Defining In Vivo Transcription Factor Complexes of the Murine CD21 and CD23 Genes

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Defining In Vivo Transcription Factor Complexes of the Murine CD21 and CD23 Genes

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The expression of the CD21 and CD23 genes is coincident with differentiation from transition 1 B cells (T1) to transition 2 B cells (T2). To define constituents controlling CD21 and CD23 expression, we conducted chromatin immunoprecipitation analyses for candidate transcription factors. We found constitutive binding of Oct-1, NFAT species, YY1, NF-κB-p52, Pax5, E2A, and RBP-Jκ to CD21 sequences and NF-κB-p52, Pax5, NFAT species, E2A, and RBP-Jκ to CD23 promoter sequences. Splenic T and B cell subsets displayed constitutive binding of YY1, NF-κB-p52, Pax5, and Oct-1 proteins to CD21 sequences in B cells but no specific binding of NFATc3 or Pax5 in T cells. Similarly, CD23 sequences demonstrated constitutive binding of NF-κB-p52 in splenic T and B cells but only Pax5 in B cells. Of the various NFAT species, only a subset were found forming constitutive DNA/protein complexes with the CD21, CD23, and IL-2 gene sequences. Maturing B cells in the marrow possess stable Pax5 complexes on CD19, CD21, and CD23 gene promoters in the nuclei of such cells, even though only CD19 is expressed. The similarity of genetic controlling elements between the CD21 and CD23 genes does not suggest a mechanism for alternative regulation of these genes; however, separation of splenic B cell subsets into T1, T2, marginal zone (MZ), and mature follicular B cells, followed by quantitative RT-PCR, demonstrated the lack of appreciable CD23 transcripts in CD21 MZ cells. We propose an alternative derivation of MZ cells as maturing directly from T1 cells, leaving CD23 transcriptionally inactive in that lineage of cells. The Journal of Immunology, 2007, 178: 7139–7150.

T he murine CD21 gene encodes two distinct proteins, known as murine CR1 and CR2, via alternative splicing (1). These proteins recognize specific complement breakdown products of C3 and C4 and are critical in generating an optimal Ab response in vivo (2–6). The gene is transcriptionally active in maturing and mature B cells and follicular dendritic cells (7, 8). Expression of the CD21 gene is induced in differentiating B cells during the transition 1 B cell (T1) to transition 2 B cell (T2) transition stage in the spleen, after expression of the B cell marker CD19 is observed in the marrow pre-B cell stage (9–11), but coincident with expression of CD23, the low-affinity IgE receptor found on B cells and some activated macrophages. B cells in peripheral lymphatic tissues such as the spleen display various levels of CD21 expression with marginal zone (MZ) B cells of the spleen expressing the highest levels, T2 cells expressing less, and follicular mature (FM) B cells the least. T1, T2, MZ, and FM cells all express CD19. CD23 is found on the surface of T2 and FM cells but is absent, or at very low levels, on the surface of MZ cells (12).
single repetitive binding sites had no affect upon silencing of reporter function; however, removal of the entire set restored CD21 promoter activity in mouse T cells (22). Intriguingly, this region of the first intron is highly conserved in sequence and position between the mouse, human, and dog CD21 genes. The expression of the CD21 gene has also been shown to be influenced by factors and conditions that alter the state of the chromatin. Demethylation of promoter sequences was shown in a variety of human B cells to be correlated with expression; inhibition of methylation induced expression in normally inactive cells (23). Additionally, increasing the level of histone acetylation in human pro-B cells and in murine T cells (via inhibiting histone deacetylase activity) induced expression of the CD21 genes (21, 23).

CD21 and the B-cell-specific low-affinity IgE receptor CD23 are expressed within the same window of B cell development. CD23 has been described as possessing an IL-4-dependent enhancer element (24) with possible binding by both STAT 1 and STAT 6 (25–27). The CD23 promoter(s) have also been shown to be directly regulated by the RBP-Jκ (Notch family), NF-κB, NFAT, and Pax5 transcription factors (27–29). Similarities between transcriptional control of CD21 and CD23 exist beyond sharing the same set of transcriptional control elements. A recent report suggested that engagement of the B cell-activating factor (BAFF) receptor with its ligand was required for CD21 and CD23 expression (30). The BAFF receptor is known to transduce its signal within B cells using an “alternative” activation pathway to allow for translocation of NF-κB subunits into the nucleus of the cell. BAFF receptor engagement has also been linked to NFAT protein activation (31,32).

The sequences and tissue-specific expression of the murine and human CD21 genes are highly conserved, yet clear discrepancies exist in the analyses of the two genetic elements. Much of the data generated in both systems has been derived from reporter analyses (either stable or transient transfections) and in vitro protein/DNA binding assays. To gain further insight into CD21 gene control, as well as to compare CD21 transcription with a similarly expressed gene, CD23, we initiated a series of in vivo analyses, via the chromatin immunoprecipitation (ChIP) assay, mapping DNA/protein binding complexes that form in the nucleus of native, nontransformed cells. We describe in this article constitutive, stable complexes of CD21 and CD23 sequences with NFAT family members, NF-κB-p52, plus Pax5 and constituents of the Notch signaling pathway. These lead us to propose a new model for the development of MZ B cells directly from a CD23+ T1-like cell instead of the CD23+ T2 intermediate.

