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Activation of NF-κB/Rel by CD40 Engagement Induces the Mouse Germ Line Immunoglobulin Cγ1 Promoter

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Interaction between CD40 on B cells and CD40 ligand (CD40L) on T cells has been shown to mediate T-cell contact help for B-cell proliferation, differentiation, and immunoglobulin isotype switching. It has recently been shown that cross-linking CD40 on mouse B cells induces germ line γ1 and ε transcripts and that interleukin-4 synergizes with CD40 signaling to further induce these germ line transcripts. Germ line transcripts have been shown to be required for class switch recombination. Here we show that signaling via CD40 increases expression of a transiently transfected luciferase reporter plasmid driven by the germ line Cγ1 promoter in M12.4.1 B-lymphoma cells. By linker-scanning mutation analysis of the promoter, we have identified a CD40-responsive region (CD40RR) which is able to confer inducibility by CD40L to a minimal c-*fos* promoter. The CD40RR contains three binding sites for NF-κB/Rel proteins which are each required for maximal induction of CD40RR activity by CD40L. Binding of the NF-κB/Rel proteins p50, p65, c-Rel, and RelB to the CD40RR is induced by CD40 signaling in M12.4.1 cells and in splenic B cells. Cotransfection of expression plasmids for p50 and p65 or p50 and RelB, but not c-Rel, into M12.4.1 cells transactivates the CD40RR and the germ line γ1 promoter. These data demonstrate that NF-κB Rel proteins activated by CD40 ligation play an important role in induction of the germ line Cγ1 promoter.

After activation, B cells can undergo antibody class, or isotype, switching, resulting in expression of a different immunoglobulin (Ig) heavy-chain constant (C_H) region while maintaining the same variable region. Antibody class switching, therefore, allows B cells to produce antibody with the same antigen binding specificity but with different effector functions. Class switching is mediated by a DNA recombination event in which the rearranged heavy-chain V(D)J region, which is initially positioned 5′ to the Cμ gene, is repositioned to a site nearer to a downstream C_H gene.

Several studies have shown that transcription of a specific unrearranged C_H gene, producing what are called germ line switch transcripts, occurs prior to class switching to that gene (9, 59, 66, 91, 92). Transcription of unrearranged C_H genes initiates at the I exon, located upstream of each switch (S) region, and continues through the S region and the C_H gene. The primary germ line transcript is spliced, resulting in deletion of the S region and sequence between the I exon and the C_H gene.

Selective switch recombination and expression of germ line transcripts have been shown to be regulated by the cytokines interleukin-4 (IL-4), gamma interferon, and transforming growth factor β, in concert with B-cell activators (reviewed in reference 90). For example, addition of IL-4 to lipopolysaccharide (LPS)-stimulated mouse B cells directs switching to IgG1 and IgE. In LPS-activated B cells, IL-4 induces transcripts of unrearranged Cγ1 and Cε genes before inducing switching to IgG1 and IgE, suggesting that these cytokines regulate isotype switching by regulating germ line transcripts (9, 24, 34, 77, 83, 91). DNase I hypersensitivity, nuclear run-on, and RNA stability measurements show that regulation of germ line transcripts by cytokines occurs at the transcriptional level (10, 64, 76, 80, 85). Gene targeting experiments have shown that germ line transcripts are required for switch recombination (11, 37, 46, 64, 101).

Induction of germ line transcription by IL-4 is mediated by the transcription factor Stat6 (IL-4 Stat). Upon IL-4 stimulation, Stat6 is phosphorylated and transported into the nucleus, where it binds to *cis*-acting elements in the Ig Cε and mouse Cγ1 promoters, mediating IL-4 inducibility of these promoters (8, 18, 42, 51, 65, 74). Other transcription factors, e.g., the C/EBP family, Pax-5/BSAP, HMG-I(Y), and NF-κB/Rel proteins, have also been shown to regulate transcription of the germ line Cγ1 and Cε promoters (18, 49, 61, 65).

Although cytokines direct isotype switching by selectively inducing germ line transcription, cytokines alone do not induce switch recombination. Induction of switching requires a B-cell activator, such as (i) LPS, (ii) a mimic of a T-cell-independent antigen, such as anti-Ig bound to Sepharose beads or anti-IgD-dextran in the presence of IL-5, or (iii) T-cell contact help (48, 71, 73, 88).

T-cell contact help is important for B-cell activation, proliferation, and isotype switching during T-dependent immune responses. T-cell contact help is primarily mediated by interaction between the CD40 molecule expressed on B cells and its ligand, CD40L, expressed on activated T cells (25, 58, 69). The central role of the CD40L-CD40 interaction in B-cell maturation and isotype switching was demonstrated by hyper-IgM syndrome patients, who have a mutated CD40L gene, and also by mice having a targeted disruption of the CD40L or CD40 gene. Hyper-IgM syndrome patients and mice lacking CD40L or CD40 do not undergo isotype switching to IgG, IgA, or IgE and lack B-cell memory responses (1, 2, 14, 19, 30, 47, 54, 99).

Engagement of CD40 by recombinant CD40L or antibody to CD40 induces germ line transcripts and in some systems also induces class switch recombination in culture in the presence of cytokines (32, 44, 86, 97). CD40 signaling appears to induce specific germ line transcripts. Membranes from activated T cells prepared from normal mice or IL-4-deficient mice have been shown to induce germ line γ1 transcripts, but no γ3, γ2b,

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or ε transcripts, in splenic B cells, suggesting that CD40 signaling in the absence of cytokines may specifically induce germ line γ1 transcripts (82). Recently, it has been shown that CD40L expressed on Sf9 insect cells induces germ line γ1 and ε transcripts and synergizes with IL-4 to further induce these transcripts (97). In human systems, the data are controversial, but in general it is found that antibody to CD40 alone does not induce germ line γ, ε, or α transcripts, although it synergizes with IL-4 to induce germ line ε transcripts (28, 33, 50). Recently, however, it has been shown that CD40L expressed on Sf9 cells, in the absence of exogenous IL-4, increases activity of the promoter for human germ line ε transcripts twofold (29).

We report here that soluble CD40L increases activity of the germ line γ1 promoter, and we identify a CD40-responsive region (CD40RR) located immediately 3′ to the IL-4-responsive region (IL-4RR) of the Cγ1 promoter. The IL-4RR is not required for induction by CD40L. Specific members of the NF-κB/Rel family bind to the CD40RR and transactivate the Cγ1 promoter.

MATERIALS AND METHODS

Soluble CD40L-CD8α chimeric protein, anti-CD40 monoclonal antibody (MAb), and anti-CD8α antibody. The CD40L used in this study is a soluble CD40L-CD8α fusion protein which is secreted by J558L mouse myeloma cells stably transfected with the CD40L-CD8α fusion gene (57). Cell culture supernatant containing secreted CD40L-CD8α was used as the source of CD40L, whereas cell culture supernatant from untransfected J558L cells was used as the control. Cells expressing CD40L-CD8α were selected in Iscove's modified Dulbecco's medium with 2% fetal bovine serum (FBS) and 2 μg of G418 per ml. After selection, cells were frozen in aliquots. For collection of CD40L-CD8α-containing supernatant, cells were transferred to RPMI 1640 medium containing 10% FBS. Control supernatant was obtained from cultures of untransfected J558L cells in RPMI 1640 medium with 10% FBS. In preliminary experiments, it was determined that 20% CD40L supernatant gives optimal induction.

Anti-CD40 MAb (rat IgG2a) was a gift of G. Klaus (National Institute for Medical Research, London, England) (38). Anti-CD8α MAb (rat IgG2a) (PharMingen, San Diego, Calif.) was used for cross-linking soluble CD40L-CD8α and also as a control antibody for anti-CD40. CD40-Ig fusion protein, in which the extracellular domain of human CD40 is fused to the CH2 and CH3 domains of human γ1 (15), was purified by C. Schrader, University of Massachusetts Medical School, Worcester.

Cells. The M12.4.1 B-lymphoma cell line is a surface (sIg)-negative variant of the M12 line, which expresses IgG2a (36). CD40 is expressed on M12.4.1 cells, as detected by staining with CD40L supernatant followed by biotin-conjugated anti-CD8α and streptavidin-phycoerythrin (PharMingen). M12.4.1 cells were maintained in a 5% CO_2 incubator in RPMI 1640 medium with 10% FBS, 50 μM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 200 U of penicillin per ml, 200 mg of streptomycin per ml, and 0.1 mg of kanamycin sulfate (GIBCO Laboratories, Grand Island N.Y.) per ml.

Splenic B cells were purified by depletion of T cells, using MAbs for Thy1.2 (HO-13-4), Thy1 (Jijo.10), CD4 (GK1.5), and CD8 (3.168.3) followed by addition of anti-rat κ-chain MAb (MAR 18.5) and guinea pig complement. Dead cells were removed by centrifugation on Ficoll-Hypaque gradients. Splenic B cells were cultured as described for M12.4.1 cells.

