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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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.

TRANSCRIPTION FACTORS of the interferon regulatory factor (IRF) family are interferon primary response genes regulate expression of a broad spectrum of secondary response genes.[1,2] The best characterized members of this family, IRF-1, IRF-2,[3] interferon-stimulated gene factor 3 (ISGF3)γ,[4] interferon consensus sequence binding protein (ICSBP),[5] and IRF-4/Pip[6-8] contain a variable C-terminal domain and a conserved N-terminal DNA-binding domain and bind to an element known as the ISRE (interferon-stimulated response element), which has a consensus sequence GAAANN.[9] The ISRE motif is present in the regulatory regions of interferons and of interferon secondary response genes, named also interferon-stimulated genes (ISG). The biological function of the majority of ISGs is not yet well understood.[1,2] Recently, ISG15 was shown to be an α/β-interferon–induced cytokine, which stimulates natural killer (NK) cell proliferation and augments γ-interferon production in lymphocytes.[10]

ICSBP is known as a negative regulator of IFN and IFN-induced genes.[11] We recently generated ICSBP-deficient mice (ICSBP−/−) by gene targeting. These mice are immunodeficient and develop disease symptoms similar to human chronic myelogenous leukemia (CML).[12,13] The main alterations compared with wild-type mice are higher counts of myeloid and B-lymphoid cells and an enhanced number of immature cells in the periphery and in hematopoietic organs. Whether ICSBP also plays a role in human CML is not yet known. Interestingly however, a lack or strongly reduced expression of ICSBP was found in 79% of CML and 66% of AML patients.[14]

A critical role of ICSBP in macrophage-mediated host defense was documented by a high sensitivity of ICSBP−/− mice to various pathogens.[13,15,16] Thus, ICSBP seems to play 2 distinct roles, 1 during development of hematopoietic cells and a second in regulating immune response. ICSBP might modulate gene transcription by a direct binding to regulatory DNA sequences or by its interaction with other transcription factors. In vitro, it has been shown that ICSBP interacts with 2 other members of the IRF family, IRF-1 and IRF-2,[11] as well as with the ets family factor PU.1.[6] Similar to ICSBP, PU.1 has also been shown to be crucial for early hematolymphoid development.[17]

In contrast to the ubiquitous expression of IRF-1 and IRF-2, the expression of ICSBP and IRF-4/Pip is more restricted. ICSBP is expressed in monocytic and lymphoid cells.[9] The expression of the IRF-4/Pip gene was reported to be predominantly in B lymphocytes and only weakly in T cells.[8,18] Of the IRF family members, IRF-4/Pip is the most closely related to ICSBP by sequence homology. Interestingly, both ICSBP and IRF-4/Pip bind only weakly to the ISRE and only in the presence of other DNA binding proteins. IRF-4/Pip was initially recognized as part of a ternary complex between PU.1 and a domain of the immunoglobulin light chain enhancer.[18,19] In conjunction with PU.1, IRF-4/Pip specifically stimulates transcription of immunoglobulin light chains.[20]

In contrast to previously published results, we show here that IRF-4/Pip is not only a lymphoid-restricted factor, but is also expressed in macrophages. Furthermore, we provide evidence that in both macrophages and B cells, IRF-4/Pip forms a complex with ICSBP, which binds to the ISRE sequence. This complex negatively regulates the expression of an IFN-inducible gene, ISG15, in a cell-specific manner.

MATERIALS AND METHODS

Mice and cells. ICSBP mutant mice were generated as described.[12] All experiments were performed with 3-month old homozygous and wild-type mice on a C57Bl/6 X 129/Sv F2 background. Peritoneal wash out cells were harvested from mice injected 4 days previously with thioglycollate medium.[16] The cell suspension was allowed to adhere to

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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% CO_2, 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 ECGRSEIEELIKEPS 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 immunoblot 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−/− cells. Antibodies against IRF-1, IRF-2, IRF-4/Pip, and horseradish-conjugated antigoat 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′GATCCTCGGGAAAGGGAAACCGAAACTGAAGCC3′.[11] The sequences for the mutated ISRE oligonucleotides are: M1-5′GATCCTCGGGAAAGGCGTACCGAAACTGAAGCC3′; M2-5′GATCCTCGGCGTAGGCGTACCGAAACTGAAGCC3′; M3-5′GATCCTCGGCGTAGGCGTACCCGTACTGAAGCC3′; M4-5′GATCCTCGGCGTAGGGAAACCGAAACTGAAGCC3′; M5-5′GATCCTCGGGAAAGGGAAACCCGTACTGAAGCC3′; GG1-5′GATCCTCGGGAAAGGGGGAAACCGAAACTGAAGCC3′; GG2-5′GATCCTCGGGAAAGGGAAACCGGGAAACTGAAGCG3′.

