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Fas Apoptosis Inhibitory Molecule Expression in B Cells Is Regulated through IRF4 in a Feed-Forward Mechanism

Hiroaki Kaku and Thomas L. Rothstein

Fas apoptosis inhibitory molecule (FAIM) was originally cloned as an inhibitor of Fas-mediated apoptosis in B cells that has been reported to affect multiple cell types. Recently, we found that FAIM enhances CD40L-mediated signal transduction, including induction of IFN regulatory factor (IRF)4, in vitro and augments plasma cell production in vivo. These results have keyd interest in the regulation of FAIM expression, about which little is known. Here, we show that Faim is regulated by IRF4. The Faim promoter contains three IRF binding sites, any two of which promote Faim expression. Faim promoter activity is lost following mutation of all three IRF binding sites, whereas activity of the full promoter is enhanced by concurrent expression of IRF4. In stimulated primary B cells, IRF4 expression precedes FAIM expression, IRF4 binds directly to the Faim promoter, and loss of IRF4 results in the failure of stimulated Faim up-regulation. Finally, FAIM is preferentially expressed in germinal center B cells. Taken together, these results indicate that FAIM expression is regulated through IRF4 and that this most likely occurs as part of germinal center formation. Because FAIM enhances CD40-induced IRF4 expression in B cells, these results suggest that induction of FAIM initiates a positive reinforcing (i.e., feed-forward) system in which IRF4 expression is both enhanced by FAIM and promotes FAIM expression. The Journal of Immunology, 2009, 183: 5575–5581.

FAIM, Fas apoptosis inhibitory molecule; AID, activation-induced cytidine deaminase; IFN, interferon; BCR, B cell receptor; CD40, B cell costimulatory receptor; IRF, IFN regulatory factor; PNA, peanut agglutinin; TNP-KLH, 2,4,6-trinitrophenyl-keyhole limpet hemocyanin; WT, wild type.

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3 Abbreviations used in this paper: AID, activation-induced cytidine deaminase; BCL-6, B cell lymphoma-6; ChIP, chromatin immunoprecipitation; FAIM, Fas apoptosis inhibitory molecule; FOB, follicular B cell; GCB, germinal center B cell; IRF, IFN regulatory factor; PNA, peanut agglutinin; TNP-KLH, 2,4,6-trinitrophenyl-keyhole limpet hemocyanin; WT, wild type.

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between 200 and 2000 bp; DNA was extracted from a fraction of the cross-linking in 1% formaldehyde for 2 min at 37°C, and quenched with /H11002 separately, (13). Briefly, primary splenic B cells (2

Chromatin immunoprecipitation (ChIP) assay

PAGE followed by immunoblotting, as previously described (12). Proteins were extracted from B cell pellets with Nonidet P-40 lysis buffer, Western blotting

Reporter plasmids

FAIM proximal promoter genomic region (1631 to 598)

Quantitative PCR

Primer Sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2-microglobulin (forward)</td>
<td>TTTTCCCGTCTTCCAGCATT</td>
</tr>
<tr>
<td>β2-microglobulin (reverse)</td>
<td>CTGACCGCCCTGTTATGCTAT</td>
</tr>
<tr>
<td>FAIM (forward)</td>
<td>TGGCAATGTCGAAAGATGGA</td>
</tr>
<tr>
<td>FAIM (reverse)</td>
<td>CGCTGCACAGCCTTTTTAG</td>
</tr>
<tr>
<td>IRF4 (forward)</td>
<td>CTACCCGCTCCAGACCATTT</td>
</tr>
<tr>
<td>IRF4 (reverse)</td>
<td>CCAAAGTCAGACAGACATT</td>
</tr>
<tr>
<td>AID (forward)</td>
<td>CAGGAAAGTGGACGAGAG</td>
</tr>
<tr>
<td>AID (reverse)</td>
<td>TCATTCTCTTTGCGACGGTCTT</td>
</tr>
</tbody>
</table>

Cloning into pGL4.10

FAIM promoter − 598–KpnI (forward) | CCGGGTGACCCCCACGCACGCTCTACCAAGGATACCAAAGC |
| FAIM promoter − 1631–KpnI (forward) | CGGCCGACCTGGACTGCTGAAGGCCC |
| FAIM promoter + 20–Khol (reverse) | ATACCCGCTGAGGTCACACAGACGGCC |