Cytokines, LPS, and PDBu. Recombinant IL-4 expressed by a baculovirus vector was a gift of W. E. Paul, National Institutes of Health, Bethesda, Md. Mouse IL-6 and anti-mouse IL-6 MAb were purchased from Endogen (Boston, Mass.). LPS (*Escherichia coli* serotype O55:B5) and phorbol dibutyrate (PDBu) were purchased from Sigma.

Plasmids. (i) Luciferase reporter plasmids. Luciferase reporter plasmids containing wild-type Ig Cγ1 promoter segments (−954WT and −150WT) and a series of luciferase reporter plasmids containing linker-scanning mutations in the segment between nucleotides −160 and −3, relative to the first RNA initiation site (−160/−3 segment), within the nucleotide −954 germ line Cγ1 promoter were described previously (100). Another series of linker-scanning mutants that contain the same mutations in the backbone of plasmid −150WT were generated by deletion of the −954/−151 segment) by *Bam*HI-*Kpn*I digestion, fill-in, and self-ligation of each mutant.

Plasmid pFL contains the mouse minimal c-*fos* promoter segment (from −71) from pFosCAT, placed upstream of the luciferase reporter gene in plasmid pXP2 (62). Plasmids containing the CD40RR inserted into plasmid pFL were created by the following method. The wild-type and mutated CD40RR fragments were amplified by PCR, using wild-type or mutated primers with addition of *Bam*HI or *Bgl*II sites at each end. Fragments, digested with *Bam*HI and *Bgl*II, were inserted into the *Bam*HI site of pFL. A plasmid containing two copies of the wild-type CD40RR in the forward direction was generated by insertion of an additional wild-type CD40RR into pCD40FL-F at the *Bam*HI site. The nucleotide sequences of all inserts were determined after cloning.

(ii) NF-κB/Rel protein expression plasmids. To create the NF-κB p50 eukaryotic expression plasmid, p50 cDNA in pBS-ATG (35) was digested with *Sph*I and *Eco*RI to excise p50 cDNA, which was inserted into pcDNAI (Invitrogen, San Diego, Calif.) digested with *Sph*I and *Eco*RI. The p65 expression plasmid was generated by excision of p65 cDNA from pSP72 (70) by *Eco*RI and *Xba*I digestion, and the fragment was inserted into pcDNAI digested with *Eco*RI and *Xba*I. The c-Rel expression plasmid was created by insertion of c-Rel cDNA, excised with *Bam*HI and *Eco*RI from plasmid Rel PCR 5′3′ (obtained from I. M. Verma, Salk Institute, San Diego, Calif.) (13), into pcDNAI digested with *Bam*HI and *Eco*RI. The RelB expression plasmid was generated by insertion of RelB cDNA, excised with *Eco*RI from pMexNeo-*relB* (from R. Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.) (78), into the *Eco*RI site of pcDNAI.

DNA transfection. Cells were electroporated by using Cell ZapII (Anderson Electronics, Boston, Mass.) at 1250 μF and 750 V/cm. Cells were washed with serum-free RPMI 1640 medium once, resuspended in 1 ml of serum-free RPMI 1640 medium, mixed with DNA, and electroporated. After transfection, cells were left at room temperature for 10 min, resuspended, and cultured in six-well plates.

Luciferase and chloramphenicol acetyltransferase (CAT) assays. Luciferase assays were performed as described previously (12). Cells were centrifuged, washed with phosphate-buffered saline (PBS) three times, and transferred to an Eppendorf tube. Lysis buffer [150 μl of 1.0% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,N′,*N*′,-tetraacetic acid (EGTA; pH 7.8), 1 mM dithiothreitol (DTT)] was added to cell pellet. The lysate was vigorously pipetted and left on ice for 30 min. The supernatant was collected after centrifugation for 5 min. To assay luciferase activity, 100 μl of cell lysate was mixed with 360 μl of assay buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 15 mM KH_2PO_4 [pH 7.8], 4 mM EGTA [pH 7.8], 2 mM ATP, 1 mM DTT). Luciferase activity was assayed in a luminometer (Analytical Luminescence Laboratory, San Diego, Calif.) by automatically injecting 100 μl of a solution containing 200 μM luciferin (Sigma) and 25 mM glycylglycine (pH 7.8). The luciferase units reported or used to calculate the fold induction were obtained after subtraction of the machine background (approximately 200 light units).

CAT assays were performed as previously described (68). Briefly, 45 μl of cell lysate for luciferase assay described above was mixed with 5 μl of 1 M Tris-HCl (pH 7.8) and incubated at 70°C for 15 min in a 7-ml scintillation vial. To the mixture was added 200 μl of cocktail containing 125 mM Tris-HCl (pH 7.8), 1.25 mM chloramphenicol (Sigma), and 0.1 μCi of ^{14}C-labeled butyryl coenzyme A (Dupont NEN Research Products, Wilmington Del.). Then 5 ml of scintillation fluid (Econofluor-2; Packard Instrument Company, Meriden, Conn.) was layered on top of the mixture. The acetylated chloramphenical was assayed in a liquid scintillation counter after incubation at 37°C for 3, 6, and 20 h, at which times the assays were linear with CAT activity.

[^{3}H]thymidine incorporation assay. Purified splenic B cells were cultured at 5×10^5/ml in 200 μl of RPMI 1640 medium with 10% FBS in 96-well flat-bottom plates and were treated with medium alone or different concentrations (20, 10, 5, and 2%) of CD40L-containing supernatant or control supernatant in the presence or absence of IL-4 (40 U/ml) for 72 h. Antibody to CD8α (0.5 μg/ml) was used to cross-link CD40L in the presence of IL-4. Proliferation was assayed by the incorporation of [^{3}H]thymidine (1 μCi per well) (2 Ci/mmol; ICN) added during the last 4 h. About 20,000 cpm was incorporated by cells treated with CD40L-CD8α, IL-4, and anti-CD8α.

Nuclear extracts and recombinant NF-κB p50 protein. The method used for small-scale nuclear protein extraction was modified slightly from the method of Schreiber et al. (81). To obtain nuclear extracts from M12.4.1 or splenic B cells, cells were cultured for 12 h with stimuli, pelleted, washed with PBS once, and resuspended in 400 μl of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μM aprotinin, 2 μM pepstatin). Cells were left on ice for 15 min, and 25 μl of 10% Nonidet P-40 was added. After vigorous vortexing for 10 s, samples were centrifuged for 30 s in a microcentrifuge. The supernatant was discarded, the pellet was washed with buffer A without Nonidet P-40, and 50 μl of solution C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μM aprotinin, 2 μM pepstatin) was added to the pellet. The mixture was vigorously vortexed every 5 min for 30 min and then pelleted by centrifugation for 10 min. The supernatant containing nuclear proteins was collected. Protein concentration in the nuclear extract was determined by the Bradford assay (Bio-Rad, Hercules, Calif.).

Purified recombinant NF-κB p50 protein expressed by a bacterial expression vector was a gift of R. Sen, Brandeis University (72).

Oligonucleotide probe labeling. The CD40RR fragment and its subfragments were labeled by phosphorylation with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Labeled fragments were gel purified. A total of 10,000 cpm of oligonucleotide probe, corresponding to approximately 0.7 ng of double-stranded oligonucleotide, was used for each sample in electrophoretic mobility shift assays (EMSAs).

