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Stage-Specific Modulation of IFN-Regulatory Factor 4 Function by Krüppel-Type Zinc Finger Proteins

Sanjay Gupta, Alissa Anthony, and Alessandra B. Pernis

Optimal humoral responses depend on the activation of Ag-specific B cells, followed by their differentiation toward a fully differentiated phenotype. Acquisition of stage-appropriate patterns of gene expression is crucial to this differentiation program. However, the molecular mechanisms used by B cells to modulate gene expression as they complete their maturation program are poorly understood. IFN-regulatory factor 4 (IRF-4) plays a critical role in mature B cell function. Using the transcriptional regulation of the human B cell activation marker CD23 as a model system, we have previously demonstrated that IRF-4 is induced in response to B cell-activating stimuli and that it acts as a transactivator of CD23 gene expression. We have furthermore found that IRF-4 function can be blocked by B cell lymphomas 6 (BCL-6) protein, a Krüppel-type zinc finger repressor normally expressed in germinal center B cells. However, CD23 expression is known to be down-regulated in plasma cells despite high level expression of IRF-4 and the lack of BCL-6, suggesting that in plasma cells the IRF-4-mediated induction of CD23 is prevented by its interaction with a distinct repressor. In this set of studies, we demonstrate that IRF-4 interacts with B lymphocyte-induced maturation protein/positive regulatory domain I-binding factor 1 (Blimp1/PRDI-BF1), a Krüppel-type zinc finger protein whose expression correlates with terminal B cell differentiation. Functional studies indicate that Blimp1, like BCL-6, can block IRF-4-transactivating ability. These findings thus support a model whereby IRF-4 function is modulated in a stage-specific manner by its interaction with developmentally restricted sets of Krüppel-type zinc finger proteins. The Journal of Immunology, 2001, 166: 6104–6111.

Effective humoral responses involve the activation of Ag-specific B cells, followed by their differentiation toward memory or plasma cells. Progression of an activated B cell along its terminal differentiation pathway requires the acquisition of stage-appropriate patterns of gene expression. Therefore, once the molecular machinery responsible for the activation program of a B cell has been implemented, it must be selectively modulated to ensure successful completion of terminal differentiation. The molecular mechanisms used by B cells to selectively regulate gene expression as they proceed along their maturation program are largely unknown.

IRF-4 is a novel member of the IFN-regulatory factor (IRF) family of transcriptional regulators, whose expression is primarily restricted to the lymphoid compartment (1–3). IRF-4 expression in B cells is induced in response to stimuli known to drive B cell activation (2, 4–6). Genetic evidence indicates that IRF-4 plays a crucial role in controlling the activation and homeostasis of immune responses (7). Despite exhibiting a normal B cell differentiation, IRF-4-deficient mice display profound defects in the function of mature B cells and are unable to mount Ab responses to either T-dependent or T-independent Ags. Functional studies have shown that IRF-4 can surveil a dual role in lymphoid cells. By itself, IRF-4 can bind to IFN-stimulated response elements and repress the expression of IFN-inducible genes (3). In the presence of a cofactor, the protooncogene PU.1, IRF-4 can act as a transactivator of the Ig κ- and λ-light chain enhancers and the CD20 promoter (1, 8, 9).

Our studies have used the regulation of the B cell membrane protein that belongs to the C-type lectin superfamily (10–12). This family includes a subset of killer-inhibitory receptors that exert inhibitory functions on NK cells (10–14). Two differentially spliced forms of CD23 (termed CD23a and CD23b) exist in humans, whereas only one form, which more closely resembles CD23a, has been consistently found in mice. The two human CD23 isoforms are the result of use of alternative transcriptional start sites and are regulated by two distinct promoters (15). CD23a and CD23b only differ by a 6/7-aa substitution, leading to a change in the CD23a and CD23b mediate distinct signaling pathways.