Introduction of mutation

FAIM promoter mutation A (forward) | GCAATTTTATAAATCTATCCAGTTGCTTATTTGAAAGGTGAC |
| FAIM promoter mutation A (reverse) | GTACCCCTCTATGAAAAAAGCCCTCTATGATAGATTTTTAAAATGC |
| FAIM promoter mutation B (forward) | ATGGTGCAAGATCCGCTGCTGCTCTGCTTCATTTAAAAATTAC |
| FAIM promoter mutation B (reverse) | GTAAATTATGAAAGCAGAAGACTGCAGACCTCTCATATACAATC |
| FAIM promoter mutation C (forward) | TCTTCCTTTATATATTAGGGAAGGTGAGTTTCTATCCGGAC |
| FAIM promoter mutation C (reverse) | GCAGCAAGATCCGCTGCTGCTTCATTTAAAAATTAC |

* All sequences are presented in the 5’ to 3’ direction.

CD40L was prepared and used as previously described (14–16). Affinity-purified Fab(1/2) fragments of goat anti-mouse IgM (anti-Ig) were obtained from Jackson ImmunoResearch Laboratories. Monoclonal anti-FcγR Ab 2.4G2, anti-B220-FITC, anti-B220-PE, anti-CD23-PE and anti-CD43-biotin were obtained from BD Pharmingen. PNA-FITC was obtained from BioLegend. Monoclonal anti-phosphotyrosine Ab 4G10 was obtained from Upstate Biotechnology. Affinity-purified anti-FAIM Ab was obtained from rabbits immunized with CYIKAVSSRKRKEGIIHTLI peptide (located near the C-terminal region of FAIM). Rabbit anti-IRF4 Ab was obtained from Santa Cruz Biotechnology. Anti-tubulin Ab was obtained from Calbiochem. Anti-actin Ab was obtained from Sigma-Aldrich.

Results

FAIM expression is regulated by IRF consensus sites

To identify the regulatory factors responsible for FAIM expression, we analyzed the putative Faim promoter sequence, upstream from the transcription start site, as described in the National Center for Biotechnology Information database. We found three putative IRF binding consensus motifs as previously defined (GAAAC(T/C)) (17), located between −1600 and −600 in relation to the transcription start site (depicted as sites “A”, “B”, and “C”, along with flanking sequences, in Fig. 1A), and one Sp1 binding site located near the transcription start site.

To determine whether the IRF binding sites, or the Sp1 site, play a role in regulating FAIM expression, we first constructed two Faim promoter firefly luciferase reporter vectors, −1631 to +20 and −598 to +20, and transfected these constructs, and empty vector, into the A20 B cell line. We then evaluated luciferase activity as an indication of Faim promoter function. We found that
the promoter sequence −1631/+20 was highly active, promoting a >50-fold increase in luciferase activity in comparison to luciferase activity produced by empty vector alone (Fig. 1B). In contrast, the truncated sequence −598/+20, which lacks the IRF binding sites (but contains the Sp1 site), was relatively inactive, promoting only a 3-fold increase in luciferase activity as compared with that produced by empty vector. These results indicate that the interval between −1631 and −598 (proximal to the Faim transcription start site) contains a strong enhancer region for Faim expression. We concluded that the three IRF binding sites contained within this sequence represent prime candidates for directing Faim expression.

To verify the role of the IRF binding sites, we introduced mutations into each motif individually and in combination by changing GAAAC(T/C) to GAggC(T/C) in the Faim promoter construct, because this mutation abolishes IRF binding (18). These mutant Faim promoter constructs were transfected into A20 cells, after which luciferase activity was determined. We found that mutation of one site at a time, be it site A, B, or C, had little effect on reporter activity driven by the −1631/+20 sequence (Fig. 1B). This suggests that none of the IRF binding sites is, by itself, determinative, and, furthermore, that all three sites need not be intact for normal promoter function. On the other hand, mutation of all three sites produced marked loss of promoter function (Fig. 1C). This indicates that the IRF sites are, in some combination, responsible for the promoter activity of the Faim −1631/+20 construct.