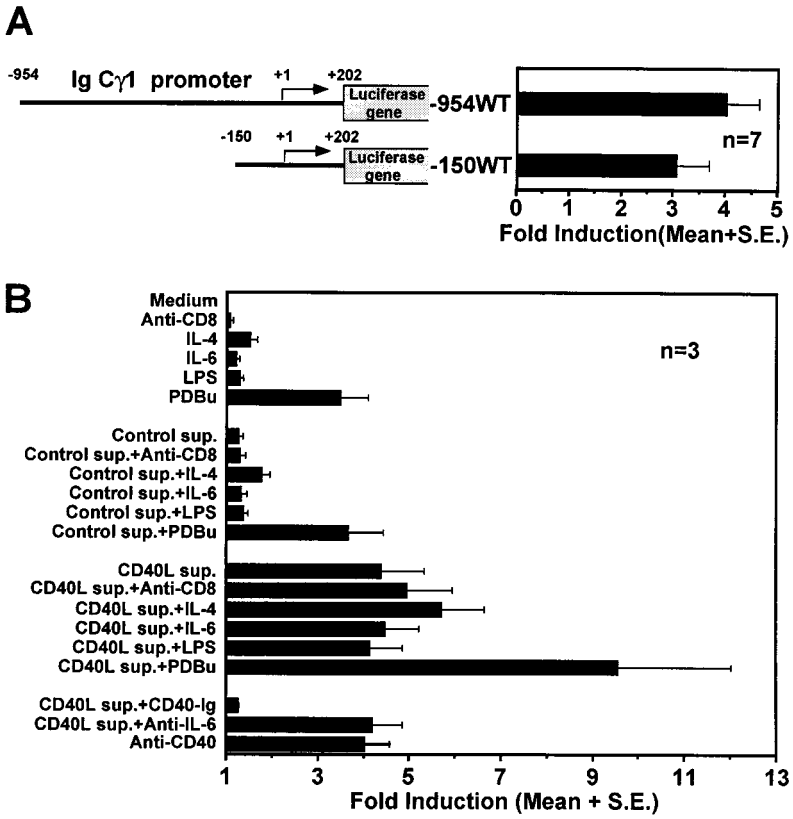

FIG. 1. Regulation of the mouse germ line Ig Cγ1 promoter activity by CD40L and other stimuli. (A) Plasmid −954WT contains the Cγ1 5′ flanking segment, from nucleotides −954 to +202 (relative the first RNA initiation site), and plasmid −150WT contains the −150/+202 segment. M12.4.1 cells were transfected with each plasmid and divided into two aliquots, which were treated with either CD40L-CD8α-containing or control supernatant (20%) for 12 h. Luciferase activity was determined, and the ratio of the activity of CD40L-treated cells relative to cells treated with control supernatant is reported. (B) M12.4.1 cells transfected with plasmid −954WT were treated for 12 h with the indicated reagents. The ratio of luciferase activity in treated cells relative to cells cultured in medium alone is reported. Concentrations of reagents: CD40L-CD8α supernatant (sup.) or control supernatant, 20%; IL-4, 400 U/ml; IL-6, 5 ng/ml; PDBu, 50 ng/ml; LPS, 50 μg/ml; anti-CD8 antibody, 0.5 μg/ml; anti-IL-6 antibody, 1 μg/ml; CD40-Ig (15), 10 μg/ml; anti-CD40 MAb (39), (0.5 μg/ml). IL-4, anti-CD8α, and CD40-Ig were used at a dose determined to be optimal in dose-response experiments (unpublished observations). Here and in Fig. 2, 6, and 7, the number of independent transfections used to obtain the data is indicated by "n," and error bars indicate the standard error of the mean.

EMSAs. DNA probe was incubated with nuclear extract (2 to 4 μg of protein) in the presence of 1 μg of poly(dI-dC) · poly(dI-dC) (Sigma Corp., St. Louis, Mo.) in reaction buffer (10% glycerol, 12.5 mM HEPES [pH 7.5], 50 mM KCl, 0.1 mM EDTA, 1 mM DTT). The mixture was incubated at room temperature for 30 min, and samples were loaded onto a 5% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) and electrophoresed in 0.5× Tris-borate-EDTA buffer at 150 V. Gels were dried and subjected to autoradiography. For antibody supershift and competition experiments, antibody or competitor fragment was mixed with nuclear extract for 15 min before addition of labeled probe.

Antibodies against NF-κB/Rel proteins and sequences of competitor oligonucleotides. Antibodies against specific members of the NF-κB protein family were used to supershift protein-DNA complexes. Antibodies against p50, p52, p65, c-Rel, and RelB were purchased from Santa Cruz Biotechnology (San Diego, Calif.). Antisera against p50 and RelB were gifts of N. Rice (National Cancer Institute, Bethesda, Md.) (75).

The competitor oligonucleotides used were (i) a double-stranded oligonucleotide with a palindromic κB site (underlined), a variant of the κB site in the human IL-2 receptor α-chain gene promoter (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) (3), (ii) a double-stranded oligonucleotide containing the *ets* site of the mouse Ig Cα promoter (5′-TGGAACAGGAAGTGGGT-3′) (62), (iii) a double-stranded oligonucleotide with the NF-AT site from the mouse IL-2 gene (5′-GCCCAAAGAGGAAAATTTGTTTCATACAG-3′) (45), and (iv) a double-stranded oligonucleotide with an AP-1 site (5′-AGCTTGGTGACTCATCCG-3′).

RESULTS

Soluble CD40L induces expression of the germ line Ig Cγ1 promoter. To examine whether CD40L-CD40 interaction can activate the promoter for mouse germ line γ1 switch transcripts, we tested whether a luciferase reporter plasmid (−954WT) driven by the germ line Cγ1 promoter can be activated by CD40 ligation after transient transfection into M12.4.1 B-lymphoma cells. This plasmid contains the germ line −954/+202 (relative to the first transcription initiation site) Cγ1 promoter segment ligated upstream of the luciferase reporter gene. After transfection, cells were divided into two equal aliquots. One aliquot was treated with culture supernatant containing soluble CD40L-CD8α fusion protein from stably transfected J558L plasmacytoma cells (57), and the other aliquot was treated with control supernatant from untransfected J558L cells. After culturing for 12 h, luciferase activity was assayed. As shown in Fig. 1A, an optimal amount of supernatant containing CD40L-CD8α protein stimulates activity of plasmid −954WT fourfold relative to control supernatant, and the control supernatant has little or no effect (Fig. 1B). We also tested the responsiveness of plasmid −150WT, which contains the −150/+202 promoter segment, and found it slightly less responsive (threefold induction) (Fig. 1A). Antibody to CD40 also induces plasmid −954WT fourfold (Fig. 1B). The optimal duration of treatment for induction of luciferase activity by CD40 ligation is 12 h (data not shown), which was used in subsequent experiments. Specificity of the CD40L activity was shown by the fact that an excess of the soluble fusion

protein CD40-Ig blocks induction of the promoter activity by CD40L (Fig. 1B).

It has been reported that the addition of anti-CD8α antibody to cross-link CD40L-CD8α enhances splenic B-cell proliferation approximately 10-fold (27). In agreement with Francis et al. (27), we found that anti-CD8α increases thymidine incorporation fivefold in splenic B cells in the presence of CD40L-CD8α protein and IL-4 (data not shown). However, anti-CD8α does not significantly increase luciferase activity of the Cγ1 promoter-containing reporter plasmid induced by CD40L-CD8α protein (Fig. 1B).

In addition to being induced by IL-4 or by cross-linking of CD40, germ line γ1 transcripts have been shown to be induced by anti-IgD-dextran in splenic B cells or by phorbol myristate acetate (PMA) (which mimics a part of the signal delivered by anti-Ig) in the B-lymphoma line A20.3 (87, 100). Expression of the −954WT reporter plasmid has also been found to be induced by anti-Ig or by PMA in two B-cell lines (L10A6.2 and A20.3), and IL-4 synergizes with PMA to further induce Cγ1 promoter activity (100). IL-4 alone does not induce the promoter in these B cell lines. We tested if CD40L synergizes with PDBu or with IL-4 to activate the Cγ1 promoter and found that the combination of CD40L with either of the other two inducers is slightly more than additive (Fig. 1B). Since M12.4.1 cells do not express sIg, we could not test the effect of anti-Ig.

As also shown in Fig. 1B, LPS alone does not induce the germ line γ1 promoter in M12.4.1 cells, nor does it synergize with CD40L, consistent with its inability to significantly induce germ line γ1 transcripts in splenic B cells (9, 24, 91). Thus, in M12.4.1 cells, CD40L may weakly synergize with phorbol ester and perhaps with IL-4, but not with LPS, to activate the γ1 promoter.

Although IL-6 seems to have no effect on switching induced by IL-4 plus LPS in vitro, neutralizing antibody to IL-6 has been shown to suppress IgG1 production in vivo, consistent with a role for IL-6 in the terminal differentiation of B cells to IgG1 secretion (96). Furthermore, IL-6 production has been shown to be induced by CD40 ligation of M12 cells transfected with human CD40 (17). Therefore, we examined whether IL-6 affects expression of the germ line γ1 promoter in our experiments. As shown in Fig. 1B, IL-6 in the presence of CD40L or control supernatant does not increase activity of the Cγ1 promoter. Furthermore, MAb to mouse IL-6 does not inhibit induction by CD40L, indicating that IL-6 does not play a role in CD40L induction of the Cγ1 promoter activity in these experiments.