Materials and Methods

Cell lines, culture conditions, and mouse strains

The murine CD21-expressing B cell lymphoma cell 2PK3 was obtained from American Type Culture Collection. It was maintained at 37°C/5% CO2 in RPMI 1640 (Invitrogen Life Technologies) with 10% bovine growth serum (HyClone), and 1% penicillin-streptomycin (Invitrogen Life Technologies). Spleenocytes were isolated from adult BALB/c mice (National Cancer Institute) (see Figs. 2–7) and maintained in single-cell suspensions with RPMI 1640 with 10% bovine growth serum and 1% penicillin-streptomycin. Cells were treated with indicated doses of cyclosporine (CSA) (Sigma-Aldrich) or an equal volume of ethanol (EtOH) vehicle in medium for indicated times. A/J and A/WySnJ mice (see Fig. 8) were used for bone marrow and spleenocyte isolation and were obtained from The Jackson Laboratory. Splenic B cells were obtained from 6- to 8-wk-old female C57BL/6 mice (see Fig. 9) purchased from the National Cancer Institute.

Antibodies

Mouse monoclonal anti-YY1 (H-10), NFATc1 (7A6), NFATc2 (4G6-G5), NFATc3 (F-11), NF-κB-p52 (C-5), Pax5 (A-11), CD3 (PC3/188A), rabbit polyclonal anti-Oct1 (C-21), PU.1 (T-21), and goat polyclonal anti-CD21 (M-19) Abs were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-BAFF (catalog no. ab1446) was purchased from Abcam. Rabbit anti-β-actin Ab (catalog no. A2066; Sigma-Aldrich) was used as the loading control in Western blot analysis.

ChIP

This protocol was adapted from published procedures (33–35). BALB/c splenocytes were isolated, and erythrocytes were lysed using RBC lysis buffer (0.15 M NH4Cl, 10 mM KHC03, and 0.1 mM Na2EDTA (pH 7.2)), washed, and resuspended in PBS with 0.1% BSA. A total of 3 × 106 cells was pelleted for each ChIP reaction, and PBS was removed. DNA/Protein cross-linking was done by resuspending cell pellets in 1% formaldehyde and incubated for 10 min at room temperature with rotation. Fifty microliters of 2.5 M glycine was added to each reaction and incubated for 5 min with rotation and then centrifuged to pellet cells. Pellet was washed once with 1× PBS with 0.1% BSA, and cells were resuspended in radiolabeled immunoprecipitation assay (RIPA) (50 mM NaCl, 25 mM Tris (pH 7.5), 1 mM EDTA, 0.1% SDS, 1% w/v deoxocholate, 1% Nonidet P-40, 1% BSA, and protease inhibitor “Complete Mini” (Roche)) and incubated for 20 min on ice with intermittent hard vortexing. Lysed samples were sonicated seven times at power setting 4 for 30 s each time. Sonicate was centrifuged for 10 min at 14,000 rpm at 4°C. Supernatant was taken and centrifuged again to pellet debris. For the input sample, 100 μl of the supernatant (named “Crude Input”) was mixed with 300 μl of ChIP elution buffer (10 mM Tris/EDTA, 1% (w/v) SDS, and 50 mM Tris-HCl (pH 8.0)), and cross-linking was resolved by heating at 65°C overnight and then purified with Qiagen PCR purification columns. Four-hundred microliters of the remaining supernatant with 100 μl of RIPA, supplemented with 1 protease inhibitor mixture (“Complete Mini”; Roche) per 10 ml of volume, was used for each immunoprecipitation reaction. Samples were precleared for 2 h at 4°C (with rotation) with sheep anti-mouse or sheep anti-rabbit IgG conjugated Dynabeads (Dynal Biotech) that were previously blocked and washed with 1× PBS with 1 mg/ml BSA.

For each immunoprecipitation reaction, 1 μg of mouse monoclonal or rabbit polyclonal Ab was used, except where indicated otherwise. Immunoprecipitated reactions were added to sheep anti-mouse or sheep anti-rabbit IgG conjugated Dynabeads blocked and washed with 1× PBS with 1 mg/ml BSA and incubated overnight. Dynabeads were washed, three times with RIPA, twice with RIPA with 0.1 mg/ml herring sperm DNA, twice with RIPA plus 0.1 mg/ml herring sperm DNA plus 500 mM NaCl, twice with RIPA plus 250 mM LiCl, then one alternate wash with RIPA plus 500 mM NaCl, one wash with RIPA plus 250 mM LiCl, and, finally, three washes with TE (10 mM Tris (pH 8)-1 mM EDTA). DNA was eluted from the bound beads by adding 200 μl of ChIP elution buffer to each reaction and incubating overnight at 65°C incubator, gentle shaking. Supernatant was then separated from Dynabeads, and soluble DNA was isolated. PCR amplification in the presence of [32P]dCTP was done for each sample (input, immunonucleotide control, and immunoprecipitated samples) and water control using primers specific for CD21, CD23, and CD19 promoter (and CD21 (30) intrinsic) elements, β-actin intragenic region and nidogen (NIDO) for 30 cycles with 6-s elongation, and 55°C annealing temperature (36). Primer sequences are listed in Table I. PCR products were subjected to electrophoresis within a sequencing gel, and the gel was dried and exposed to x-ray film overnight.

For PhosphorImager quantification of ChIP assay, the gel was dried and was exposed to a phosphor screen overnight and scanned with a PhosphorImage (STORM820; Molecular Dynamics). Intensity values for each PCR product were analyzed with Image Quant software (Molecular Dynamics). For relative quantification, a 4-fold dilution series of input was done. The concentration of output samples was taken as “x” (as 400 μl of crude input used per immunoprecipitation), and accordingly, the concentration of the input was “0.25x” (as 100 μl of crude input used per input). The data were normalized to the input and to the reference gene NIDO to determine the fold enrichment. The fold enrichment value was obtained by dividing the ratio of relative values by the ratio of input to reference gene NIDO.