In separate experiments these same Faim promoter constructs were transfected into two additional B cell lines, BRD2 (19) and BAL-17 (20), yielding results that faithfully reproduced our findings with A20 cells in all key aspects (the −1631/+20 construct was highly active, the −598/+20 construct was little active, the ABC mutant lost virtually all activity, and mutation of either A, or B, or C sites had little or no effect; data not shown). Thus, our findings with these key Faim promoter constructs are not limited to A20 cells.

Next, we introduced mutations into the three IRF binding sites two at a time and evaluated promoter activity of the doubly mutated −1631/+20 promoter sequences in A20 cells. We found that mutation of two sites markedly reduced resultant luciferase activity; moreover, this was true regardless of which two sites were mutated (Fig. 1C). Thus, mutation of the AB, the
AC, or the BC sites substantially blocked promoter function in each case, with mutation of the AC and BC sites having the largest effect. Thus, these results strongly suggest that two (of the three) IRF sites act together to drive full activation of the FAIM promoter.

Among the nine-member IRF family, IRF4 is most likely to play a role in activated B cells (21, 22), for which reason we introduced a CMV-driven IRF4 expression vector together with the aforementioned luciferase vectors into A20 cells. We reasoned that introduction of promoter-reporter plasmids into A20 cells might produce an excess of binding sites over available, endogenous IRF4, in which case introduction of IRF4, if it plays a role, would act to enhance reporter gene activity. In fact, cotransfection of CMV-driven IRF4 increased luciferase activity produced by the native Faim −1631/+20 promoter by 1.5- to 2-fold, providing further evidence of a role for the IRF4 binding sites within the −1631/+20 sequence. Conversely, as might be expected, coexpression of IRF4 had little effect on the Faim promoter construct in which all three IRF binding sites had been mutated. These data suggest that IRF4 regulates Faim gene transcription in a positive manner.

**IRF4 expression precedes FAIM expression in stimulated B cells**

We previously showed that FAIM expression is up-regulated by anti-Ig stimulation of CD40L-pretreated murine splenic B cells (1). To further evaluate the correlation between IRF4 and FAIM, we examined the time course of Irf4 and Faim mRNA and protein expression in stimulated primary B cells. As expected, we found that Faim gene expression was induced by anti-Ig stimulation of CD40L-pretreated cells; Faim mRNA was increased over baseline within 3 h and peaked at 6 h, declining somewhat by 18 h. We found that Irf4 gene expression was induced and peaked earlier, preceding induction of Faim; Irf4 mRNA was markedly increased over baseline at 1 h, which was also the time of peak induction, and had declined to background levels by 18 h (Fig. 2A). Similar results were obtained when we monitored IRF4 and FAIM protein expression by Western blotting. As with mRNA expression, we found that Irf4 protein was induced hours before FAIM protein when CD40L-treated B cells were stimulated with anti-Ig (Fig. 2B). Thus, the time course of stimulated IRF4 and FAIM expression in primary B cells is consistent with a model in which IRF4 plays a key role in directing FAIM expression.

**IRF4 binds to the FAIM promoter in vivo**

To directly test the role of IRF4 in regulating the Faim promoter, we carried out ChIP in primary (murine splenic) B cells pretreated with CD40L and then stimulated with anti-Ig to induce FAIM expression. With separate primers we evaluated the Iga 3′ enhancer region as a positive control (23). As expected, we found that IRF4 bound to the Iga 3′ enhancer region (data not shown). Importantly, we found that IRF4 bound to sites within the Faim promoter after, but not before, anti-Ig was added to CD40L-treated B cells (Fig. 3), the same conditions required for induction of FAIM expression. Thus, IRF4 binding to the Faim promoter is induced specifically by conditions that induce FAIM expression in primary B cells, indicating that Faim is an IRF4 target gene and further implicating IRF4 in the regulation of FAIM expression.

