methods are as described in Table I.



**FIGURE 4.** B cells lacking p50 or both p50 and p65 switch normally to IgG1. B cells lacking p50 or both p50 and p65 were stimulated with sCD40L (10 μg/ml), αδ-dex (0.3 ng/ml), IL-4 (3000 U/ml), and IL-5 (150 U/ml). Flow cytometry was performed 4 days after initiation of culture and data is expressed as the log of fluorescent intensity of staining with the indicated Ab on the x-axis and linear representation of forward scatter on the y-axis. Numbers in the right upper corner of each plot refer to the percentage of cells expressing the indicated isotype on the cell surface. Data is representative of two similar experiments.

type switching to IgG1. This combination of activating agents induces switching to IgG1 but does not stimulate appreciable switching to other Ig isotypes except for IgE. Switching to IgE under these conditions is defective in the absence of p50 alone (15). After stimulation, B cells lacking both p50 and p65 secreted as much if not more IgM than p50-deficient B cells suggesting that cells lacking both subunits mature to Ab secretion as well as cells lacking p50 alone (Table II). Furthermore, p50/p65-deficient B cells appear to secrete similar levels of IgG1 as p50-deficient cells, arguing that B cells lacking p50 and p65 are able to switch to IgG1 (Table II). To examine isotype switching directly, we analyzed surface expression of IgG1 on stimulated B cells and found that a similar percentage of p50/p65-deficient and p50-deficient B cells express surface IgG1 (Fig. 4). This demonstrates that B cells lacking both p50 and p65 can efficiently switch to IgG1, and argues that, under these conditions, p50 and p65 are not involved in isotype switching to IgG1.

Our detailed analysis of B cell activation programs in cells lacking p65 or both p50 and p65 has demonstrated that p50 and p65 have both redundant and nonredundant functions. This suggests that to fully appreciate important functions of NF-κB during these programs it will be necessary to evaluate B cells lacking multiple different family members. Relating the particular functional properties of the subunits to their structure and regulation is a future challenge.

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