Separation and analysis of splenic lymphocyte populations

For ChIP analysis, splenic B and T cell subsets were purified using Miltenyi Biotec magnetic beads, according to the manufacturer’s protocol. B220 and CD90 magnetic beads were used for labeling B and T cells, respectively. The purity of fractionated cells was confirmed by FACs analysis.

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For B cell subset separation, total splenocytes were isolated from 6- to 8-wk-old C57BL/6 female mice. After RBC lysis, a B cell-enriched population was obtained via B220<sup>+</sup> magnetic bead depletion. B220<sup>+</sup> cells were stained with Abs against CD21, CD23, and CD24 and resuspended at 2 x 10<sup>6</sup> cells/ml. Cells were sorted into various B cell subsets with the FACS Vantage SE.

**RNA preparation, cDNA synthesis, and RT-PCR**

Total RNA from cells was isolated using the RNeasy mini kit (Qiagen) and quantified. cDNA was synthesized by mixing 2 μg of RNA, 10 μl of 5× first-strand buffer, 5 μl of 5 mM dNTP, 5 μl of 0.1 M DTT, 1 μl of 1.25 mM random primers, 2 μl of Maloney murine leukemia virus virus-reverse transcriptase (all were from Invitrogen Life Technologies), and water to a final volume of 50 μl. The reaction mixture was incubated at 37°C for 2 h. Two microliters of DNase-free RNase (1 mg/ml from Invitrogen Life Technologies) was then added, and the reaction mixture was incubated for additional 5 min at room temperature, followed by cDNA isolation, using the Qiagen PCR purification kit. Radioactive RT-PCR using <sup>32</sup>P was performed as described previously (36). Quantitative real-time RT-PCR was performed as described previously (21, 37). Primer sequences for RT-PCR analysis are listed in Table 1.

**Western blot analysis**

Splenic or bone marrow B and T cell subsets were purified as described above. Live cells were counted by trypan blue dye exclusion, and 5 x 10<sup>6</sup> cells were resuspended to 100 μl of RIPA (50 mM NaCl, 25 mM Tris (pH 7.5), 1 mM EDTA, 0.1% SDS, 1% w/v deoxycholate, and 1% Nonidet P-40) with one protease inhibitor “Complete Minipill” (Roche) and 100 μl of PMSF per 10 ml of lysis buffer. The reaction mixture was mixed and incubated 15 min on ice with intermittent vigorous agitation. Lysed cells were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatants were separated and transferred to membrane for Western blot analysis using standard protocols.

**Results**

*Defining in vivo factor binding sites in the CD21 and CD23 genes via ChIP*

The murine CD21 gene has previously been scrutinized for transcription factor binding sites via sequence scanning and EMSA analysis. These two procedures are useful in defining possible factors binding sites but do not always reflect relevant in vivo binding. Therefore, we sought to increase the stringency of our analyses by using ChIP analysis with Abs against the various candidate transcription factors.

The mouse CD21 promoter element possesses a variety of potential sites, including those for Oct-1 (defined by EMSA analysis) (38), Pax5, E-box proteins, and NF-κB (by sequence scanning) (Fig. 1). Studies on the human CD21 promoter identified binding sites specific for E-box binding proteins (E2A) (14). The CD23a promoter contains defined binding sites (from the human and mouse genes) for RBP-J<sub>K</sub>, the Stat1/Stat6 IL-4 response element, Pax5, NF-κB, E proteins, and NFAT family members (27–29).

ChIP analysis was performed on total BALB/c splenocytes (Fig. 2). Cells used in these analyses were freshly harvested from the spleen and immediately cross-linked with formaldehyde without any cell stimulation or activation. Samples were amplified using primers specific for sequences from the CD21 and β-actin genes (Table I). As shown in Fig. 2A, titrating the amount of anti-Oct-1 Ab increased the immunoprecipitation of sequences from the CD21 gene but not that of the β-actin control, which does not possess Oct-1 binding sites. This data validated our previous EMSA observations of Oct-1 binding sites within the CD21 gene by showing that these are bound by Oct-1 in vivo. We had expected that moving away from the core Oct binding sites within the CD21 gene would diminish the immunoprecipitation of distal regions of the gene (our ChIP protocol generates sonicated DNA fragments of ~500–1000 bp in size). This was not the case for Oct-1 (or precipitations of the CD21 gene using other transcription factors), suggesting that the gene is highly condensed, perhaps due to its telomeric location, giving rise to large cross-linked complexes.