Identification of a CD40RR in the Cγ1 promoter. To attempt to determine which nucleotides in the Cγ1 promoter are required for induction by CD40L, luciferase reporter plasmids containing a series of linker-scanning mutations spanning the −160/−3 segment of the Cγ1 promoter were tested for responsiveness to CD40L. The mutations are within the −954/+202 Cγ1 promoter segment in the pXP2 vector (100). M12.4.1 cells, transiently transfected with each plasmid indicated in Fig. 2A, were treated with CD40L supernatant or control supernatant for 12 h, and luciferase activity was assayed. The effects of the linker-scanning mutations on induction of luciferase activity by soluble CD40L are shown in Fig. 2A. Mutations of nucleotides at −101 to −95, −90 to −83, −77 to −56, or −62 to −53 reduce induction of the germ line γ1 promoter by CD40L, whereas mutations at other sites either have no effect or increase induction relative to the wild-type promoter in plasmid −954WT. The data suggest that a CD40RR is located at −99 to −43 in the germ line Cγ1 promoter. This CD40RR is located 3′ to the previously identified IL-4RR at −124 to −100 (8, 65, 100). Mutations in the IL-4RR do not inhibit CD40L inducibility, indicating that the IL-4RR is not required for induction of the Cγ1 promoter by CD40L. Similar results were obtained in assays using the same series of linker-scanning mutations in the −150/+202 Cγ1 promoter (data not shown).

The CD40RR is sufficient to transfer inducibility to a heterologous promoter. To determine if the CD40RR is sufficient to mediate promoter induction by CD40L, the CD40RR was inserted 5′ to a different promoter, the minimal c-*fos* promoter, driving a luciferase reporter gene. In addition, to determine whether the orientation of CD40RR affects the induction, the CD40RR was inserted in forward and reverse directions in pCD40FL-F and pCD40FL-B, respectively. As shown in Fig. 2B, CD40L increases luciferase activity threefold if the CD40RR is inserted in the forward direction and nearly as well when the CD40RR is inserted in the reverse direction, but it does not increase luciferase activity of the plasmid containing only the minimal c-*fos* promoter (pFL). Two copies of the CD40RR do not further increase activity. Induction of promoter activity by antibody to CD40 is similar to that by CD40L (Fig. 2B). Neither LPS or PDBu increases the activity of pCD40FL-F in the presence or absence of CD40L (data not shown). Thus, the CD40RR is sufficient to mediate promoter induction by CD40L, and PDBu does not act through the CD40RR to induce Cγ1 promoter activity.

CD40L induces binding of protein complexes to the CD40RR. To examine whether CD40L induces nuclear proteins that bind to the CD40RR, we performed EMSAs using the CD40RR fragment (−99/−43) as a probe. One major slowly migrating complex is formed in EMSAs with nuclear extracts from M12.4.1 cells (Fig. 3A), but two additional less abundant, faster-migrating complexes are also evident in some experiments (Fig. 4). These complexes are not significantly induced by control supernatant but are induced by CD40L-containing supernatant (Fig. 3A and Fig. 4). The fact that the complexes are present in uninduced M12.4.1 cells is consistent with the relatively modest induction of Cγ1 promoter activity by CD40 signaling. Binding is specific, as shown by competition with excess unlabeled CD40RR fragment (Fig. 3A). Time course experiments starting with 30 min of treatment in M12.4.1 cells demonstrate that the major nuclear protein-DNA complex is induced after 4 to 6 h of CD40L treatment, is maximally induced by 24 h, and is maintained at least until day 3 (data not shown).

Treatment of splenic B cells with CD40L for 12 h results in a much greater induction of complexes binding to the CD40RR than in M12.4.1 cells, presumably as a result of the resting state of uninduced splenic B cells (Fig. 3B). As shown in Fig. 4, the two most slowly migrating complexes formed with splenic nuclear extracts comigrate with complexes in M12.4.1 cells. Antibody to CD40 also induces the same complexes in both splenic B cells (Fig. 3B) and M12.4.1 cells (data not shown). Addition of antibody to CD8α slightly augments the amount of complexes in both splenic and M12.4.1 cells but does not alter the complexes qualitatively (data not shown). Treatment of splenic B cells with control supernatant, in addition to anti-CD40 antibody, does not affect anti-CD40-induced binding activity, indicating that the J558L myeloma cell culture supernatant does not contribute an additional component which affects binding activity (Fig. 3B). IL-4 does not increase the binding activity induced by CD40 signaling in splenic B cells, nor does IL-4 alone significantly increase binding to the CD40RR.

NF-κB proteins bind to the CD40RR. The transcription factors NF-κB/Rel, NF-AT, and AP-1 have been shown to be induced by CD40 ligation in splenic B cells and B-cell lines (7, 16, 27, 43, 56). To identify nuclear proteins that bind the γ1

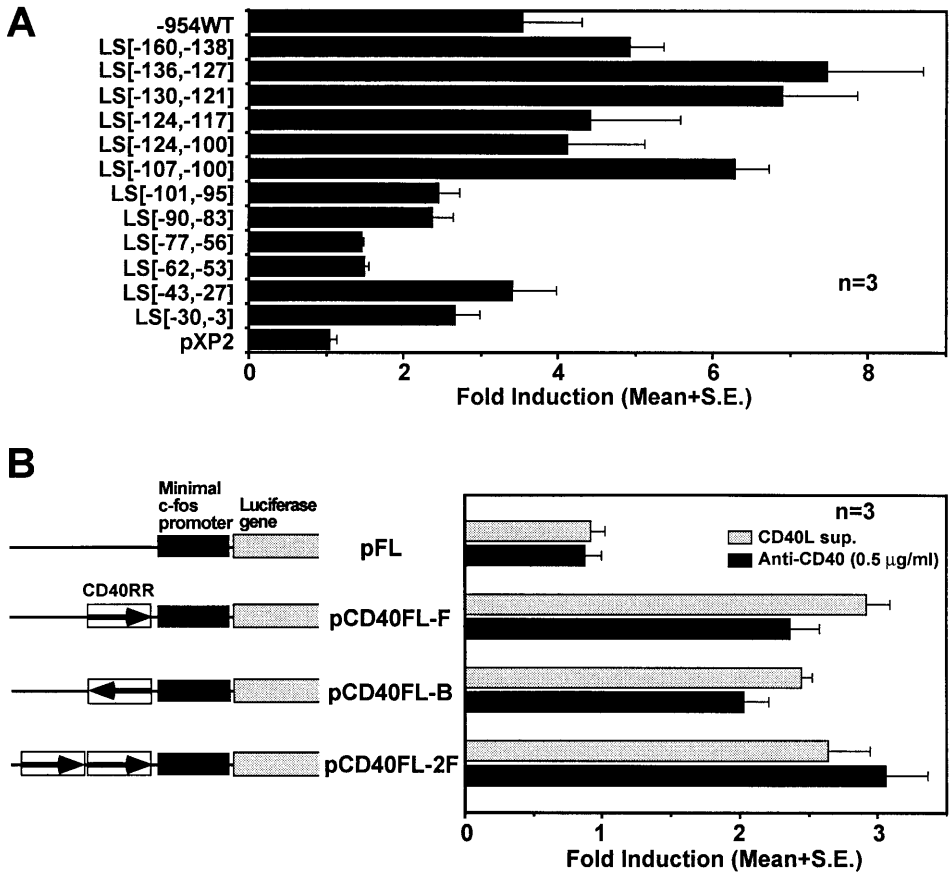

FIG. 2. Identification of the CD40RR in the germ line Ig Cγ1 promoter. (A) Twelve linker-scanning mutants within the −954/+202 promoter segment (100) were analyzed in the search for the CD40RR. Numbers in the names represent the boundaries of the mutated nucleotides. M12.4.1 cells transfected with each linker-scanning mutant were treated with CD40L-CD8α or control supernatant for 12 h, and then luciferase activities of cell lysates were determined. −954WT is the wild-type Cγ1 promoter control plasmid. pXP2 is the vector containing no promoter. Data are calculated as described for Fig. 1A. Constitutive luciferase activities vary among the mutants, as found previously for L10A6.2 and A20.3 cells (100). (B) The γ1 CD40RR is sufficient to transfer inducibility by CD40 signaling to a heterologous promoter. Plasmid pFL contains a minimal (−71) c-*fos* promoter upstream of the luciferase reporter gene. pCD40FL-F contains one copy of the CD40RR inserted 5′ to the c-*fos* promoter in the forward direction, and pCD40FL-B contains one copy in the reverse direction. pCD40FL-2F contains two copies of the CD40RR inserted in the forward direction. M12.4.1 cells were transfected with each construct and treated with CD40L-CD8α supernatant (sup.; 20%), control supernatant (20%), anti-CD40 antibody (0.5 μg/ml), or control antibody against CD8α (0.5 μg/ml) for 12 h. The mean values of the ratio of luciferase activity of CD40L-treated cells relative to cells treated with control supernatant or the ratio of luciferase activity of anti-CD40-treated cells relative to anti-CD8α-treated cells from three experiments are indicated.