We have focused our attention on the regulation of CD23b, the ITIM-less isoform of CD23. We have previously found that IRF-4 participates in a multiprotein or “enhanceosome-like” complex that targets the CD23b IFN-γ activation site (GAS), a critical regulatory element in the promoter of CD23b (4). The IRF-4-mediated transactivation of CD23b is blocked by B cell lymphomas 6 protein (BCL-6), a Krüppel-type zinc finger transcriptional repressor present in germinal center B cells (18, 19). Because BCL-6
expression is down-regulated upon B cell activation (4, 18, 20), we have proposed that induction of high levels of IRF-4 coupled with the loss of BCL-6 plays a key role in the activation of activated B cells to up-regulate CD23b expression. Interestingly, upon terminal differentiation of a B cell into a plasma cell, CD23 expression is normally down-regulated (21), although plasma cells continue to express high levels of IRF-4 (1) and lack BCL-6 (19). This observation led us to hypothesize that, in plasma cells, the IRF-4-mediated induction of CD23b might be repressed by a different mechanism.

Previous work has demonstrated that another Krüppel-type zinc finger protein, Blimp-1 (B lymphocyte-induced maturation protein), is mainly detected in late B and plasma cells and is a critical regulator of terminal B cell differentiation (22). Although the full extent of B lymphocyte-induced maturation protein (Blimp1) functions is not known, Blimp1 is a critical transcriptional repressor of the c-myc oncogene (23) and the MHC class II transactivator (CIITA) (24), both of which are down-regulated upon terminal B cell differentiation. Although Blimp1 is believed to play a key role in terminal B cell differentiation, its expression is not confined to lymphoid cells. Indeed, the human homologue of Blimp1, PRDL-BF1 (positive regulatory domain I-binding factor 1), is induced upon viral infection of fibroblasts and was cloned because of its ability to target the IRF binding site within the IFN-β enhancer, and to repress β-IFN gene expression (25). Interestingly, recent immunohistochemical analysis has revealed that plasma cells as well as a subset of germinal center B cells with a partial plasma cell phenotype strongly express both Blimp1 and IRF-4 (26).

In this study, we report that PRDL-BF1/Blimp1 can bind to the same functional element in the human CD23b promoter to which BCL-6 and IRF-4 had previously been shown to bind, and that, like BCL-6, Blimp1 can repress IRF-4-transactivating ability. Thus, IRF-4 function can be modulated in a stage-specific manner by its interaction with developmentally restricted sets of Krüppel-type zinc finger proteins.

Materials and Methods

Cell lines and cultures

The human B cell line Ramos (obtained from Dr. S. Lederman, Columbia University, New York, NY) is an EBV-negative Burkitt’s lymphoma. U266 (American Type Culture Collection (ATCC), Manassas, VA) is derived from a multiple myeloma cell line. U937 (obtained from Dr. K. Calame, Columbia University) is a monocytic cell line. Human embryo kidney 293T cells (obtained from Dr. S. Lederman) were cultured in DMEM supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA). All other cells were grown in IMDM supplemented with 10% FCS (Atlanta Biologicals), as previously described (4). U266 cells (10-20 × 10^6) were stimulated with 1 μg/ml of either anti-CD40 or an isotype-matched control Ab in a final volume of 10 ml at 37°C for 24 h.

Antibodies

The rabbit polyclonal antiserum against IRF-4 used in EMSA experiments was a generous gift of Hisamaru Hirai (University of Tokyo, Tokyo, Japan) (3). We subsequently generated our own rabbit polyclonal anti-IRF-4 antiserum using a similar GST-IRF-4 (nucleotides 441–924) fusion protein as the immunogen (Berkeley Antibody, Richmond, CA). This antiserum was used for immunoprecipitations and immunoblot analyses (4). Blots were also reprobed with a commercially available anti-IRF-4 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), which gave identical results. Rabbit polyclonal antiserum against human Stat6, or BCL-6, were purchased from Santa Cruz Biotechnology. The mAb against hemagglutinin (HA) epitope (clone 12CA5) was from Boehringer Mannheim (Indianapolis, IN). The hybridomas secreting the anti-CD40 mAb G28-5 (IgG1) or an isotype-matched control mAb were obtained from ATCC.