<table>
<thead>
<tr>
<th>ChIP oligos</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introns I0 (CD21)</td>
<td>CACCATGTCAGCCATAGTACCA</td>
<td>CTGACATAGTGGAGTGAAGAGATG</td>
</tr>
<tr>
<td>Introns I0 (CD21)</td>
<td>CACCATGTCAGCCATAGTACCA</td>
<td>CTGACATAGTGGAGTGAAGAGATG</td>
</tr>
<tr>
<td>Introns I0 (CD21)</td>
<td>CACCATGTCAGCCATAGTACCA</td>
<td>CTGACATAGTGGAGTGAAGAGATG</td>
</tr>
<tr>
<td>Promoter P2 (CD21)</td>
<td>CGATATCTACTCACTTCCTAG</td>
<td>CACGATACAGTGGAGTGAAGAGATG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CCATATCTACTCACTTCCTAG</td>
<td>CACGATACAGTGGAGTGAAGAGATG</td>
</tr>
<tr>
<td>NFATc1</td>
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<td>GTTGGTCCGCTTGGTGCAGAGAGAC</td>
</tr>
<tr>
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</tr>
<tr>
<td>ADAM10</td>
<td>GCCATCCCGCCGAGATGGCG</td>
<td>GTTGGTCCGCTTGGTGCAGAGAGAC</td>
</tr>
</tbody>
</table>

Table I. Oligonucleotides used in the ChIP and RT-PCR assays
Similar data to that of the Oct-1 ChIP analysis were derived for NFATc3, YY1, and NF-κB-p52. These data thus validate the in vivo binding of Oct-1, NFATc3, YY1, and NF-κB-p52 to the CD21 gene and suggest such analyses can be used to differentially analyze various subsets of splenocytes (see below).

We expanded these ChIP assays with naive total splenocytes for additional transcription factors and analyzed the products with a different control sequence (NIDO) and sequences specific for the CD23 promoter (see Fig. 1). As shown in Fig. 3, the CD21 promoter and intronic elements were specifically precipitated with Abs specific for YY1, NF-κB-p52, Pax5, NFATc3, E2A, RBP-Jκ, and Oct-1. The CD23 promoter sequence was isolated with Abs specific for all of these transcription factors with the exception of YY1 and Oct-1. The NIDO control sequence was not precipitated by any of these Abs.

**Splenic T and B cells demonstrate differential transcription factor binding to the CD21 gene**

CD21 is expressed in B cells, not in splenic T cells; therefore, we sought to determine which of the transcription factors identified in

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**FIGURE 1.** Schematic diagram of murine CD21 promoter and intronic regulatory regions and the murine CD23a promoter. The CD21 promoter is shown divided arbitrarily into 6 overlapping fragments of −200 bp each (P6-P1). The first half of intron 1 is arbitrarily divided into 12 overlapping fragments of −200 bp each (i1-i12) (22). The arrows denote the genomic fragments of the CD21 and CD23 genes used in this article. Binding sites for specific factors on the CD21 and CD23 genetic elements are shown (see text for explanation). The scale of each gene sequence is noted.

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**FIGURE 2.** ChIP of Oct-1 (A), NFATc3 (B), YY1 (C), and NF-κB-p52 (D), binding to CD21 intronic and promoter fragments. Splenocytes were treated with 1% formaldehyde, lysed, and sonicated. H2O sample is a negative water control (no DNA added). Input sample is the cross-linked, sonicated cell lysate. A total of 0.1, 1, and 5 μg of normal mouse IgG or normal rabbit IgG was used as the nonspecific Ab controls (Control Ig). A total of 0.1, 1, and 5 μg of rabbit polyclonal anti-Oct-1 or mouse monoclonal anti-NFATc3, -YY1, and -NF-κB-p52 Abs was used. PCR was done using specific oligonucleotides (Table I) to amplify CD21 intronic fragments (i8, i10, and i12) (see Fig. 1), promoter (P2), and β-actin.

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**FIGURE 3.**
Figs. 2 and 3 were specific for B cells. Splenic B and T cells were positively selected by magnetic bead sorting using anti-B220 for B cells and anti-CD90 for T cells. Greater than 90% of the selected cells were of the desired lineage as determined by FACS analysis (data not shown). Selected cells were immediately fixed, cross-linked, and used for ChIP analysis. As shown in Fig. 4A, NF-κB-p52 binding to the CD21 genetic elements was evident in both T and B cell populations, whereas Pax5 binding was only evident in the B cell samples. This result illustrated differential binding patterns between T and B cells because Pax5 is not expressed by T cells (39). Fig. 4B shows that Oct-1 bound to the CD21 sequences in both T and B cells, whereas PU.1, as expected (40), demonstrated no binding in either cell type. Fig. 4C shows that YY1 also constitutively bound to the CD21 sequences in both T and B cells but not the control β-actin and IL-2 gene sequences. NFATc3 showed specific binding to the CD21 gene only within the B cell samples, not the T cell subset. As a control, the IL-2 promoter sequence was analyzed for NFATc3 binding. ChIP analysis demonstrated NFATc3 binding to the IL-2 gene (whose transcription is NFAT dependent) was evident in the B cell subset but lacking in the T cell sample. These data indicate that NFATc3 is present in the nucleus of resting B cells (but not resting T cells) bound to defined NFAT binding sites (see below). Similar data for the CD23 gene were obtained from T and B cell subsets (Fig. 4D) in that NF-κB-p52 and Pax5 showed constitutive binding to CD23 sequences in the nuclei of B cells but not the CD23 promoter. As a control, the IL-2 promoter sequence was analyzed for NFATc3 binding. ChIP analysis demonstrated NFATc3 binding to the IL-2 gene (whose transcription is NFAT dependent) was evident in the B cell subset but lacking in the T cell sample. These data indicate that NFATc3 is present in the nucleus of resting B cells (but not resting T cells) bound to defined NFAT binding sites (see below).