The sequences of the murine IRF-4/Pip primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) are: upper strand-5′CTCAGAGTTCGGCATGAGCGCA3′; lower strand-5′CTCCAGCTCCTGTCATGGGG3′, whereas the sequences of the human IRF-4/Pip primers are: upper strand-5′GCAACGACCGGCCCAACAAAC3′; lower strand-5′GCAAGACCCCGTATCCCCGTATCA3′. These primers do not cross-react with the ICSBP gene. The sequences of the ISG15 RT-PCR primers are: upper strand-5′TGGCCTGGGACCTAAAGGTGAAGA3′; lower strand-5′TGCACTGGGGCTTTAGGCCATACT3′.

RT-PCR analysis. RT-PCR analysis was performed according to the methods of Schmidt et al.[14] For the ISG15 gene analyses in B lymphocytes, 30 cycles were used, whereas in macrophages, 25 cycles of the following parameters were used: 24 seconds, 94°C; 36 seconds, 60°C; 36 seconds, 72°C.

Gel electrophoretic mobility shift assay. Nuclear extracts were harvested according to the method of Schreiber et al.[21] Mobility shift assays were performed by incubating 5 to 10 μg of nuclear extract in 6 mmol/L HEPES, pH 7.9, 30 mmol/L potassium chloride, 6% glycerol, 0.1 mmol/L EDTA, 0.3 mmol/L dithiotreitol (DTT), 2 μg salmon sperm, and 20 μg bovine serum albumin for 10 minutes on ice. After this, 2 ng of labeled probe was added and the samples were incubated at room temperature for 10 minutes. One microgram of the antibody was added to the reactions before the addition of the probe for the supershift reactions. To exclude unspecific binding, a 200-fold excess of the unlabelled ISG15 oligonucleotide was used. After incubation, the reaction mixtures were run on a 1X TBE gel at 4°C.

Transfections. K562 cells were transfected by electroporation exactly according to the conditions of Waring et al.[22] K562 cells were transfected with 15 μg of pGL-ISG15p and 15 μg of pcDNA or pcDNA-ICSBP or pcDNA-IRF-4. In addition, cells were transfected with 3 μg of the internal control pRL-TK. The relactive light units from the pGL-ISG15p vector were standardized relative to the expression of pRL-TK. Vectors pRL-TK, pcDNA, and pGL3-Promoter were purchased from Promega (Madison, WI). The pGL3-Promoter vector had 150 bp of the ISG15 promoter cloned into the SmaI restriction site. The ICSBP expression vector was made by inserting an EcoRI fragment of the ICSBP cDNA, which includes all of the coding exons into the vector pcDNA. The IRF-4/Pip expression vector was a gift from Drs H.-W. Mittrücker and Tak W. Mak (Department of Immunology, University of Toronto, Toronto, Ontario, Canada).

Western blotting and immunoprecipitation. A total of 3×10^7 cells were washed twice with phosphate-buffered saline (PBS) and lysed in 1 mL ristocetin-induced platelet agglutination (RIPA) buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 200 mmol/L Pefabloc SC, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin) and were precleared for 1 hour with 40 μL agarose. Immunoprecipitations were performed with 750 μg whole cell lysate in 500 μL RIPA buffer using 2 μg anti-ICSBP (rabbit) or anti-IRF-4/Pip (goat) at 4°C overnight. Immunocomplexes were separated by 40 μL A/G-Sepharose (2 hours, 4°C) and were analyzed by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes, and immunoblottings were performed with anti-IRF–4/Pip (1:2,000) or anti-ICSBP (1:2,000) followed by horseradish peroxidase-conjugated antigoat IgG (1:3,000) or antirabbit IgG $F(ab')_2$ fragment (1:3,000).

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 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 spleenic 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

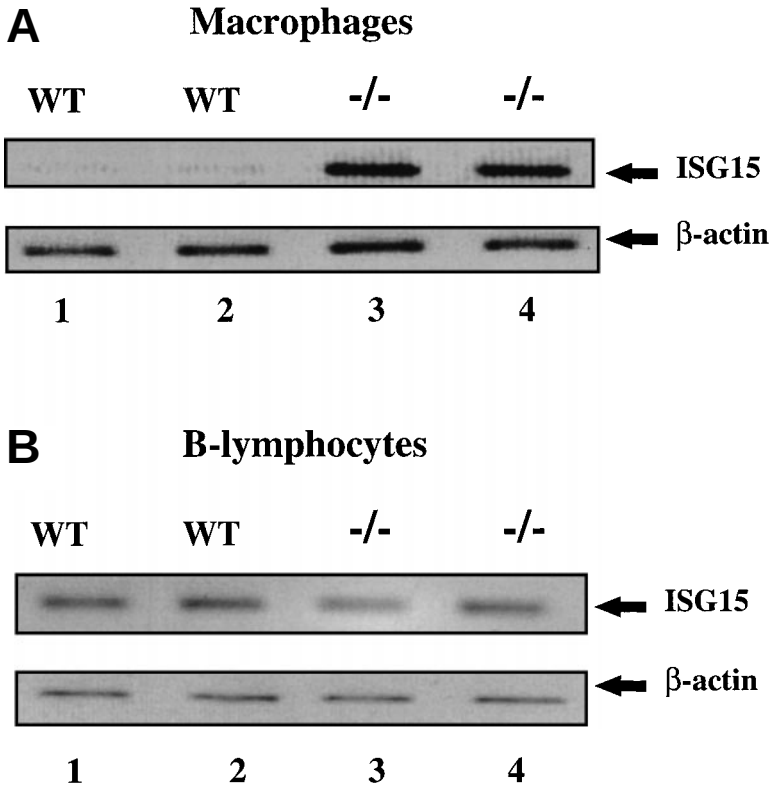

Fig 1. ISG15 mRNA expression levels in macrophages and B lymphocytes. Semiquantitative RT-PCR analysis showing the mRNA expression levels of the ISG15 gene in primary macrophages (A) and B lymphocytes (B) from wild-type and ICSBP-deficient mice. The PCR protocols were standardized for both ISG15 and the internal control β-actin to ensure that PCR amplification was in the linear phase.