DNA constructs

The human IRF-4 expression plasmid (pCEP4-IRF-4) and the GST-IRF-4 expression plasmid were previously described (4). Various deletion mutants of IRF-4 were prepared by Pfu PCR from pBSKS-IRF-4 plasmid template using appropriate primers. The cDNA segments encoding these deletion mutants were then subcloned, in frame, into the filled EcoRI site of pGEX-2X vector to generate GST-IRF-4 deletion mutants. The in-frame junction in the GST-IRF-4 fusion constructs was confirmed by DNA sequencing in an automated cycle sequencer (Perkin-Elmer, Norwalk, CT). The full-length human BCL-6 expression vector was a kind gift of R. Dalla-Favera (Columbia University, New York, NY) (27). HA epitope-tagged murine Blimp1 cDNA cloned into pHBluescript vector (pBSKS-HA-Blimp1) was a kind gift of K. Calame (23). The Blimp1 expression construct (pCEP4-HA-Blimp1) was generated by cloning the coding region of the HA-Blimp1 cDNA into pCEP4 expression vector. The full-length human Blimp1/PRDL-BF1 cDNA cloned into pX mammalian expression vector was a kind gift of T. Maniatis (Harvard University, Cambridge, MA) (25). The CD23b promoter firefly luciferase reporter construct in the pGL3-enhancer vector was previously described (4).

DNA-binding assays and cell extracts

The preparation and employment of DNA oligonucleotides probes for EMSAs have been described previously (4). The double-stranded oligonucleotides used as probes or cold competitors in these studies were as follows: CD23b GAS (wild-type, wt), 5’-gatcGGGTGAAATTCTAAGAAAGGGAC-3’; CD23b GAS M1, 5’-gatcGGGTGAAATTCTAAGAAGGGAC-3’; CD23b GAS M2, 5’-gatcGGGTGCTTCTAAGAAAGGGAC-3’; CD23b GAS M3, 5’-gatcGGGTGAAATTCTAAGAAGGGAC-3’; CD20, 5’-gatcGGGTGAAATTCTAAGAAGGGAC-3’; CIITA, 5’-gatcAACAGTGAAGAAAGGGAC-3’; IκBα, 5’-gatcACAGTGAAGAAAGGGAC-3’; MHC class II enhancer, 5’-gatcCCGCTGACAAAGGGAAAGGACTGAC-3’; IκBα, 5’-gatcACAGTGAAGAAAGGGAC-3’ (28); CIITA, 5’-gatcACAGTGAAGAAAGGGAC-3’ (29). Oligonucleotide competition and Abs interference assays were performed as previously described (4). Nuclear extracts were prepared as previously described (4).

Immunoprecipitations and Western blot analysis

Cell extracts were immunoprecipitated with an anti-IRF-4, or an anti-HA Ab, as previously described (4). The immunoprecipitates were resolved by 7% SDS-PAGE. The gel was transferred to a nitrocellulose membrane, and then immunoblotted with an IRF-4 or HA epitope Ab. The bands were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

GST pull-down assays and transient transfections

GST pull-down assays were conducted as previously described (4). The bound proteins were eluted from the beads by boiling them in SDS-PAGE sample buffer, fractionated on a 7% SDS-polyacrylamide gel, and then blotted onto a nitrocellulose membrane. The blot was probed with either a BCL-6, HA epitope, or Stat6 Ab.

For expression of recombinant proteins, 293T cells were transfected with expression plasmids by calcium phosphate precipitation method. After 24 h of incubation, the transfected cells were harvested for nuclear extract preparation. Transient transfection assays for reporter experiments were performed as previously described (4).

