Interferon consensus sequence binding protein (ICSBP), a transcription factor of the interferon (IFN) regulatory factor (IRF) family, binds to the IFN-stimulated response element (ISRE) in the regulatory region of IFNs and IFN-stimulated genes (ISG). To identify target genes, which are deregulated by an ICSBP null-mutation in mice (ICSBP⁻/⁻), we have analyzed transcription of an ISRE-bearing gene, ISG15. We have found that although ISG15 expression is unchanged in B cells, it is upregulated in macrophages from ICSBP⁻/⁻ mice. Three factors, ICSBP, IRF-2, and IRF-4/Pip interact with the ISRE in B cells, however only ICSBP and IRF-4/Pip were found to bind this sequence in macrophages of wild-type mice. Although IRF-4 was considered to be a lymphoid-specific factor, we provide evidence for its role in macrophage gene regulation. Our results suggest that the formation of cell-type-specific heteromeric complexes between individual IRFs plays a crucial role in regulating IFN responses.

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plastic overnight in complete RPMI 1640 medium (10% fetal calf serum, 1.5 mmol/L L-glutamine, 100 U/mL penicillin/streptomycin, nonessential amino acids (GIBCO-BRL, Gaithersburg, MD), and 50 mmol/L 2-mercaptoethanol) at 37°C in 5% CO2, and nonadherent cells were removed before further use. The macrophages used in our preparations were checked for B-cell or other cell contamination, and they were confirmed to be composed of over 95% macrophages with no detectable lymphocytes. CD19+ B lymphocytes were isolated from mouse spleens using magnetic analyzed cell sorting (MACS) as described.14 The K562 and A 20.2j cell lines were maintained in complete RPMI 1640 medium.

Antibodies. Generation of ICSBP antiserum (designated S 183): the peptide ECGSIEEELKES corresponding to residues 137–151 of murine ICSBP (no homology to any known IRF family member) was cysteine-conjugated to keyhole limpet hemocyanin and injected into rabbits followed by booster immunizations. Rabbits were bled sequentially and sera assayed for specific Ig via Western blot. High-titer serum obtained 14 days after the second boost was used in a routine immuneblot at a 1:2,000 dilution. The antiserum was affinity-purified by chromatography on antigenic peptide immobilized to Sulfolink (Pierce, Rockford, IL) according to the instructions of the manufacturer. Column-bound antibody was washed sequentially in buffers containing 150 mmol/L NaCl at pH 7.5, 6.0, and 5.0, and eluted at pH 3.0, followed by immediate realkalinization. Specificity was documented by competition with antigenic peptide. No ICSBP was detected in ICSBP-deficient cells. Antibodies against IRF-1, IRF-2, IRF-4/Pip, and horseradish-conjugated anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Synthetic oligonucleotides. The sequence of the ISG15 ISRE oligonucleotide used in binding assays is 5'-GATCCTCGGGAAGGG-GAACAAGAATGGGACCTA-3'. The sequences for the mutated ISRE oligonucleotides are: M1-5'-GATCTCCTGGGAAGGTTACAGCAGGAGG-3'; M2-5'-GATCTCCTGGGAAGGTTACAGCAGGAGG-3'. The sequences of the human ISRE oligonucleotides are: M1-5'-GATCTCCTGGGAAGGTTACAGCAGGAGG-3'; M2-5'-GATCTCCTGGGAAGGTTACAGCAGGAGG-3'. The results show that the expression level of the ISG15 gene in ICSBP−/− mice. To investigate whether the lack of ICSBP affects the expression of ISRE-containing genes, we have analyzed the transcription of the ISG15 gene in primary B cells and peritoneal macrophages. Both cell types are known to express ICSBP constitutively in wild-type mice. ISG15 is a prototype gene containing an ISRE-sequence that has been used in several previous studies.5,11

RESULTS

Cell type-specific disregulation of the ISG15 gene in ICSBP−/− mice. To investigate whether the lack of ICSBP affects the expression of ISRE-containing genes, we have analyzed the transcription of the ISG15 gene in primary B cells and peritoneal macrophages. Both cell types are known to express ICSBP constitutively in wild-type mice. ISG15 is a prototype gene containing an ISRE-sequence that has been used in several previous studies.5,11

The results show that the expression level of the ISG15 gene was consistently 3-fold to 5-fold higher in macrophages from ICSBP−/− than in wild-type mice (Fig 1A). In contrast to the situation in macrophages, in B cells, the expression of the ISG15 was essentially the same in wild-type and ICSBP−/− mice (Fig 1B). The results indicate that in the ICSBP−/− mice, there is a cell type-specific disregulation of the ISG15 gene.