CD40RR, competition and antibody supershift EMSAs were performed. As shown in Fig. 4, a double-stranded oligonucleotide containing a palindromic κB site competes with the labeled CD40RR fragment for binding to CD40L-inducible nuclear proteins in nuclear extracts from splenic B cells and M12.4.1 cells, indicating that all of these CD40L-induced complexes contain NF-κB/Rel proteins. Oligonucleotides containing NF-AT or AP-1 binding sites do not compete (data not shown), nor does an oligonucleotide containing a binding site for Ets family proteins (lanes labeled PU.1 fragment) (Fig. 3A). These data suggest that NF-κB/Rel proteins are the major proteins involved in binding to the CD40RR of the Cγ1 promoter.

To determine which members of the NF-κB/Rel family are in these complexes, antibodies against specific NF-κB/Rel proteins were used to supershift protein-DNA complexes in EMSAs. As shown in Fig. 4, antibodies against p50 and RelB supershift or inhibit formation of the most slowly migrating complex in CD40L-treated M12.4.1 and splenic B-cell nuclear extracts, indicating that this complex consists of p50 and RelB proteins. Anti-p50 and anti-RelB also supershift the major complex in untreated or control supernatant-treated M12.4.1 nuclear extracts (data not shown), suggesting the p50-RelB complex is constitutively expressed in M12.4.1 cells and further induced by CD40 signaling. Antibody to RelB, but not antibodies for other NF-κB Rel family proteins, abolishes formation of the second complex in M12.4.1 cells, indicating this complex also contains RelB. RelB appears to be unable to bind κB sites as a homodimer or as a heterodimer with p65 or c-Rel, being found only in association with p50 or p52 (21). Thus, this band may contain p52, although the antibody to p52 that we tested had no effect on any complex. Another possibility is that this complex contains a protein that is not a member of the NF-κB family.

Antibodies to p50 and to p65 inhibit formation of the third band in CD40L-treated M12.4.1 cells (Fig. 4). The comigrating band formed with splenic B-cell extracts may also contain p50-p65 heterodimers, because anti-p50 slightly inhibits its formation and anti-p65 appears to inhibit the upper part of this band. Antibodies to c-Rel and RelB appear to inhibit formation of the lower portion of this splenic band, suggesting it may also contain c-Rel and RelB. Antibody to c-Rel does not affect any complex in M12.4.1 extracts (two different anti-c-Rel antibodies were used in several experiments). The fastest-migrating

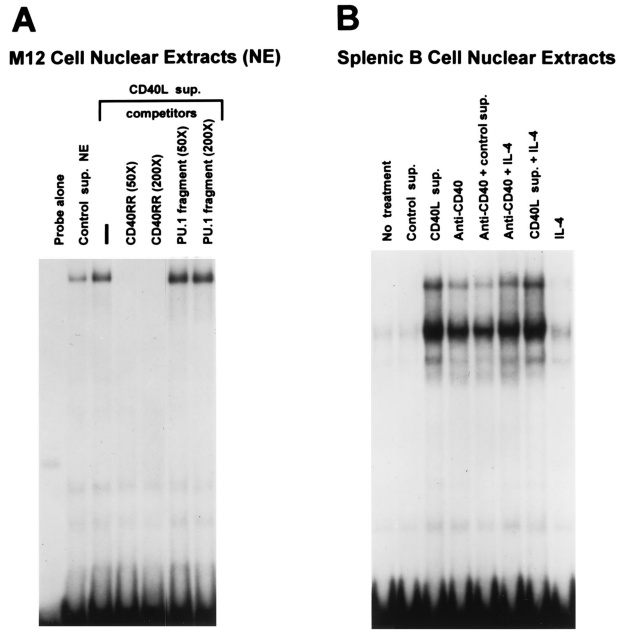

FIG. 3. EMSAs with the γ1 CD40RR demonstrate that CD40L-inducible complexes form with nuclear extracts from M12.4.1 and splenic B cells. (A) Four-microgram aliquots of nuclear extracts from M12.4.1 cells treated with control supernatant or CD40L-CD8α supernatant for 12 h were incubated with end-labeled CD40RR (−99/−43) probe. Unlabeled CD40RR fragment or a double-stranded oligonucleotide containing an *ets* binding site (lanes labeled PU.1 fragment) was added at a 50- or 200-fold excess to compete with labeled CD40RR probe. (B) Two-microgram aliquots of nuclear extracts from splenic B cells untreated or treated with control supernatant (sup.), CD40L-CD8α supernatant, or antibody to CD40 (0.5 μg/ml), in the presence or absence of recombinant mouse IL-4 (400 U/ml), were incubated with the labeled CD40RR probe.

complex in splenic B-cell nuclear extracts, which cannot be detected in M12.4.1 cell nuclear extracts, may consist of p50 homodimers, since it is supershifted only by antibody to p50.

Three NF-κB-binding sites in the γ1 CD40RR. On the basis of a comparison of the CD40RR sequence with the 10-bp consensus NF-κB binding sequence, GGGGYNNCCY (reviewed in reference 53), we identified three potential κB sites in the CD40RR (Fig. 5A). The first and second κB sites (κB1 and κB2) fit the consensus better in the reverse direction. To begin to localize the binding sites for the NF-κB complexes that bind the CD40RR, we used four subfragments from the CD40RR to compete with labeled CD40RR probe in EMSAs. As shown in Fig. 5B, three subfragments, −99/−82, −73/−56, and −60/−43, containing the three putative κB-binding sites, compete with the CD40RR probe, whereas subfragment −86/−69, which does not contain a putative κB site, does not compete. However, unlike the CD40RR oligonucleotide with all three κB sites, each of the three κB sites at a 100-fold excess does not completely compete the NF-κB binding activity of the CD40RR, suggesting that having three κB sites together increases their avidity.

We next used each of the three subfragments with κB sites as probes in EMSAs with M12.4.1 cell nuclear extracts. As shown in Fig. 5C, each of the three subfragments binds a complex induced by CD40L, and these complexes can be competed for by the palindromic κB site oligonucleotide, demonstrating that each of these three putative κB sites binds NF-κB proteins induced by CD40 signaling. Further support for this conclusion was obtained by the demonstration that purified recombinant NF-κB p50 binds these three subfragments and that the binding activity is abolished when subfragments with mutations in the putative κB sites, indicated in Fig. 5A, are used as probes in the EMSA (Fig. 5D). Mutations of all three predicted κB sites in the CD40RR eliminate formation of all of the CD40L-inducible complexes with nuclear extracts from M12.4.1 cells and splenic B cells (data not shown).

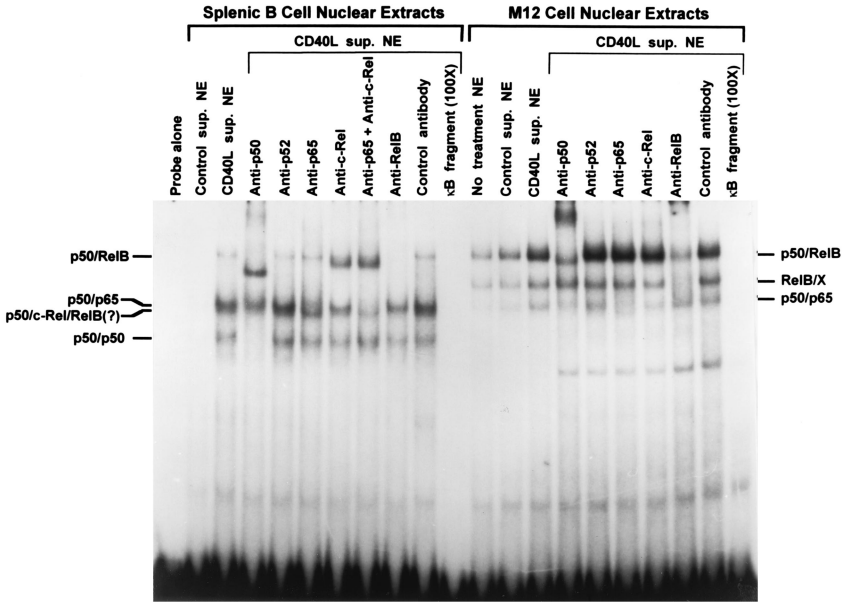

FIG. 4. EMSA to demonstrate that NF-κB proteins are components of CD40L-inducible complexes that bind the γ1 CD40RR. The CD40RR (−99/−43) probe was incubated with the indicated antibodies and nuclear extracts (NE; 2 μg) from splenic B cells or M12.4.1 cells, treated as indicated. Antibodies against p50, p52, p65, c-Rel, and RelB were purchased from Santa Cruz Biotechnology. Antibody against PU.1 (Santa Cruz) was used as the control serum. Similar results have been obtained with antisera for p50 and RelB from N. Rice (75) (data not shown). A double-stranded oligonucleotide with a palindromic κB site at a 100-fold excess was used in the competition assay. sup., supernatant.