Results

Blimp1 binds to the CD23b GAS

The human homologue of Blimp1, PRDL-BF1, has previously been shown to target the IRF binding site in the IFN-β enhancer (25). Furthermore, the known DNA-binding elements for murine IRF-4 (1, 8) and Blimp1 (23, 24) are strikingly similar (Table I). We thus decided to investigate whether Blimp1/PRDL-BF1 can target the IRF-4 binding site in the human CD23b promoter (4). Therefore, we performed EMSAs with a CD23b GAS probe on extracts from U266. This is a myeloma cell line, representative of a terminally differentiated plasma cell (30), which lacks BCL-6 and expresses high levels of Blimp1/PRDL-BF1 (data not shown). As shown in Fig. 1A, the pattern of CD23b GAS-binding complexes in U266 extracts was markedly different from that detected in extracts from Ramos cells, which phenotypically resemble germinal center B cells and contain BCL-6, but not Blimp1/PRDL-BF1 (data not shown) (31). As we previously demonstrated (4), in Ramos cells, IRF-4 participates in the formation of a slow mobility complex, whose visualization is normally obscured by the presence of a
strong BCL-6-containing complex. In contrast, extracts from U266 cells contained a clearly visible IRF-4 complex and lacked the BCL-6 complex. Interestingly, U266 contained a distinct CD23b GAS-binding complex (complex X), which was absent in Ramos cells. The mobility of this complex was faster than that of the BCL-6-containing complex. Furthermore, BCL-6 expression is normally down-regulated upon CD40 stimulation (4, 18, 20). In contrast, stimulation of U266 cells with an anti-CD40 Ab did not affect the appearance of complex X, despite appropriately decreasing the intensity of the BCL-6 complex in Ramos cells (Fig. 1A). Supershifting experiments confirmed the presence of IRF-4 in U266 cells and demonstrated that complex X did not contain either IRF-4 or BCL-6 (Fig. 1B and data not shown). EMSA analysis of three additional myeloma cell lines revealed a similar pattern of CD23b GAS-binding complexes (data not shown).

To determine whether the faster CD23b GAS-binding complex present in U266, but not in Ramos cells contained Blimp1/PRDI-BF1, we subjected U266 extracts to oligonucleotide competition experiments using the known Blimp1 binding site from the myc promoter (MYC-PRF site) (23) as a cold competitor of the radio-labeled CD23b GAS wt probe. These oligonucleotide competition experiments revealed that the MYC-PRF element could efficiently compete both the IRF-4-containing complex as well as the faster mobility complex, suggesting that the latter complex might contain Blimp1/PRDI-BF1 (Fig. 2A, left panel). Competition of IRF-4 was not surprising because MYC-PRF has been described as an IFN-stimulated response-like element (23). To confirm this observation, we then proceeded to directly test whether rBlimp1 could target the CD23b GAS. 293T cells (which lack BCL-6, Blimp1, and IRF-4) were transiently transfected with an expression plasmid encoding either Blimp1 or BCL-6. Cells transfected with an empty vector were included as control. Extracts from the various 293T transfectants were then subjected to EMSA experiments using the CD23b GAS as a probe (Fig. 2A, right panel). These experiments clearly revealed that 293T transfectants expressing Blimp1 or BCL-6 contained CD23b GAS-binding complexes, which were absent in 293T cells transfected with an empty vector. The mobility exhibited by rBlimp1 was similar to that of the CD23b GAS-binding complex, which we had detected in U266 cells, but not in Ramos, and was clearly distinct from that displayed by rBCL-6.