ICSBP and IRF-4/Pip form complexes that bind to the ISG15 ISRE in B cells and macrophages. We analyzed whether an altered formation of DNA protein complexes is directly responsible for the observed disregulation of ISG15 expression in ICSBP−/− mice. Mobility shift assays were performed using the ISRE sequence from the ISG15 gene promoter and protein extracts from primary spleen B cells and peritoneal macrophages isolated from ICSBP+/+ and ICSBP−/− mice.

Figure 2A shows the result of a mobility shift assay obtained by incubating the ISG15 ISRE with nuclear extracts from sorted B cells of wild-type and ICSBP-deficient mice. Two complexes, designated B1 and B2, were seen in B-cell nuclear extracts from wild-type mice (Fig 2, lane 2). When an antibody against ICSBP was added to the binding reaction, the slower migrating B1 complex disappeared. These results are consistent with the hypothesis that ICSBP and IRF-4/Pip form ISRE-specific protein complexes.
complex disappeared completely, and a supershifted band could be seen, indicating that ICSBP binds to the ISG15 ISRE in B-cell extracts (Fig 2, lane 3). The addition of an antibody against IRF-2 resulted in a supershift of both B1 and B2 complexes (Fig 2, lane 4). It has been previously shown that IRF-2 can bind to the ISRE by itself, and it is very likely that B2 represents IRF-2 binding alone to the ISRE, whereas the slower migrating B1 complex is composed of IRF-2 complexed to ICSBP and/or other ISRE binding factors. Remarkably, the addition of an antibody against IRF-4/Pip also caused a complete supershift of the B1 complex, which contained ICSBP and IRF-2 (Fig 2, lane 5). Our results suggest that in B cells, ICSBP, IRF-2, and IRF-4/Pip form a ternary complex that binds to the ISG15 ISRE, which is of interest because it has not been shown previously that ICSBP complexes with IRF-4/Pip.

Incubation of the ISG15 ISRE with B-cell nuclear extracts from ICSBP-deficient mice also resulted in the formation of the 2 binding complexes, B1 and B2, as well as a third faster migrating band labeled B3, which has not yet been characterized (Fig 2A, lane 7). As seen in Fig 2, lanes 9 and 10, IRF-2– and IRF-4/Pip–specific antibodies both gave rise to a complete supershift of the B1 complex, indicating that IRF-4/Pip and IRF-2 form a ternary complex with ISRE in the absence of ICSBP. We did not detect the IRF-2 protein in spleen cells from ICSBP−/− mice in our previous experiments. This difference is probably due to an improved preparation of extracts from primary cells, which contain high concentrations of proteases. Taken together, the above results show that ICSBP, IRF-2, and IRF-4/Pip all bind to the ISG15 ISRE in B-cell nuclear extracts and suggest a complex formation between all 3 proteins.

Different protein-DNA complexes were observed in nuclear extracts from macrophages. Only 1 ISRE-binding complex, labeled M1, which migrates at the same position as B1 in B-cell extracts, was detected in macrophage extracts from wild-type mice. This complex was supershifted with the ICSBP antibody (Fig 2, lane 3). Unlike the results in B cells, despite its presence (Fig 3C), no IRF-2 binding was seen in nuclear extracts from macrophages (data not shown). However, the addition of an IRF-4/Pip–specific antibody did result in a supershift of the M1 band, indicating that IRF-4/Pip binds to the ISG15 ISRE in macrophages (Fig 2B, lane 2). Previous studies using cell lines had suggested that IRF-4/Pip expression is limited to lymphoid cells. The observation that IRF-4/Pip is present in macrophages suggests that IRF-4/Pip expression is not as restricted as previous results indicated. Similar to the results in B cells, the results in macrophages suggest that a complex is formed between ICSBP and IRF-4/Pip and implicate IRF-4/Pip as a new binding partner for ICSBP. Analyses of protein DNA complexes in macrophage nuclear extracts from ICSBP−/− mice showed the absence of the M1 complex. Antibodies against ICSBP and IRF-4/Pip did not result in a supershift (Fig 2B, lanes 5 and 6). These results suggest a cell-specific complex formation of the IRFs on the ISG15 promoter.

The IRF-4/Pip mRNA and protein is expressed in both wild-type and ICSBP−/− mouse macrophages. The absence of IRF-4/Pip binding to the ISRE in ICSBP−/− macrophages would suggest that either IRF-4/Pip is not present or that in macrophages, it requires the presence of ICSBP to bind the ISRE. To distinguish between these 2 possibilities, we analyzed IRF-4/Pip mRNA and protein expression in macrophages by RT-PCR and by immunoprecipitation followed by Western blotting. Figure 3A and B shows that IRF-4/Pip mRNA and
protein are present in wild-type and ICSBP−/− macrophages, suggesting that IRF-4/Pip requires the presence of ICSBP to bind to the ISG15 ISRE.