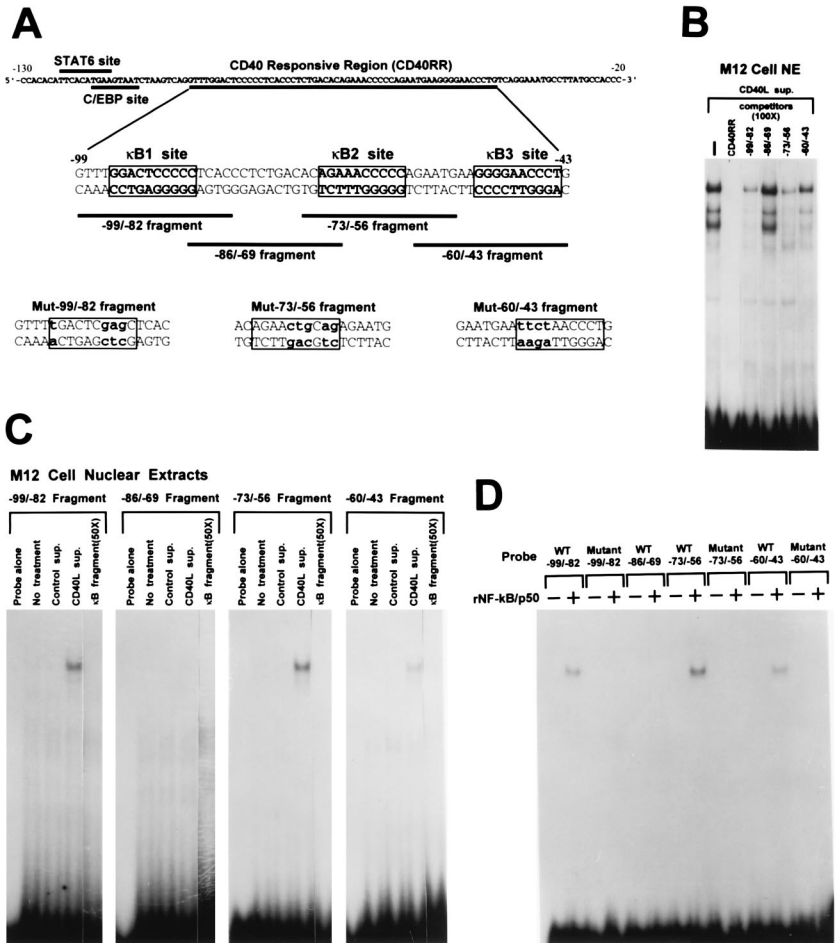

FIG. 5. Sequence of the CD40RR in the Cγ1 promoter and EMSAs which identify three κB binding sites within the CD40RR by EMSAs. (A) The sequence of the Cγ1 promoter from nucleotides −134 to +1 (relative to the first RNA initiation site), with the CD40RR sequence expanded and the putative κB sites marked. Stat6 (IL-4 Stat) and C/EBP binding sites are indicated upstream of the CD40RR (8, 65). Heavy lines below the sequence of the CD40RR indicate the four wild-type subfragments, −99/−82, −86/−69, −73/−56, and −60/−43, used as competitor fragments and probes in EMSAs. Subfragments Mut-99/−82, Mut-73/−56, and Mut-60/−43, containing mutations at the putative κB sites, are shown, with the substituted nucleotides indicated by lowercase letters. (B) EMSA using the CD40RR as a probe, competing with wild-type CD40RR or wild-type subfragments of the CD40RR at a 100-fold excess. Nuclear extract (NE; 4 μg) from CD40L-treated M12.4.1 cells was used. The first lane contains no competitor. sup., supernatant. (C) EMSA in which the four labeled wild-type subfragments indicated were incubated with nuclear extracts from M12.4.1 cells treated with medium alone, with control supernatant, or with CD40L supernatant. The last lane in each set shows competition with the κB double-stranded oligonucleotide, using extracts from CD40L-treated cells. (D) Purified recombinant NF-κB p50 (0.3 μg) binds to labeled wild-type (WT) −99/−82, −73/−56, and −60/−43 subfragments but not to labeled wild-type −86/−69 subfragment or subfragments with mutated κB sites.

Three NF-κB-binding sites are required for full induction of transcriptional activity of the Cγ1 promoter by CD40L. To understand the functional roles of these three κB sites in activation of the CD40RR by CD40L, we mutated each κB site individually and determined the effects of mutations on CD40L inducibility in reporter gene assays. Each CD40RR fragment with the mutations shown in Fig. 5A in one of the three κB sites was inserted upstream of the minimal c-*fos* promoter in plasmid pFL. Transient transfection of M12.4.1 cells and CD40L treatment were performed as described above. As shown in Fig. 6, mutation of the first κB site reduces induction by CD40L, and mutation of either the second or the third κB site almost completely abolishes induction. Therefore, although the first κB site is less important, all three sites are required for full induction of promoter activity by CD40L, indicating these three κB sites are not redundant but appear to cooperate with each other for induction by CD40L.

NF-κB p50 in combination with either p65 or RelB transactivates the CD40RR and the germ line γ1 promoter. To determine whether NF-κB/Rel proteins can transactivate the minimal c-*fos* promoter through the CD40RR in the absence of CD40 signaling, we cotransfected eukaryotic expression vectors encoding NF-κB/Rel proteins along with pFL luciferase reporter plasmids containing the wild-type CD40RR (pCD40FL-F) or CD40RR with all three κB sites mutated (pMutCD40FL-F). Each of the NF-κB/Rel expression plasmids alone or the p50 expression plasmid in combination with a p65, c-Rel, or RelB expression plasmid was transiently cotransfected with one of the luciferase reporter plasmids into M12.4.1 cells. As shown in Fig. 7A, expression of p50 in combination with p65 or RelB induces the activity of plasmid pCD40FL-F 10- to 12-fold but does not induce the activity of pFL or pMutCD40FLF.

To determine if overexpression of NF-κB/Rel proteins also induces the intact germ line γ1 promoter, the NF-κB/Rel expression plasmids were cotransfected along with plasmids −954WT and −150WT (Fig. 7B). Similar to their effects on pCD40FL-F, overexpression of p50 in combination with p65 or RelB transactivates each Cγ1 promoter about 11-fold, whereas overexpression of c-Rel in combination with p50 does not transactivate the promoter.

To determine if induction of the γ1 promoter by NF-κB/Rel

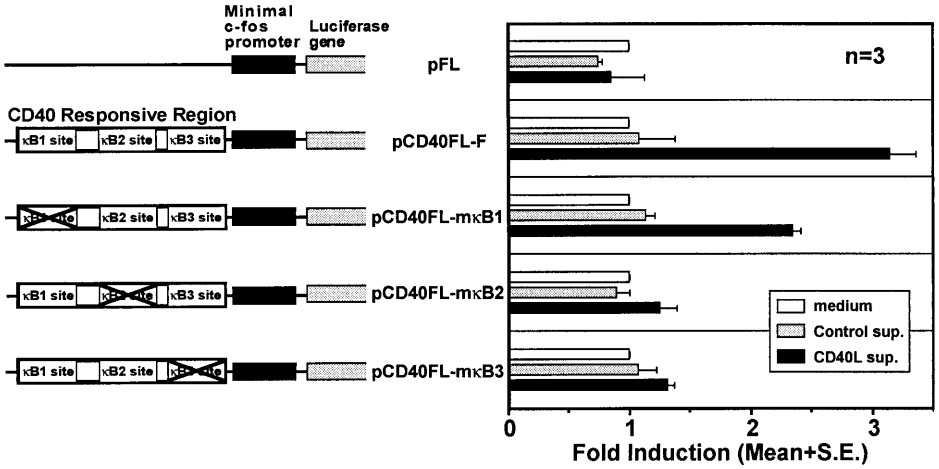

FIG. 6. Three κB binding sites are required for full induction of the CD40RR in transfection assays. M12.4.1 cells were transfected with plasmid pCD40FL-F (containing the wild-type CD40RR), plasmid pCD40FL-mκB1 (with the first κB site mutated), plasmid pCD40FL-mκB2 with the second κB site mutated, or plasmid pCD40FL-mκB3 (with the third κB site mutated). The transfection mixture contained 3×10^7 cells, 50 μg of each plasmid, and 10 μg of pSV2CAT as the control for transfection efficiency. After transfection, cells were separated into three aliquots, and each aliquot was cultured in medium alone, with control supernatant (sup.), or with CD40L-CD8α supernatant for 12 h. The fold induction is calculated relative to luciferase activity of lysates of cells incubated in medium alone.

requires an intact CD40RR, we tested the effects of cotransfecting the p50 and p65 or p50 and RelB expression plasmids along with a germ line γ1 promoter-luciferase plasmid containing a linker-scanning mutation of the second κB site within the CD40RR (LS[−77,−56]). The other two κB sites are intact in this mutant. As shown in Fig. 7B, mutation of this single κB site greatly reduces constitutive expression (14-fold), slightly reduces induction by p50-p65 (to 8-fold), and eliminates induction by p50-RelB. These data indicate that this κB site is important for basal expression of the γ1 promoter and for induction by p50 and RelB. The fact that induction by p50-RelB is more dependent on this particular κB site than is induction by p50-p65 may be due to differences in binding specificity of these two Rel proteins.