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>IRF-4</td>
<td>ATAAAA GCCAACTGAAAA ACAGA</td>
</tr>
<tr>
<td>Blimp1</td>
<td>ATAAA GGAAGTGAAAA CCAAG</td>
</tr>
<tr>
<td>MYC-PRF</td>
<td>GGTTACA GGAAGGGGGAAAA GGACCT</td>
</tr>
<tr>
<td>CIITA</td>
<td>CAGTAA GGAAGTGAAAA TTAAT</td>
</tr>
</tbody>
</table>

Table I. Sequence comparison of IRF-4 and Blimp1 binding sites

Oligonucleotide competition experiments with a panel of CD23b GAS mutant elements (Fig. 2B) revealed that the CD23b GAS M2 mutant, which contains mutations in the IRF-4 binding site (4), is unable to efficiently compete the Blimp1 complex. The competition pattern for the Blimp1 complex is furthermore distinct from that we have previously shown for BCL-6, which is competed by the M2, but not by the M1 or M3 mutants (4). These results thus suggest that at different stages of B cell differentiation, distinct Kruppel-type zinc finger proteins target the CD23b GAS.

To assess whether the ability of BCL-6, Blimp1, and IRF-4 to target a common DNA element was confined to the CD23b GAS, we then subjected extracts from 293T transfectants expressing either BCL-6 or Blimp1 to an oligonucleotide competition experiment with a panel of DNA elements from different promoters/enhancers.

To determine whether the ability of BCL-6, Blimp1, and IRF-4 to target a common DNA element was confined to the CD23b GAS, we then subjected extracts from 293T transfectants expressing either BCL-6 or Blimp1 to an oligonucleotide competition experiment with a panel of DNA elements from different promoters/enhancers. The elements used in this experiment included: 1) sites known to bind Blimp1 (MYC-PRF, CIITA) (23, 24, 2) sites known to bind IRF-4 (κ3’ enhancer, CD20) (8, 9), and 3) a site known to bind BCL-6 (Iκ GAS) (32). Self-competition with the CD23b GAS element was included as control. As shown in Fig. 2C, this experiment indicated that all three known IRF-4 binding sites (κ3’ enhancer, CD20, and CD23b GAS) could efficiently block the binding of both the BCL-6 as well as the Blimp1 complexes to the CD23b GAS. In contrast, the known Blimp1 binding sites (MYC-PRF, CIITA) only competed the Blimp1, but not the BCL-6 complex, whereas the addition of an oligonucleotide containing the BCL-6 binding site (Iκ GAS) only blocked binding of the BCL-6 complex, but not that of the Blimp1 complex. These data thus suggest that both BCL-6 and Blimp1 can also target other IRF-4 binding sites in addition to the CD23b GAS. However, the targets of the two Kruppel-type zinc finger proteins do not completely overlap. Of particular interest is the finding that the Blimp1 complex was not competed by the Iκ GAS, which is known to bind Stat6 in addition to BCL-6 (32). In contrast to the CD23b GAS, the Iκ GAS does not appear to be targeted by IRF-4, because it fails to compete IRF-4 complexes in oligonucleotide competition assays (data not shown). This result thus suggests that the known ability of BCL-6 to modulate Stat6 activity may not be shared by Blimp1.

**IRF-4 physically interacts with Blimp1**

In our previous studies, we had detected a very strong interaction between BCL-6 and IRF-4 (4). To determine whether Blimp1 could also physically associate with IRF-4, we first performed pull-down assays with a GST-IRF-4 fusion protein. As shown in Fig. 3A, incubation of a GST-IRF-4 fusion protein with extracts from 293T cells transfected with an HA epitope-tagged Blimp1 expression vector revealed that IRF-4 can indeed associate with Blimp1. No interaction was observed with the GST moiety alone or upon incubation of the GST-IRF-4 with a control extract (Ramos) or with 293T cells transfected with an empty vector. We