**ICSBP and IRF-4/Pip form stable complexes in vivo in the absence of DNA.** Because IRF-4/Pip is unable to bind to the ISG15 ISRE in macrophages in the absence of ICSBP, it is likely that these 2 proteins form a complex in macrophages. However, in B cells, IRF-4/Pip binds to the ISG15 ISRE in the absence of ICSBP; thus it is not clear if these 2 proteins complex in B cells or if, as in the case with PU.1, ICSBP and IRF-4/Pip compete for IRF-2 binding. To determine if ICSBP and IRF-4/Pip form a complex in B cells, we depleted ICSBP from wild-type B-cell nuclear extracts and used the depleted extracts in a mobility shift assay. ICSBP, IRF-4/Pip, and IRF-2 bound to the ISG15 ISRE in mock-depleted extracts (Fig 4A, lanes 1 to 4). However, when ICSBP was depleted from the B-cell wild-type extracts, binding of IRF-4/Pip to the ISG15 ISRE was abrogated, although IRF-2 binding is still present (Fig 4A, lanes 7 and 8). In addition, the slower migrating complex B1, which contains ICSBP, IRF-2, and IRF-4/Pip antibody; lanes 2 and 5, anti-IRF-4/Pip antibody. The arrow shows the specific complex binding to the ISG15 ISRE. The slowly migrating bands seen in the ICSBP−/− extracts are nonspecific bands. (C) Western blot indicating the presence of IRF-2 in mouse macrophages.

**Fig 2.** ICSBP and IRF-4/Pip form a complex on the ISG15 ISRE. (A) ICSBP, IRF-2, and IRF-4/Pip form a complex on the ISG15 ISRE in B lymphocytes. Mobility shift assay with the ISG15 ISRE probe and B-lymphocyte nuclear extracts from wild-type (lanes 1 to 5) and ICSBP-deficient mice (lanes 6 to 10). Lanes 1 and 6, competition with unlabelled ISG15 ISRE oligonucleotides; lanes 3 and 8, anti-ICSBP antibody; lanes 4 and 9, anti-IRF-2 antibody; lanes 5 and 10, anti-IRF-4/Pip antibody. The position of the bands, B1, 2, and 3, formed on the ISG15 ISRE are indicated with arrows. (B) IRF-4/Pip is present in macrophages and forms a complex with ICSBP. Mobility shift assay showing complex formation of the ISG15 ISRE and proteins from primary macrophage nuclear extracts from wild-type (lanes 1 to 3) or ICSBP-deficient mice (lanes 4 to 6). Lanes 3 and 6, anti-ICSBP antibody; lanes 2 and 5, anti-IRF-4/Pip antibody. The arrow shows the specific complex binding to the ISG15 ISRE. The slowly migrating bands seen in the ICSBP−/− extracts are nonspecific bands. (C) Western blot indicating the presence of IRF-2 in mouse macrophages.
DNA. The existence of such a complex in both B cells and macrophages was confirmed by coimmunoprecipitation, followed by Western blotting (Fig 4B and C).

The number and spacing of the consensus ISRE sites within the ISG15 ISRE motif are critical for ICSBP and IRF-4/Pip binding. As shown previously, the consensus binding site for the ISRE is GAAANN. The ISG15 ISRE is somewhat unique in that it contains 3 consensus binding sites next to each other with no spacing in between. To determine if the number and spacing of the ISRE consensus sites is important for IRF binding, we made a series of mutations in the ISG15 ISRE, either mutating or inserting base pairs between the consensus sites (Table 1). These oligonucleotides were used for mobility shift assays with macrophage and B-cell nuclear extracts from wild-type mice. When the mutated ISG15 ISRE oligonucleotides GG1 or M4 (Table 1) were used in a mobility shift assay, the binding of ICSBP, IRF-2, and IRF-4/Pip strongly decreased. ICSBP, IRF-2, and IRF-4/Pip were unable to bind to the oligonucleotides, M1-3, M5, and GG2, which have a disruption in the middle or the 3' ISRE consensus sites. The results of these experiments suggest that the middle and 3' consensus ISRE sites are more crucial for binding of the ICSBP protein complexes than the 5' ISRE. Nonetheless, binding of the IRF family protein complexes was significantly weaker when any of the ISRE consensus sites was mutated.

A similar result was seen in mobility shift assay with the mutated ISRE oligonucleotides and macrophage nuclear extracts. Using the mutated ISRE oligonucleotides GG1 or M4, the binding of both ICSBP and IRF-4/Pip was reduced. Similar to the results in B cells, the mutated oligonucleotides, M1-3, M5, or GG2, were unable to bind ICSBP or IRF-4/Pip (Table 1). The results in both B cells and macrophages show that the number and spacing of the ISRE consensus sites are crucial for generation of the ICSBP and IRF-4/Pip complexes.