**Up-regulation of Faim expression requires IRF4**

To further test the role of IRF4 in regulating the Faim promoter, we evaluated stimulated Faim mRNA expression in follicular B cells sort-purified from the spleens of Irf4-null mice and littermate control mice. B cells were cultured with CD40L for 48 h alone or were cultured with CD40L for 48 h, during which time anti-Ig was added for the last 6 h before the end of the 48-h culture period. We then determined Faim gene expression in these cells by real-time PCR. As expected, we observed that Faim mRNA expression was enhanced (by 2.4-fold) in CD40L- and anti-Ig-stimulated wild-type (WT) B cells (Fig. 4A). In contrast, enhancement of FAIM expression by anti-Ig stimulation was substantially

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**FIGURE 2.** Stimulated FAIM expression is preceded by induction of IRF4 expression in primary B cells. A, Primary B cells were stimulated by CD40L for 48 h alone (NS), or were stimulated by CD40L for 48 h during which time anti-Ig, LPS, or IL-4 was added for the final number of hours indicated. From these cells, RNA was prepared, reverse transcribed, and evaluated for Faim and Irf4 gene expression by real-time PCR. Expression levels were normalized to β2-microglobulin. Mean values for two independent experiments are shown, along with lines corresponding to the SEM. B, Primary B cells were stimulated by CD40L for 48 h alone (0 h), or were stimulated by CD40L for 48 h during which time anti-Ig was added for the final number of hours indicated. From these cells, lysates were prepared and evaluated for expression of FAIM-S and IRF4 by Western blotting. Blots were stripped and reprobed for tubulin content as a loading control. One of two comparable experiments is shown.

**FIGURE 3.** IRF4 binds the Faim promoter in stimulated primary B cells. Primary B cells were stimulated by CD40L for 48 h alone (0 h) or were stimulated by CD40L for 48 h during which time anti-Ig was added for the final number of hours indicated and ChIP assay was carried out. Cells were lysed and DNA was sheared by sonication, after which chromatin was immunoprecipitated with anti-IRF4 Ab or control IgG, and Faim promoter sequence was amplified by real-time PCR. Results are expressed as the degree of amplification relative to input DNA. Mean values for two independent experiments are shown, along with lines corresponding to the SEM.
impaired in IRF4-null B cells amounting to less than a third that of WT B cells (\(p/H110210.01, n/H110053\)) (Fig. 4A). To rule out the possibility that BCR signaling is blocked in IRF4-null B cells, we examined general protein tyrosine phosphorylation. We found no difference in BCR-triggered inducible phosphorylation between IRF4-null B cells and WT B cells (Fig. 4B). Thus, inducible expression of \textit{Faim} largely fails in the absence of IRF4, indicating that IRF4 is required for normal up-regulation of \textit{Faim} gene expression in primary B cells.

It has been reported that FAIM mRNA expression is elevated in human germinal center B cells (24). To examine whether FAIM is expressed in mouse germinal center B cells (GCB), we sort-purified germinatinal center (B220\(^*\), PNA\(\text{high}\), GL7\(\text{high}\)) and follicular B cells (FOB; B220\(^*\), PNA\(\text{low}\), GL7\(\text{low}\)) from mouse splenocytes immunized with TNP-KLH in alum. Isolated GCB and FOB were >94% and >99% pure, respectively (Fig. 5A). We then analyzed GCB for \textit{Aid} and \textit{Faim} gene expression. We found that \textit{Aid}, a marker of GCB, was highly expressed in GCB but little expressed in FOB, confirming the efficiency of sort purification. Using the same cDNA samples, we found that \textit{Faim} expression was up-regulated in GCB (by ~4–5-fold) as compared with FOB (\(p < 0.01, n = 5\)) (Fig. 5B). We further monitored FAIM protein expression in these populations by Western blotting and found that FAIM expression was increased in GCB as compared with FOB at the protein level (Fig. 5C). These data indicate that FAIM mRNA and protein expression are preferentially up-regulated in mouse GCB.