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Different patterns of CD23b GAS-binding complexes are displayed by human cell lines corresponding to distinct stages of B cell differentiation. A. Nuclear extracts from U266 (a myeloma cell line) were compared with those from Ramos (a Burkitt’s lymphoma) by EMSA using a 32P-labeled CD23b GAS wt probe. Ramos and U266 cells were either unstimulated or stimulated with an anti-CD40 Ab (1 μg/ml) or a control Ab (1 μg/ml) for 24 h at 37°C. B. AB interference mobility shift assays were conducted by addition of antisera against IRF-4 or control. All antisera were added at a final dilution of 1/20 for 30 min at 4°C before incubation with the probe for 20 min at 25°C. U266 cells were unstimulated.
also cotransfected 293T cells with both IRF-4 as well as HA epitope-tagged Blimp1 expression vectors and then subjected the extracts to immunoprecipitation assays with either an anti-IRF-4 or anti-HA Ab (Fig. 3B). Consistent with our GST pull-down assays, presence of the IRF-4 protein could be detected in anti-HA immunoprecipitates of 293T cells cotransfected with both IRF-4 and HA epitope-tagged Blimp1 (Fig. 3B, upper panel), but not of control transfectants. Stripping and reprobing of the filter with an anti-HA Ab demonstrated the presence of HA-Blimp1 in the IRF-4 immunoprecipitates of 293T transfectants expressing both IRF-4 or Blimp1 and then assayed as described above. Oligonucleotide competition assays were performed either in the absence or in the presence of a 100-fold molar excess of cold wt or mutant CD23b GAS oligonucleotides added to the shift reaction as indicated. C, 293T cells were transiently transfected with an empty vector or with an expression vector encoding either BCL-6 or Blimp1 and then assayed as described above. Oligonucleotide competition assays were performed either in the absence or in the presence of a 100-fold molar excess of cold oligonucleotides corresponding to previously described Blimp1, IRF-4, or BCL-6 binding sites. Cold competitors were added to the shift reaction as indicated.

To further dissect the interaction of IRF-4 with the two Krüppel-type zinc finger proteins, we proceeded to map the IRF-4 domains involved in the association of IRF-4 with BCL-6 and with Blimp1. We thus generated a panel of different GST-IRF-4 mutant proteins, and compared them with wt IRF-4 in GST pull-down assays (Fig. 4A). Incubation of the different GST-IRF-4 deletion mutants with extracts from 293T transfectants expressing HA epitope-tagged Blimp1 revealed that interaction of IRF-4 with Blimp1 requires the aa 1–150 region of IRF-4, which is known to contain its DNA binding domain (Fig. 4A, upper panel) (1). Contribution of the proline-rich region of IRF-4 to its interaction with Blimp1 is also likely because deletion of this region (∆151–199) resulted in a diminished interaction of the mutant protein with Blimp1. When the GST-IRF-4 deletion mutants were incubated with extracts from Ramos cells and the IRF-4/BCL-6 interaction visualized by Western blotting with an anti-BCL-6 antiserum, the aa 1–150 region of IRF-4 was again found to be essential for its interaction with BCL-6 (Fig. 4A, middle panel). Similarly to the Blimp1/IRF-4 interaction, the IRF-4 mutant lacking the proline-rich region (∆151–199) also displayed a decreased ability to associate with BCL-6. However, in striking contrast to the results obtained with Blimp1, association of IRF-4 with BCL-6 also required the aa 200–360 region of IRF-4. We have previously shown that, in Ramos cells, IRF-4 can interact not only with BCL-6, but also with Stat6 (4). Interestingly, stripping and reprobing of this Western blot with a Stat6 Ab (Fig. 4A, lower panel) demonstrated that the IRF-4 ∆1–150 interacted normally with Stat6. Association of Stat6 with IRF-4 was instead found to require the aa 200–360 region. As summarized in Fig. 4B, this experiment thus revealed that the aa 1–150 region of IRF-4, which contains the DNA binding domain of IRF-4, is required for its interaction with both BCL-6 and Blimp1, but not with Stat6. In contrast, the aa 200–360 region of IRF-4, which contains residues previously shown to be critical for its interaction with PU.1 (33), is necessary for the association of IRF-4 with BCL-6 and Stat6, but not with Blimp1.
Blimp1 selectively blocks the ability of IRF-4 to transactivate CD23b