The previous figure suggested that NFATc3 is constitutively bound to the CD21 gene in B cells but is excluded from the gene in T cells. To further analyze this finding, the effect of CsA on the ChIP analyses of the CD21 and CD23 genes was performed. CsA blocks the ability of NFAT proteins to move from the cytoplasm to the nucleus; therefore, we anticipated that CsA treatment would block binding of the NFATc3 to the CD21 and CD23 genes. As shown in Fig. 5A, treatment of total splenocytes with CsA, but not the ethanol control, resulted in a loss of NFAT binding to the CD21 sequences. This analysis was expanded in Fig. 5B by comparing the effect of CsA treatment on total splenocytes with CsA, but not the ethanol control, resulted in a loss of NFAT binding to the CD21 sequences. This analysis was expanded in Fig. 5B by comparing the effect of CsA treatment on total splenocytes with that of

**FIGURE 3.** ChIP of transcription factors on the CD21 and CD23 genes. ChIP analysis was performed on total splenocytes. The H2O sample is a water control, and INP is a sample of the sonicated cell lysate (input). M. control and R. control are mouse- and rabbit-nonspecific Ab controls, respectively. The mouse mAbs (M.YY1, M.NF-κB-p52, M.Pax5, and M.E2a) and the rabbit polyclonal Abs (R.RBP-Jκ/H9260 and R.Oct-1) are as noted. PCR products are from the CD21 first intron (i8) and promoter (P2), the CD23 promoter (CD23), and the mouse NIDO gene.

Figs. 2 and 3 were specific for B cells. Splenic B and T cells were positively selected by magnetic bead sorting using anti-B220 for B cells and anti-CD90 for T cells. Greater than 90% of the selected cells were of the desired lineage as determined by FACS analysis (data not shown). Selected cells were immediately fixed, cross-linked, and used for ChIP analysis. As shown in Fig. 4A, NF-κB-p52 binding to the CD21 genetic elements was evident in both T and B cell populations, whereas Pax5 binding was only evident in the B cell samples. This result illustrated differential binding patterns between T and B cells because Pax5 is not expressed by T cells (39). Fig. 4B shows that Oct-1 bound to the CD21 sequences in both T and B cells, whereas PU.1, as expected (40), demonstrated no binding in either cell type. Fig. 4C shows that YY1 also constitutively bound to the CD21 sequences in both T and B cells but not the control β-actin and IL-2 gene sequences. NFATc3 showed specific binding to the CD21 gene only within the B cell samples, not the T cell subset. As a control, the IL-2 promoter sequence was analyzed for NFATc3 binding. ChIP analysis demonstrated NFATc3 binding to the IL-2 gene (whose transcription is NFAT dependent) was evident in the B cell subset but lacking in the T cell sample. These data indicate that NFATc3 is present in the nucleus of resting B cells (but not resting T cells) bound to defined NFAT binding sites (see below). Similar data for the CD23 gene were obtained from T and B cell subsets (Fig. 4D) in that NF-κB-p52 and Pax5 showed constitutive binding to CD23 sequences in the nuclei of B cells but not the CD23 promoter. As a control, the IL-2 promoter sequence was analyzed for NFATc3 binding. ChIP analysis demonstrated NFATc3 binding to the IL-2 gene (whose transcription is NFAT dependent) was evident in the B cell subset but lacking in the T cell sample. These data indicate that NFATc3 is present in the nucleus of resting B cells (but not resting T cells) bound to defined NFAT binding sites (see below). Similar data for the CD23 gene were obtained from T and B cell subsets (Fig. 4D) in that NF-κB-p52 and Pax5 showed constitutive binding to CD23 sequences in the nuclei of B cells but not the CD23 promoter. As a control, the IL-2 promoter sequence was analyzed for NFATc3 binding. ChIP analysis demonstrated NFATc3 binding to the IL-2 gene (whose transcription is NFAT dependent) was evident in the B cell subset but lacking in the T cell sample. These data indicate that NFATc3 is present in the nucleus of resting B cells (but not resting T cells) bound to defined NFAT binding sites (see below). Similar data for the CD23 gene were obtained from T and B cell subsets (Fig. 4D) in that NF-κB-p52 and Pax5 showed constitutive binding to CD23 sequences in the nuclei of B cells but not the CD23 promoter.
Redundant expression and chromatin binding of NF-AT family members

We focused on the NFATc3 protein in the previous assays because it is expressed in murine B cells, and the Ab reagents specific for the protein generated robust data in EMSA supershift and ChIP assays. However, others have shown that animals lacking NFATc3 demonstrate normal levels of B cell CD21 expression, indicating that the NFATc3 gene product is dispensable for CD21 transcription (41). To determine whether other members of the NFAT family are also expressed in murine B cells allowing for redundant control of the CD21 gene, NFAT transcript and protein quantification was performed on splenic T and B cells. In Fig. 6A, RT-PCR analysis clearly showed the expression of NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5 in both T and B cell subsets. The accessory signaling protein NIP45 is also equally expressed between the two lymphocyte types. In Fig. 6B, the presence of NFATc1, NFATc2, and NFATc3 protein was also identified by Western blot analysis. The Ab regents specific for the NFATc4 and NFAT5 proteins could not resolve those species in either of these cell samples (data not shown). However, based on the previous transcript analysis, it is likely that these proteins are also present within T and B cells and that the Ab reagents are deficient for Western blot analysis.

ChIP analysis was performed on purified, unstimulated splenic B and T cells with a variety of anti-NFAT Abs (Fig. 7). A diverse pattern of NFAT family member binding was evident when comparing binding to the CD21, CD23, and IL-2 genetic elements. B cells demonstrated complexes with all five NFAT family members, although preferential complex formations were clearly evident. Thus, the IL-2 promoter sequence was clearly isolated with NFATc1, NFATc2, and NFATc3 reagents, whereas CD23 showed little complex formation with NFATc2. Similarly, the CD21 genomic elements were identified with a variety of the NFAT family members, including NFATc3. No NFAT complexes were found associated with the control NIDO gene.