\section*{Discussion}

FAIM affects B cells in two important ways: (1) FAIM opposes Fas-mediated apoptosis in B cells, an activity emphasized by the reported Fas sensitivity of FAIM-null immune cells, which in turn demonstrates the nonredundant nature of FAIM function; and (2) FAIM enhances CD40 signaling in B cells, augmenting NF-\(\kappa\)B activation, BCL-6 loss, and IRF4 expression, as well as boosting the plasma cell population (1, 6, 7). These immune system effects, along with reported activities in neuronal cells and other cell types, have heightened interest in elucidating how FAIM expression is regulated in vivo. To address this important issue we examined the Faim upstream region computationally and experimentally. We concluded that Faim promoter activity is regulated by IRF4 because: (1) the Faim promoter contains 3 IRF binding sites; (2)
Faim promoter activity is lost following deletion or mutation of all three IRF binding sites (Fig. 1); (3) Faim promoter activity is enhanced by concurrent expression of IRF4 (Fig. 1); (4) IRF4 expression precedes FAIM expression in primary B cells stimulated ex vivo (Fig. 2); (5) IRF4 binds directly to the Faim promoter in primary B cells in vivo (Fig. 3); and (6) absence of IFN-γ impairs induction of FAIM expression in primary B cells ex vivo (Fig. 4). Because FAIM enhances CD40-induced IRF4 expression in B cells, our results in this study suggest that induction of FAIM initiates a positive reinforcing (i.e., feed-forward) system in which IRF4 expression is both enhanced by FAIM and promotes FAIM expression. It may be speculated that once induced, FAIM can then perpetuate itself through enhancement of IRF4 expression, until such point as, through mechanisms unknown at the present time, FAIM expression declines, CD40 signaling ceases, or B cells become fully differentiated. The FAIM-influenced level of IRF4 would then be expected to determine B cell fate, inasmuch as the level of IRF4 has been shown to determine the level of plasma cell differentiation (23), which we have demonstrated elsewhere is enhanced by FAIM (7).

All three IRF binding sites in the Faim promoter appear to be active. A similar situation exists in the IL-4 promoter, in that all of the three tandem IRF1 and IRF2 binding sites are functional for IL-4 gene repression induced by IFN-γ in T cells (25). In the Faim promoter, however, individual IRF sites alone produce little promoter activity, whereas two promoter sites together generate virtually full activity and there is little preference for one combination over another; that is, any two will do. The mechanism for this remains unclear, particularly in view of the varied intersite distances involved with various binding site combinations, but presumably some interaction is required. It would appear that IRF4 is not the sole determinant of FAIM expression because FAIM is poorly stimulated by CD40L alone, which induces IRF4 expression, but is instead strongly stimulated by the combined action of CD40L and anti-Ig, and because FAIM is expressed in germinal center B cells (Fig. 5) but not in CD138+ plasma cells (data not shown), which abundantly express IRF4 (26). In addition, it is important to note that non-IRF4 factors could be involved in regulating FAIM expression. For example, the potential participation of long-range elements beyond −1631 has not been ruled out. Furthermore, elements within −1631 to +20 could play a role, particularly in terms of the constitutive level of FAIM that was not affected by loss of IRF4. In preliminary experiments, we carried out EMSA binding assays using as probes seven different 240-bp sequences that spanned the region −1631 to +49, and we found evidence for binding with at least one probe (−671/−423), although it is impossible at this stage, without knowledge of a target motif, to rule out nonspecific binding. However, if this binding does represent a specific nucleoprotein complex, the factor involved most likely plays a role only in constitutive IRF4 expression insomuch as binding did not change with B cell stimulation for FAIM induction (data not shown).

Like FAIM, activation-induced cytidine deaminase (AID) is expressed in GC B (27) but not in plasma cells. AID is responsible for somatic hypermutation within germinal centers, resulting in the generation of Ig molecules that express a spectrum of binding affinities for specific Ag (28). The phenomenon of affinity maturation depends on positive selection of B cells bearing high-affinity Ag-binding Ig for further development and differentiation and on negative selection of outcompeted B cells bearing low-affinity Ig. Positive B cell selection within germinal centers depends on signals generated by BCR and CD40 engagement (29), signals that together lead to induction of FAIM, which is shown here and elsewhere to be up-regulated in GCB. FAIM in turn appears to foster the continued development of Ag-binding B cells in two ways, both of which may depend on the ability of FAIM to enhance CD40 signaling for NF-κB (7). On the one hand, FAIM enhances up-regulation of FLIP expression (data not shown and Ref. 6), which opposes death receptor-mediated apoptosis (30). On the other hand, FAIM enhances down-regulation of BCL-6 and up-regulation of IRF4, which propel B cell differentiation (7). Our new finding that FAIM expression is regulated by IRF4, levels of which are enhanced by FAIM, suggests a mechanism whereby, once induced, FAIM promotes its own expression, resulting in a stable push in favor of continued GCB development and differentiation.

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Disclosures
The authors have no financial interests of conflict.

References