To determine whether Blimp1/PRDI-BF1 could repress the ability of IRF-4 to transactivate the CD23b promoter, we performed transient transfection assays in U937 cells, a monocytic cell line that is capable of activating Stat6 in response to IL-4, but lacks IRF-4, BCL-6, and Blimp1 (25). Consistent with previous results (4), cotransfection of an IRF-4 expression vector with the CD23b reporter construct driven by the CD23b promoter resulted in a 3-fold induction in luciferase activity. As we previously demonstrated, similar inducibility of the CD23b reporter construct can be achieved upon IL-4 stimulation of U937 or upon cotransfection of IRF-4. However, when cotransfection of IRF-4 is conducted in the presence of IL-4 stimulation, higher levels of luciferase activity are detected, suggesting that optimal CD23b inducibility requires cooperation between Stat6 and IRF-4 (4). Consistent with previous results, addition of BCL-6 blocked the IRF-4-mediated transactivation of this reporter construct as well as the IL-4 inducibility of CD23b (4, 34). Cotransfection of Blimp1 blocked the ability of IRF-4 to drive CD23b promoter activity and to augment the IL-4 inducibility of this reporter construct. However, in contrast to BCL-6, absence of Blimp1 did not affect the IL-4 inducibility of CD23b, regardless of the presence of IRF-4. These findings thus suggest that, in contrast to BCL-6, Blimp1 selectively targets IRF-4 function and does not interfere with Stat6 activity.

Discussion

IRF-4, an IRF family member whose expression is mostly restricted to lymphocytes (1–3), has been shown by genetic studies to play a crucial role in mature B cell function (7). Consistent with the phenotype displayed by the IRF-4-deficient mice, IRF-4 expression is up-regulated in response to B cell activation stimuli (2, 4–6). Our previous studies have indicated that IRF-4 function can be regulated by BCL-6, a Krüppel-type zinc finger transcriptional repressor (27, 35, 36). In this study, we report that IRF-4 function can also be modulated by a distinct Krüppel-type zinc finger protein, Blimp1/PRDI-BF1, previously shown to be a critical regulator of terminal B cell differentiation (22). Whereas expression of BCL-6 is mostly restricted to germinal center B cells (19), high levels of Blimp-1 are primarily found in terminally differentiated cells (22). Thus, the ability of IRF-4 to pair with developmentally restricted sets of Krüppel-type zinc finger proteins may regulate IRF-4 activity in a stage-appropriate manner. Furthermore, because Blimp1 expression can be detected in nonhemopoietic cells (25), interaction of Blimp1 with IRF-4 may confer lineage specificity to the actions of Blimp1.

The interaction of IRF-4 with Blimp1 may have broad biological implications. Indeed, our competition experiments suggest that this interaction is not restricted solely to the regulation of CD23 gene expression, but may extend to other IRF-4 target genes. Interestingly, one of these genes is CD20, a B cell surface marker, which, in humans, is differentially expressed in memory cells (CD20<sup>+</sup>) vs plasma cells (CD20<sup>−</sup>) (37). Because IRF-4 has been shown to participate in the control of CD20 (9), this finding suggests that modulation of IRF-4 function by Blimp1 may constitute one of the molecular events driving activated B cells toward a specific cell fate.

Our studies reveal not only similarities between the two Krüppel-type zinc finger proteins, but also profound differences between them. In both cases, association with IRF-4 maps to a region of IRF-4 that contains its DNA binding domain (1). However, physical interaction of IRF-4 with BCL-6, but not with Blimp1, also requires a region of IRF-4 that mediates its association with cofactors such as PU.1 (33) or Stat6 (Fig. 4A). This finding suggests that BCL-6 may modulate not only the DNA-binding ability of IRF-4, but also its ability to interact with cofactors and possibly to assemble into multiprotein complexes. In contrast, Blimp1 may primarily target the IRF-4 DNA binding domain. Because plasma
cells express high levels of both IRF-4 and Blimp1, we suspect that the interaction of IRF-4 with Blimp1 does not necessarily lead to repression of IRF-4 function, but it may rather direct IRF-4 toward stage-appropriate targets by modulating its DNA-binding properties. Thus, one may predict that, in a different promoter context, cooperative interactions between IRF-4 and Blimp1 may also be observed. Another important distinction uncovered by our studies is that, in contrast to BCL-6 (34), Blimp1 selectively represses IRF-4 function and neither targets Stat6 binding sites nor blocks Stat6-mediated transactivation. Thus, it will be interesting to determine whether terminally differentiated B cells possess distinct mechanisms to modulate Stat6 activity.