Consistent with the known inability of RelB to form homodimers (20), overexpression of RelB in the absence of cotransfected p50 does not transactivate the CD40RR or germ line γ1 promoter. Overexpression of p65 alone activates plasmids −954 and −150WT about 50% as well as p50 and p65 together. This may be due to the formation of p65 homodimers (31) or heterodimers with endogenous p50 in the nuclei of untreated M12.4.1 cells. The effect of p65 alone is greater on the intact promoter than on the CD40RR during action as an enhancer for the c-*fos* promoter, possibly as a result of interaction with factors bound at other sites in the promoter.

Expression of c-Rel alone or in conjunction with p50 has little effect on activity of the CD40RR or γ1 promoter, although c-Rel binding activity can be detected in nuclear extracts from CD40L-induced splenic B cells. This result is in agreement with those of other studies showing that the transactivation activity of c-Rel is much weaker than that of p65 (22, 63).

To obtain further evidence that the mechanism by which CD40 signaling induces the transcription of germ line γ1 transcripts is by activating NF-κB, we attempted to inhibit CD40L induction of the γ1 promoter by treatment of transfected M12.4.1 cells with an inhibitor of NF-κB. M12.4.1 cells were transfected and treated with CD40L supernatant in the presence or absence of the antioxidant pyrrolidine dithiocarbamate (PDTC), which has been shown to prevent phosphorylation and subsequent degradation of IκB-α (95). Treatment with PDTC at a low dose (1 μM) completely eliminated induction of the germ line γ1 promoter (954WT) by CD40L and greatly reduced induction of NF-κB in nuclear extracts, as shown by Western blotting (immunoblotting) (data not shown). We also tested the effect of okadaic acid, a phosphatase inhibitor which has been shown to induce NF-κB (93), and found that okadaic acid (50 ng/ml) induced expression of the 954WT Cγ1 promoter 3.2-fold after 12 h of treatment (data not shown). Although we have been unable to induce germ line γ1 transcripts in M12.4.1 cells, perhaps because of a gene deletion, we have recently demonstrated that the mouse B-cell line 1B4.B6 (98a) can be induced by CD40L to express germ line γ1 transcripts and that this induction can be completely inhibited by PDTC (50 μM) (61a). Thus, these data indicate that activation of NF-κB by CD40 signaling provides a mechanism for induction of transcription of germ line γ1 RNA.

DISCUSSION

CD40 signaling regulates the germ line Cγ1 promoter. T-cell contact help plays an important role in B-cell proliferation and isotype switching during B-cell differentiation in T-dependent immune responses. Although the mechanism by which CD40L-CD40 interaction regulates isotype switching is not yet clear, CD40 signaling may be involved in all three components required for induction of isotype switching: induction of B-cell proliferation, induction of the switch recombination machinery, and induction of germ line transcription. The role of CD40 engagement in conjunction with cytokines in induction of B-cell proliferation and switch recombination has been documented (reviewed in references 4 and 86). We have demonstrated here that CD40 engagement in the absence of added cytokines induces the promoter for germ line γ1 transcripts in the M12.4.1 B cell line. These results confirm and extend a recent report indicating that CD40L expressed on Sf9 cells induces germ line γ1 transcripts in splenic B cells and also induces the germ line γ1 promoter threefold in BCL_1-3B3 mouse B-lymphoma cells and that this induction does not require the Stat6 binding site (97). IL-4 and phorbol ester further upregulate the CD40L-induced promoter activity, suggesting that signaling through the IL-4 receptor, sIg, and CD40 con-

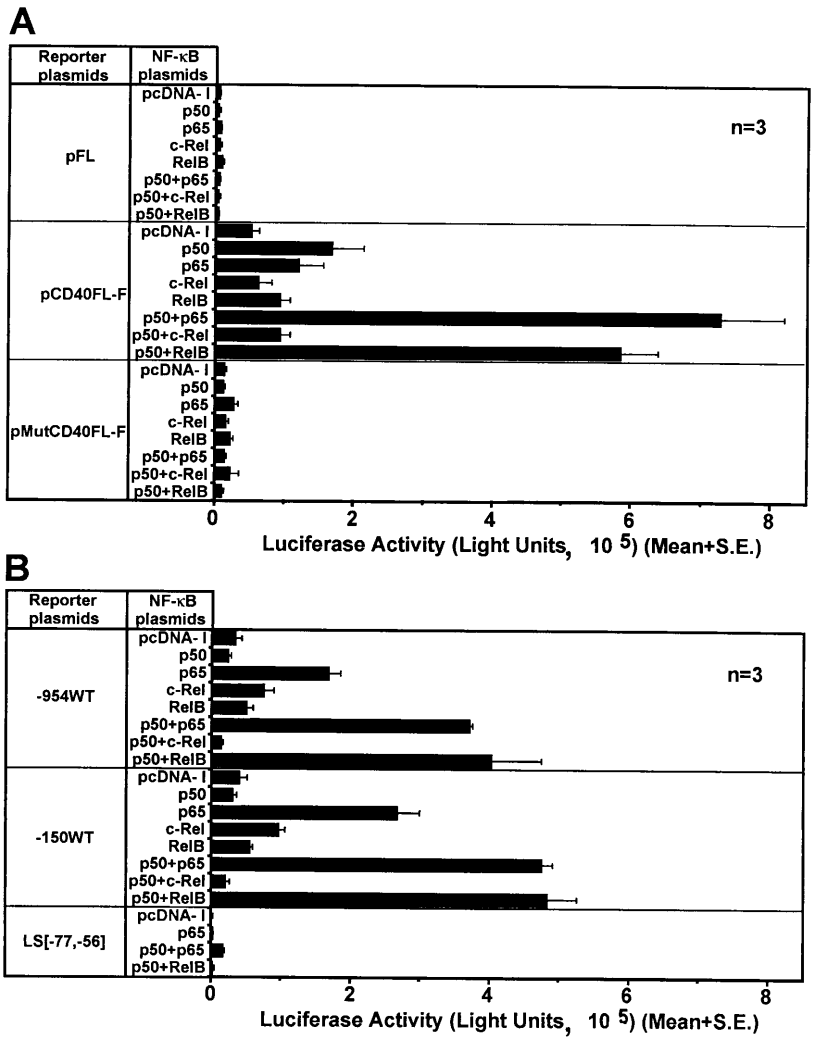

FIG. 7. Cotransfection of NF-κB/Rel proteins transactivates the CD40RR and Cγ1 promoter. (A) Plasmids expressing p50, p65, c-Rel, or RelB were cotransfected into M12.4.1 cells along with luciferase reporter plasmid pFL (vector control), pCD40FL-F, or pMutCD40FL-F (bearing one copy of the CD40RR mutated at all three κB sites). For each transfection, 2×10^7 cells were electroporated with 20 μg of each NF-κB/Rel protein-expressing plasmid, 20 μg of reporter gene plasmid (pFL, pCD40FL-F, or pMutCD40FL-F), and 10 μg of pFosCAT as the transfection efficiency control. The total amount of expression plasmid was kept constant at 40 μg by adding empty expression vector, pcDNAI. Cells were harvested 14 h after transfection. Another c-Rel expression plasmid in pEVRF-2 (a gift of R. Sen), previously shown to activate the IL-2 receptor α-chain gene promoter (72), was also transfected, giving the same result (data not shown). (B) Cotransfection experiments using the Cγ1 promoter luciferase plasmids −954WT and −150WT were performed as described above except only one c-Rel expression plasmid was used. Cotransfection experiments using the −954 Cγ1 promoter containing the linker-scanning mutation at −77 to −56, which disrupted the second κB site of the CD40RR, were performed as for panel A but with only the indicated NF-κB expression plasmids. The mean luciferase activity of LS[−77,−56] when cotransfected with the control plasmid pcDNAI was 2,464, well above the machine background of 200.

tribute to induction of germ line γ1 transcription during T-dependent immune responses.