**DISCUSSION**

We have shown that the ISG15 gene promoter is a target for several IRFs that form different heteromeric complexes in macrophages and in B cells. While 3 IRF members, ICSBP, IRF-2, and IRF-4/Pip, interact with the ISRE in B cells, only ICSBP and IRF-4/Pip were found to bind this sequence in macrophages from wild-type mice. The presence of IRF-4/Pip in macrophages was surprising and suggests that IRF-4/Pip expression is not as restricted as previously indicated.

Mutational analyses of the ISRE indicated that the sequence requirements for binding of the ICSBP and IRF-4/Pip complex are highly specific. The strongest binding of the complex is seen with the native ISG15 sequence, which is composed of 3 consensus ISRE sequences placed together with no intervening base pairs. Therefore, it is likely that only a limited number of genes with ISRE promoter elements are regulated by the ICSBP and IRF-4/Pip complex.

Both ICSBP and IRF-4/Pip do not bind strongly to the ISRE, and it has been reported that complex formation with other transcription factors improves their binding significantly. Both ICSBP and IRF-4/Pip binding to the ISRE of the lambda B site is dependent on the presence of the ets family member, PU.1. In contrast, the other IRF proteins, IRF-1, IRF-2, and ISGF3γ, bind to the same site independently from PU.1. The binding of ICSBP to the ISG15 ISRE in vitro is enhanced by IRF-1 or IRF-2. Although ICSBP and IRF-4/Pip are closely related by sequence homology, cooperative binding between IRF-4/Pip
and IRF-1 or IRF-2 has not yet been reported. Therefore, it was somewhat surprising to find IRF-4/Pip complexing with 2 other IRF-members, ICSBP and IRF-2. The above results extend previous observations indicating a high frequency of heterocomplex formation within the IRF family.11

Our observations define novel interactions between IRF-4/Pip and ICSBP and show that these 2 factors downregulate the transcription of an ISRE-promoter from the ISG15 gene upon its transfection into monocytic cells. The fact that the expression of ISG15 is enhanced in macrophages from ICSBP−/−

Table 1. Binding of IRF Complexes to Mutated ISG15 ISRE Oligonucleotides

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>B Lymphocyte</th>
<th>Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISRE</td>
<td>GATCCCTCGGAAAGGAAACCGAATGGAAGCC</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M1</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M2</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M3</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M4</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M5</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>GG1</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GG2</td>
<td>GATCCCTCGGAAAGGAGCAGCCTCAGAATGGAAGCC</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Abbreviations: ++, strong binding; +, weak binding; −, no binding.

*The presence of individual IRF proteins was concluded from positive supershifts with the corresponding antibody. The mobility of protein complexes formed with mutated oligonucleotides is in part altered.
mice, in which the ICSBP and IRF-4/Pip complex binding to the ISRE is absent, strongly suggests that these factors also regulate ISG15 expression in physiological conditions. Thus, the transcriptional repressor ICSBP, which is strongly induced by γIFN, could mediate γIFN responsiveness by interacting with a variety of transcription factors, which by themselves do not respond to γIFN.

The identification of IRF-4/Pip as a new binding partner of ICSBP is of considerable interest. Cotransfection experiments suggested that ICSBP is a negative regulator of genes induced by interferons. IRF-4/Pip may function in controlling both the transcriptional activity and the recombinational specificity of immunoglobulin light-chain genes in B cells. Finally, the evidence that ICSBP and IRF-4/Pip are important and nonredundant transcriptional regulators in vivo was provided by analyses of knock-out mice. The lack of either of the factors causes profound changes in the development and function of the hematolymphoid system. Our finding of IRF-4/Pip expression in macrophages and its cooperative interaction with ICSBP provokes the question on the additional roles of IRF-4/Pip in macrophages. Whether macrophage functions are altered in IRF-4/Pip-deficient mice has not yet been investigated.

Interferons are pleiotropic regulators of defense mechanisms against pathogens, immune responses, and cell growth. The production of α/β-interferons is triggered by many external stimuli (eg, viral infection). ISG15 has been described as an α/β-interferon–stimulated cytokine secreted by macrophages and lymphocytes that augments γ-interferon production in lymphocytes. γ-Interferon is a key regulator of inflammatory responses, and its uncontrolled activity may lead to deleterious pathological changes. Previous observations and results presented here suggested that 1 possible mechanism terminating γ-interferon responses is the ICSBP and IRF-4/Pip–mediated downregulation of ISG15.

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Interferon Consensus Sequence Binding Protein and Interferon Regulatory Factor-4/Pip Form a Complex That Represses the Expression of the Interferon-Stimulated Gene-15 in Macrophages

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