Although our studies suggest that all of the IRF-4 binding sites tested could serve as targets for both BCL-6 and Blimp1 (Fig. 2C), the regulation of IRF-4-mediated gene expression is likely to display additional complexities. In particular, different subsets of IRF-4 target genes may exist, which can be selectively targeted by different combinations of Krüppel-type zinc finger proteins. Consistent with this notion, although the IRF-4 binding sites in both CD23a and CD23b promoters can efficiently compete Blimp1, we have so far been unable to detect any targeting of the CD23a isoform by BCL-6 (S.G., unpublished observations). A selective effect of BCL-6 only on the CD23b (ITIM-less) isoform may thus explain the failure of microarray analysis to detect CD23 as a BCL-6 target gene (38). It is intriguing to speculate that employment of different mechanisms for regulating the two CD23 isoforms may allow a B cell to modulate the ratio, and thus the signaling outcome, of this pair of potentially inhibitory/activating receptors.

Consistent with the central role exerted by IRF-4 in B cell activation, deregulation of IRF-4 expression has been postulated to play a role in a variety of lymphoid malignancies (5, 39). Translocations of the BCL-6 gene leading to inappropriate expression of BCL-6 are a common event in non-Hodgkin’s lymphomas (35, 40–42). Interestingly, deletion of chromosome 6q21-q22.1, which contains the PRDI-BF1/Blimp1 gene, has been consistently detected in high-grade non-Hodgkin’s lymphoma and PRDI-BF1/Blimp1 has been suggested to be a candidate B-NHL suppressor.

*FIGURE 4.* Identification of IRF-4 domain sequences important for interaction with Blimp1 and BCL-6. A, Binding of various GST-IRF-4 mutant proteins with Blimp1 or BCL-6. To examine the IRF-4 domains mediating the IRF-4/Blimp1 interaction, nuclear extracts from 293T cells transfected with an HA epitope-tagged Blimp1 expression vector were incubated with various GST-IRF-4 mutant proteins immobilized onto reduced glutathione-agarose beads, as indicated. Bound proteins were eluted, fractionated by 7% SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with an anti-HA Ab (upper panel). Binding to immobilized GST alone is shown as a control. To assess the IRF-4 regions involved in the IRF-4/BCL-6 interaction, nuclear extracts from unstimulated Ramos cells were incubated with the same panel of GST-IRF-4 mutant proteins as above. Bound proteins were eluted, fractionated by 7% SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with an anti-BCL-6 Ab (middle panel). The blot was then stripped and reprobed with a Stat6 Ab (lower panel). B, Summary of the deletion mutation analysis. The IRF-4 mutant proteins used in the GST pull-down assays are diagrammed with the deleted sequences indicated on the left of each mutant. The positions of the DNA binding domain (DBD), the proline-rich region (PRO-RICH), and the glutamine-rich region (GLN-RICH) are indicated. The relative ability of each mutant to interact with Blimp1, BCL-6, or Stat6 is shown on the right of each mutant. ++ indicates binding comparable with that demonstrated by wt IRF-4; + indicates a diminished interaction; – indicates no interaction.
Our findings suggest that either persistence of BCL-6 expression or loss of Blimp1 may lead to the inappropriate regulation of IRF-4 function. This may result in an activated or partially activated B cell, which cannot successfully complete its differentiation program. In addition to the recently described ability of BCL-6 to repress Blimp1 expression and thus block terminal B cells differentiation (38), disturbances in the IRF-4/Krüppel interaction may thus also contribute to lymphomagenesis.

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