The analysis of NFAT complexes formed in T cells was more restricted than that seen in the B cell populations. Although NFATc3 binding was robust in B cells, it was absent for all of the genes examined in the T cell populations. Instead, NFATc1, c4, and NFAT5 binding was detected with the NFATc1, NFATc2, and NFATc3 reagents, whereas CD23 showed little complex formation with NFATc2. Similarly, the CD21 genomic elements were identified with a variety of the NFAT family members, including NFATc3. No NFAT complexes were found associated with the control NIDO gene.

Pax5 binds the CD21 and CD23 genes independent of active transcription

Pax5 has been described as a master transcriptional activator required for B cell lineage commitment and induction of B cell-specific genes (42, 43). One of the primary targets of Pax5 is CD19, whose promoter possesses multiple Pax5 binding sites (44). Expression of CD19 is coincident with the expression of Pax5 at
Animals deficient in Pax5 show B cell arrest at the early pro-B cell stage (45). As shown in Fig. 1, there are also Pax5 binding sites in the CD21 and CD23 promoters, yet the expression of these genes is much later in B cell maturation. Therefore, we were curious whether we could detect Pax5 binding to CD21 and CD23 sequences in marrow B cells (which do not transcribe these genes) or whether Pax5 was blocked from binding to these inactive genes.

One problem in analyzing mature mouse marrow is that it not only possesses maturing B cell precursors but also mature B cells that recirculate from the spleen and other sites of activation. Thus, FACS analysis of normal, mature bone marrow (Fig. 8A, top two quadrants) shows a small percentage of CD21- and CD23-expressing cells (~3% of total cells). Animals lacking a functional BAFF receptor (the A/WySnJ strain) (46) produce normal B cells to the splenic T2 stage where they arrest development and differentiation. Thus, mature marrow cells from A/WySnJ mice possess normal immature B cell populations but lack recirculating CD21/CD23 positive mature B cells (Fig. 8A, bottom two quadrants).

B220⁺ B cells, representing immature and mature B cell populations, were obtained from the marrow and spleen of the A/J mouse and A/WySnJ mice and evaluated for the possession of the Pax5 protein by Western blot analysis. As shown (Fig. 8B), comparable levels of Pax5 protein is evident in marrow and spleen samples from the A/J and A/WySnJ mouse. As controls, YY1 protein is also evident in cell subsets from both animals, but CD21 is primarily found in the A/J splenic sample, which is consistent with the defective BAFF receptor phenotype of A/WySnJ splenic B cells.

ChIP analysis was performed on the bone marrow cells and splenocytes from the A/J and A/WySnJ mouse for Pax5 binding to the CD19, CD21, and CD23 genes. Nonspecific binding to NIDO

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**FIGURE 7.** ChIP with NFAT family members. ChIP analysis was done as described using freshly isolated splenic B and T cells. One microgram of nonimmune mouse IgG (M.Control) or nonimmune rabbit IgG (R.Control) was used as Ab controls. The two different controls were required because the Abs specific for NFATc1, NFATc2, and NFATc3 were mouse mAbs, while those specific for NFATc4 and NFAT5 were rabbit polyclonal Abs. One microgram of Abs was used for each immunoprecipitation. PCR was done using oligonucleotides specific for CD21 intronic (i8), promoter (P2) sequences, CD23 promoter, and the IL-2 and NIDO genes. Input is the cross-linked sonicated cell lysate from each sample. H₂O represents a negative PCR control with no DNA added.

**FIGURE 8.** Pax5 is associated with CD21 and CD23 gene sequences in developing bone marrow B cells. A, FACS analysis of total bone marrow from 10-wk-old A/J (upper panels) and A/WySnJ (A/W) mice (lower panels) double stained with CD19/CD21 or CD23/CD21. B, Western blot analysis on purified B220⁺ splenic and bone marrow B cell populations from 10-wk-old A/J and A/WySnJ (A/W) mice. Total protein (15 μg) was loaded into each well. Actin was used as loading control. C, ChIP of Pax5 binding to CD21, CD23, and CD19 promoter fragments from bone marrow and spleen. ChIP assay for Pax5 was performed on total bone marrow cell population from 10-wk-old A/J (A/J BM) and A/WySnJ (A/W BM) mice. PCR was done for CD21, CD23, and CD19 promoter fragments. Total splenocytes from A/J mice (A/J SP) was used as a positive control for Pax5 binding to CD21, CD23, and CD19 promoters. PhosphorImager analysis and quantification of the PCR products were performed as described in Materials and Methods; fold enrichment = (ChIP target/ChIP reference)/(input target/input reference). □, Precipitation with anti-Pax5 Ab; ○, precipitation with nonspecific Ab control.
was subtracted from each sample (see Materials and Methods). The fold enrichment of Pax5 binding vs nonspecific binding was very similar for the different samples examined, regardless of the gene. Thus, CD19, which is expressed in marrow and spleen, showed, as expected, specific binding of Pax5 to the CD19 promoter in all three samples. If the Pax5 protein was excluded from binding to the inactive CD21 and CD23 genes in the immature marrow B cells, then no specific binding should have been evident in marrow samples compared with the splenic samples, which was not the case. Therefore, the CD21 and CD23 promoters are accessible to Pax5 binding, even though the genes are transcriptionally silent. The relative fold precipitation of the spleen samples is higher than the marrow for the three genes because a higher percentage of cells in the spleen express Pax5 than in the marrow.