NF-κB activation by CD40 signaling. The data presented in this report indicate that induction of the germ line Cγ1 promoter by CD40L is mediated by activated NF-κB/Rel proteins that bind the three κB sites of the CD40RR. Although AP1 and NF-AT have also been shown to be induced by CD40 engagement, they do not appear to bind the γ1 CD40RR. Members of the NF-κB/Rel family in mammalian cells which bind DNA include p50, p52, p65(RelA), c-Rel, and RelB. NF-κB/Rel proteins form dimers that are sequestered in the cytoplasm as a result of the binding of IκB inhibitor proteins to the complexes or as a result of the presence of p105 or p100 (precursor for p50 or p52, respectively) in the complexes (reviewed in reference 67). Activation of NF-κB/Rel involves degradation of IκB proteins followed by translocation to the nucleus. This activation process is induced within minutes by activators such as tumor necrosis factor alpha, PMA, LPS, or IL-1 (5, 40, 41, 94).

Binding of NF-κB p50-p65 heterodimers was found to be induced within 10 min after treatment of the human B-cell line Daudi with antibody to CD40 or within 1 h in mouse splenic B cells in response to CD40L plus anti-CD8α antibody (7, 27). Surprisingly, we find that it takes 4 to 6 h for soluble CD40L to induce binding of the RelB-p50 complex to the γ1 CD40RR in M12.4.1 cells, although binding of the p50-p65 complex to the CD40RR in these cells is induced within 30 min by soluble CD40L (data not shown). The mechanism of activation of RelB is not understood (21, 60). Although we found that antibody to CD8α does not significantly increase the amount of binding activity at 12 h, we have not investigated whether it would increase the kinetics of induction of binding of RelB to the CD40RR.

Total NF-κB binding activity induced by CD40L in M12.4.1

and splenic B cells was maximal at about 24 h but still very high at 3 days, consistent with results reported for Daudi and splenic B cells (7, 27). Therefore, like LPS, CD40L induces persistent NF-κB activation, unlike tumor necrosis factor alpha and PMA, which transiently activate NF-κB (26). The mechanism by which CD40 signaling induces persistent NF-κB activation is unknown, although a possibility is that, like LPS, CD40L induces degradation of IκBβ in addition to IκBα (94). Another possibility is that CD40L induces synthesis of new NF-κB/Rel proteins which maintain the binding activity in the nucleus. The levels of IκB and NF-κB/Rel proteins in CD40L-treated cells must be determined to address these questions.

Role of NF-κB Rel proteins in T-dependent immune responses in vivo. Different binding affinities for different κB sites, tissue-specific expression of NF-κB/Rel proteins, and interaction with other transcription factors all indicate that different NF-κB/Rel proteins mediate different biological functions (67). Targeted mutations of individual NF-κB/Rel family members in mice allow evaluation of their roles in immune responses, except for p65, because targeted disruption of the p65 gene causes embryonic lethality (6). Mice deficient in either p50, RelB, or c-Rel each have normal T- and B-cell development but display different phenotypes during immune responses (52, 84, 98).

In p50-deficient mice, serum IgG1 levels are reduced 10-fold, but antigen-specific IgG1 production in a T-dependent immune response is reduced 40-fold. These data may be explained by our findings that induction of germ line transcripts by CD40L involves induction of p50-p65 and p50-RelB complexes and that mutation of the κB sites in the germ line Cγ1 promoter prevents induction by CD40L. Also consistent with this hypothesis, p50 knockout mice do not express IgE antibodies and a κB site in the germ line Cε promoter is required for expression of the germ line Cε promoter in reporter gene assays (18). The finding that p50 disruption does not inhibit serum IgG1 levels in unimmunized mice as much as it inhibits IgG1 produced in response to a T-dependent antigen may be explained by the facts that germ line γ1 transcripts and the promoter for germ line γ1 transcripts can be induced by cross-linking sIg or by phorbol ester and that induction by phorbol ester does not require the κB sites (87, 100).

A recent report may appear to contradict this hypothesis for the phenotype of the p50 knockout mice, since it has been reported that splenic B cells from p50 knockout mice can be induced in culture to express germ line γ1 transcripts by treatment with CD40L, IL-4, and IL-5 and to secrete almost normal levels of IgG1 in response to these inducers (89). The findings of Snapper et al. (89) can be explained by the fact that IL-4 can induce germ line γ1 transcripts in cultured splenic B cells and that this induction does not require p50 (9, 89). Furthermore, neither cell proliferation in response to CD40 nor switch recombination requires p50 (84). In vivo, the responses are probably more carefully regulated. T-cell contact help is perhaps limited and locally delivered and probably synergizes with locally delivered IL-4 to induce germ line γ1 transcripts and switching to IgG1. We propose that the amount of IL-4 available in vivo to compensate for the lack of induction of germ line γ1 transcripts may be inadequate to induce normal levels of germ line γ1 transcripts in the absence of signaling by CD40.

Further support for the hypothesis of the importance of CD40 induction of germ line γ1 transcripts in vivo comes from the finding that in IL-4 knockout mice, serum IgG1 expression is reduced sixfold and expression of IgG1 in a T-dependent immune response is reduced only twofold (55). This result indicates there is a mechanism(s) for induction of germ line γ1 transcripts which does not require IL-4.

B-cell function and humoral immune response in RelB-deficient mice have not been reported (98). c-Rel-deficient mice show impaired T- and B-cell activation (52). The serum levels of IgM and all IgG subclasses are lower in c-Rel-deficient mice than in wild-type mice; in particular, levels of IgG1 and IgG2a are lower (100- and >100-fold, respectively). IgG1 production in a T-dependent response is also 100-fold lower. It is unknown if the defect in IgG1 production is due to the inability to activate the Cγ1 promoter. Although we detect a complex containing c-Rel that binds the γ1 CD40RR in splenic B-cell extracts, overexpression of c-Rel in the presence or absence of p50 does not transactivate the germ line γ1 promoter. Köntge et al. (52) speculate that the defects in IgG1 and IgG2a production are due to defective production of a cytokine(s) from T cells required for the synthesis of IgG1 and IgG2a.

LPS and PDBu do not induce promoter activity through the CD40RR. LPS and anti-Ig (or phorbol ester) are polyclonal B-cell activators which activate NF-κB/Rel proteins. Both of these activators induce binding of NF-κB complexes to the CD40RR in EMSAs (data not shown). However, in the absence of IL-4, LPS does not induce germ line γ1 transcripts or induce the Ig Cγ1 promoter. Although phorbol ester activates the Cγ1 promoter, it does not activate via the CD40RR. Various possibilities might explain why LPS and phorbol ester cannot induce promoter activity through the CD40RR in M12.4.1 cells. One possibility is that other transcription factors that interact with NF-κB/Rel proteins and are required for CD40RR activity are induced by CD40L but not by LPS or PDBu. Another possibility is that different NF-κB/Rel proteins are activated by different inducers and have different transactivation potentials for induction of the Cγ1 promoter. This latter possibility is supported by the fact that induction of NF-κB binding activity by LPS is abolished in the p50 knockout mice, although the response to anti-IgM is only partially reduced (84).

Interaction between transcription factors for promoter regulation. Despite the fact that only NF-κB/Rel proteins were found to be involved in induction of Cγ1 promoter activity by CD40L, NF-κB/Rel proteins may cooperate with other transcription factors for Cγ1 promoter activity. NF-κB/Rel proteins have been shown to cooperate with several different transcription factors to regulate other promoters. Transcription factors that interact with NF-κB/Rel proteins include ATF-2 for E-selectin expression induced by tumor necrosis factor alpha, C/EBPβ(NF-IL6) at the IL-6 and IL-8 promoters, Sp1 at the human immunodeficiency virus long terminal repeat, and interferon regulatory factor 1 at the VCAM-1 promoter (67). In addition, HMG-I(Y) facilitates the binding of NF-κB/Rel proteins to κB sites (23). Binding sites for Stat6 (IL-4 Stat) and C/EBP proteins are located just upstream of the CD40RR, in the IL-4RR of the Cγ1 promoter (Fig. 5A) (8, 65). It is possible that NF-κB/Rel proteins interact with these transcription factors when bound to the Cγ1 promoter, since mutations within the CD40RR reduce the basal activity and both IL-4 and PMA responsiveness of the γ1 promoter (reference 100 and this report).

Function of multiple κB sites in the CD40RR. Maximal induction of the Cγ1 promoter by CD40 ligation or by cotransfected NF-κB expression plasmids requires all three κB sites in the CD40RR. Multiple κB sites have also been shown to be required for activation of the E-selectin promoter (79). Together, these data suggest that cooperative interaction of NF-κB/Rel proteins with each other contributes to promoter activity, although the exact mechanism needs to be explored. Individual κB sites in the CD40RR appear to bind NF-κB/Rel proteins with low affinity, because each site competes poorly

with the intact CD40RR and has weak binding activity when used as a probe in EMSAs, suggesting that multiple κB sites increase the avidity of NF-κB.

Altogether, the data in this report indicate that induction of germ line γ1 transcripts by CD40 signaling occurs via induction of binding of the NF-κB proteins p50 and p65 and/or p50 and RelB to the three adjacent κB elements of the Cγ1 promoter.

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