The CD21 and CD23 transcription profiles in spleen B cell subsets

The data in this article and that described by others suggests that CD21 and CD23 share many common transcriptional control regulators. However, the CD21 and CD23 proteins vary in the level of surface expression during peripheral B cell development. During B cell maturation, the CD21 and CD23 proteins are first found on cells making the T1 to T2 transition in the

![Diagram](http://www.jimmunol.org/)

**FIGURE 9.** Quantification of CD19, CD21, CD23, and ADAM10 transcripts in defined splenic B cell populations. A. Splenic B cells were stained and sorted into discrete populations. Staining protocol was based on using CD21, CD24, and CD23 to elucidate T1 (CD21^high^CD24^low^CD23^+^), T2 (CD21^high^CD24^high^CD23^+^), MZ (CD21^high^CD24^high^CD23^+^), and FM (CD21^low^CD24^low^CD23^+^) B cell subsets. Cells were gated with CD23^+^ or CD23^−^ populations and then sorted by a combination of CD21 and CD24 surface expression. B. Cells from each representative B cell subset were analyzed for transcriptional expression of CD21, CD23, CD19, and ADAM10. Levels of expression for each gene are shown relative to β-actin. The data shown are averages of three independent cell sorts.
spleen (9, 47). T2 cells express both CD21 and CD23. The CD21 proteins are expressed on the FM B cells and, to an elevated level, on the surface of MZ cells. The CD23 product is expressed on the surface of T2 cells and FM B cells but not by the MZ population (9).

The CD23 protein, found on the surface as a homotrimer, is known to be cleaved from the cell by the ADAM10 protease (48); therefore, the MZ cells could conceivably lose CD23 from the cell surface by elevated expression of ADAM10.

Previously, it had been shown that CD23 transcripts are depressed in MZ cells compared with FM and T2 CD23 levels (49) using a semiquantitative RT-PCR analysis. We chose to reanalyze this question using real-time quantitative RT-PCR for CD19, CD21, CD23, and ADAM10 transcripts in sorted T1, T2, MZ, and FM cell populations. Splenic B cells were enriched with B220+ magnetic beads and stained with Abs specific for CD21, CD23, and CD24 (Fig. 9). CD23-positive (the R5 population) and CD23-negative cells (the R2 population) were then analyzed for relative expression of CD21 and CD23. The expression of CD23 in MZ cells was much lower than in FM cells, and the least in the T1 immature B cells. These data match that of the cell surface expression levels. The CD23 transcripts also matched the cell surface staining data in that the T1 and MZ cells had very low levels of transcripts whereas T2 and FM cells had roughly equivalent levels. CD19 was abundantly expressed in all four splenic B cell types as was the ADAM10 gene. These data confirm that CD23 transcript levels are depressed in MZ cells and that the absence of CD23 on the surface of MZ cells is due to the absence of CD23 mRNA, not heightened ADAM10 production. Thus, even though the CD21 and CD23 genes share a number of key regulatory mechanisms, the stage-specific transcriptional control of these two genes is distinct.

**Discussion**

This article has focused on defining in vivo binding of candidate transcription control factors to the CD21 and CD23 genes in expressing and nonexpressing cells using ChIP. In previous reports, we have identified a number of potential protein/DNA complexes based on conserved sequence motifs and EMSA shift data (20, 22). Such assays are capable of cataloguing the potential of such complexes forming yet do not address whether such complexes actually develop in the nucleus of the cell.

The ChIP assay defined constitutive binding of NFAT and NF-κB species to the CD21 and CD23 (and IL-2) genetic control regions. These families of transcription factors are notable for their cytoplasmic localization until cellular activation. However, we observe nuclear binding complexes of NF-κB-p50 and NFAT proteins in the absence of specific activation. One argument to explain our findings could be that the spleen consists of mixed populations of activated and unactivated cells, and the sensitivity of the ChIP procedure allows for the detection of a small subset of activated cells. We were very careful in these ChIP analyses to prevent cellular activation. Fresh cells were isolated and immediately cross-linked with formaldehyde or quickly purified and formaldehyde treated. Because the same results were obtained from purified cell populations or total splenocytes, it is not likely that our purification schemes resulted in cellular activation. If we were identifying DNA/protein complexes from a small subset of activated cells then, for example, we should have observed NFATc3 binding to gene sequences in the T cell populations because activated T cells clearly translocate NFATc3 into the nucleus, and CD21, CD23, and IL-2 gene sequences are recognized by that protein. Because this was not observed in this report, the simplest interpretation of our data is that there are low levels of different NF-κB and NFAT species that are constitutively present within the nucleus of resting splenic B and T cells, and these proteins can establish binding complexes on target genes.

Investigations into the role of the NFAT proteins in the regulation of CD5 expression may provide clues pertaining to CD21 and CD23 regulation. The expression of CD5 is clearly up-regulated by NFAT activation following calcium flux (via cross-linking the BCR-BCR) due, in part, to the presence of multiple NFAT binding sites in the promoter of the gene (50–53). Normal B-1a cells, however, constitutively express CD5 without requiring exogenous activation. We have previously shown that the expression of the CD21 gene is sensitive to CsA treatment (thus blocking NFAT protein migration into the nucleus) (22). However, the expression of CD21 on B cell surfaces is not increased following BCR-mediated activation (our unpublished data). One difference between CD5 and CD21 is that NFAT binding sites of the former gene are within the promoter while those in CD21 are within the intronic enhancer/silencer.

Whether the NFAT proteins are directly facilitating transcriptional induction (or repression) at the CD21 intronic site or are altering the organization state of the chromatin by recruiting histone modification enzymes is not known (7). Our previous observation that CD21 transcriptional control is influenced by the state of histone acetylation (21) plus our data in this report showing the exclusion of YY1 and Oct-1 proteins to the CD21 gene following CsA treatment in splenocytes (Fig. 5) may suggest a role for the NFAT binding proteins to alter chromatin structure in the CD21 gene. A low level of constitutive NFAT movement into the nucleus of T and B cells may be enough to satisfy chromosomal organization duties, but large-scale transcriptional induction of NFAT target genes would require the translocation of the cytoplasmic stores of the proteins.

Animals deficient in NFATc2 and NFATc3 express CD21 and CD23 on the surface of splenic B cells (41). MZ B cells are reduced in such animals based on the selective loss of CD21highIgMhigh cells. In addition, animals lacking NFATc1 express normal levels of CD21 and CD23 (54). In contrast, animals deficient in NFATc1 show an absence of CD5+ B cell populations (but those deficient in NFATc2 possess normal CD5 expression), suggesting that the NFATc1 protein is either directly responsible for constitutive CD5 expression or the outgrowth of the B-1a cells capable of expressing that surface protein (54). We demonstrate in this article that multiple NFAT family members do bind the intronic NFAT binding sites of the CD21 and CD23 genes and thus may functionally complement different family members. Indeed, our data showing expression of all five NFAT family members in splenic B cells suggest that, to accurately define the role of NFAT family members via mouse knockout models, strains may need to be developed lacking B cell expression of all of the family members (thus the genetic equivalent of CsA treatment).

The binding of the B cell-specific transcription factor Pax5 to the CD21 and CD23 promoters could help define the B cell-specific expression of these genes. CD19 has been defined as relying on Pax5 for B cell stage-specific expression, yet CD19 is expressed...
The signaling pathway ascribed to BAFF is that of the NF-κB pathway, requiring receptor interacting protein and NF-κB-inducing kinase activation and generating the p52 subunit (31, 32). The constitutive binding of the NF-κB-p52 subunit to the CD21 and CD23 genes in T and B cells (Figs. 3 and 4) (56) requires a model of low-level translocation of NF-κB species into the nucleus of both cell types. We have previously shown that NF-κB-p52 binds to the CD21 and CD23 genes in splenic B cells from BAFF receptor-defective animals (A/WySnJ) as the wild-type controls (A/J) (56), indicating lack of this transcription factor is not blocking CD21 and CD23 expression in A/WySnJ mouse.

The current model of B cell development in the mouse involves a linear maturation pathway of pre-T1 B cell release from the marrow and seeding of such cells into peripheral lymphatic sites such as the spleen (Fig. 10A) (47). T1 cells in the spleen possess low levels of Bcl2 and are susceptible to BCR-induced apoptosis. Our (and other’s) analysis of T1 cells suggests this group possesses two subsets, one which expresses CD23 but not CD21, a subset that then gives rise to the T2 and FM populations. In this scenario, CD23 expression does not need to be elevated and then extinguished but instead is repressed in the entire lineage. The T1b lineage, in contrast, allows for CD23 and CD21 expression in the entire lineage, in contrast, allows for CD23 and CD21 expression in both cell types. We have previously shown that NF-κB-p52 binds to the CD21 and CD23 genes in splenic B cells from BAFF receptor-defective animals (A/WySnJ) as the wild-type controls (A/J) (56), indicating lack of this transcription factor is not blocking CD21 and CD23 expression in A/WySnJ mouse.

FIGURE 10. Alternative pathway for the generation of MZ B cells. A, Schematic for generation of MZ and FM cells from a T2 splenic precursor. B, Schematic for generation of MZ cells from a lineage of T1 cells distinct from the population of T1 cells giving rise to T2 and FM cells.

much earlier in the B cell lineage than CD21 and CD23 (42, 44). As shown in Fig. 8, Pax5 binding to the CD21 and CD23 genes was as evident in immature marrow cells not transcribing those genes as it was to the CD19 gene, which is being transcribed. This finding suggests that either additional positive regulatory components are missing for the CD21 and CD23 genes to be activated or that Pax5-induced transcription is repressed. Because CD23 is differentially expressed in splenic B cell maturation compared with CD21 (55, 56), two distinct regulatory components must exist, one for CD21 and the other for CD23. The presence of an inhibitory protein binding to the CD21 promoter, such as the E2 complex described for the human CD21 gene (17), could mitigate the action of an active Pax5 protein in maturing marrow B cells.

It was recently reported that BAFF-dependent activation of B cells was required for the expression of CD21 and CD23 (30). The signaling pathway ascribed to BAFF is that of the NF-κB pathway, requiring receptor interacting protein and NF-κB-inducing kinase activation and generating the p52 subunit (31, 32). The constitutive binding of the NF-κB-p52 subunit to the CD21 and CD23 genes in T and B cells (Figs. 3 and 4) (56) requires a model of low-level translocation of NF-κB species into the nucleus of both cell types. We have previously shown that NF-κB-p52 binds to the CD21 and CD23 genes in splenic B cells from BAFF receptor-defective animals (A/WySnJ) as the wild-type controls (A/J) (56), indicating lack of this transcription factor is not blocking CD21 and CD23 expression in A/WySnJ mouse.

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Disclosures

The authors have no financial conflict of interest.