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,[23] 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 heteromeric 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.[12] 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.[6-8] 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

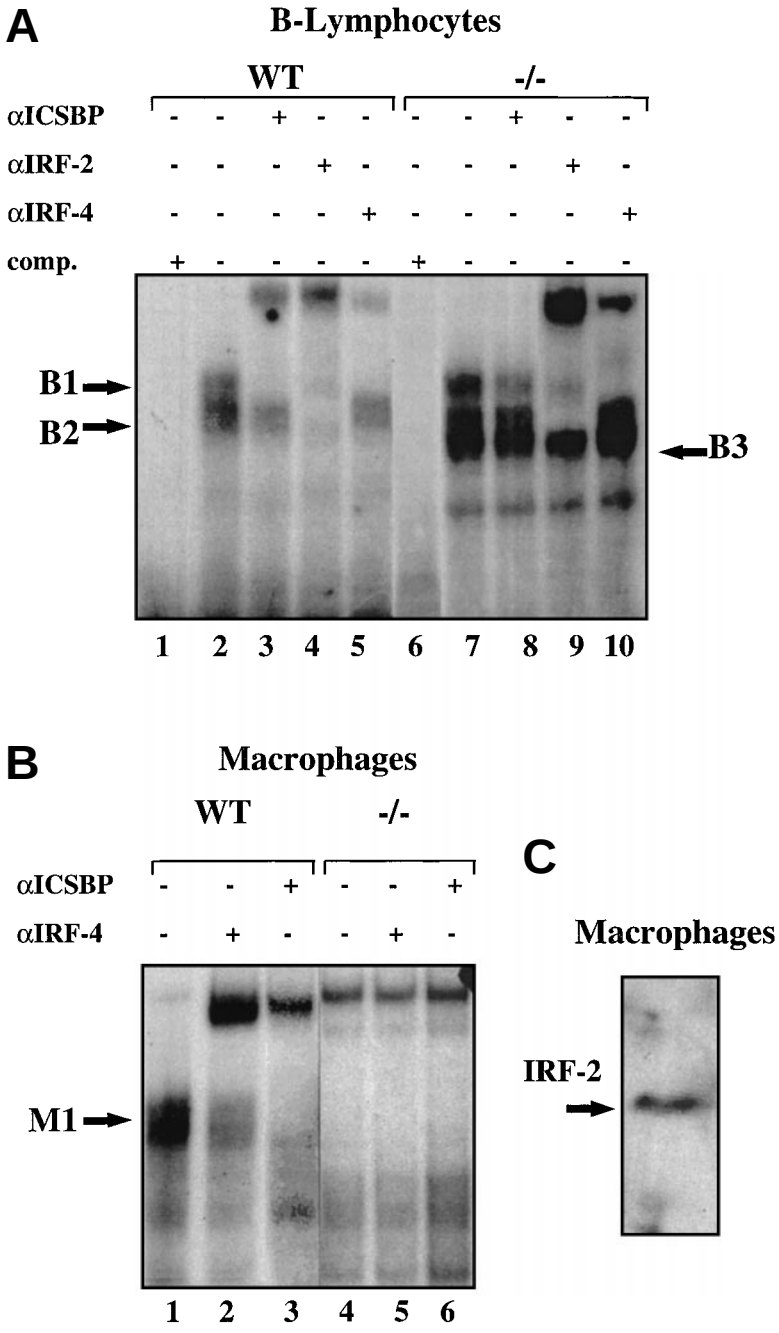

Fig 2. ICSBP and IRF-4/Pip form a complex on the ISG15 ISRE. (A) ICSBP, IRF-2, and IRF-4/Pip form a complex on the ISG15 ISRE in B lymphocytes. Mobility shift assay with the ISG15 ISRE probe and B-lymphocyte nuclear extracts from wild-type (lanes 1 to 5) and ICSBP-deficient mice (lanes 6 to 10). Lanes 1 and 6, competition with unlabelled ISG15 ISRE oligonucleotide; 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.

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, was no longer present (Fig 4A, compare lane 1 *v* lane 5). Because IRF-4/Pip was previously shown to bind to the ISG15 ISRE in the absence of ICSBP in B cells (Fig 2A), this raised the possibility that IRF-4/Pip was coimmunoprecipitated from the wild-type B-cell extracts by the ICSBP antibody, suggesting the existence of an ICSBP-IRF-4/Pip complex in the absence of

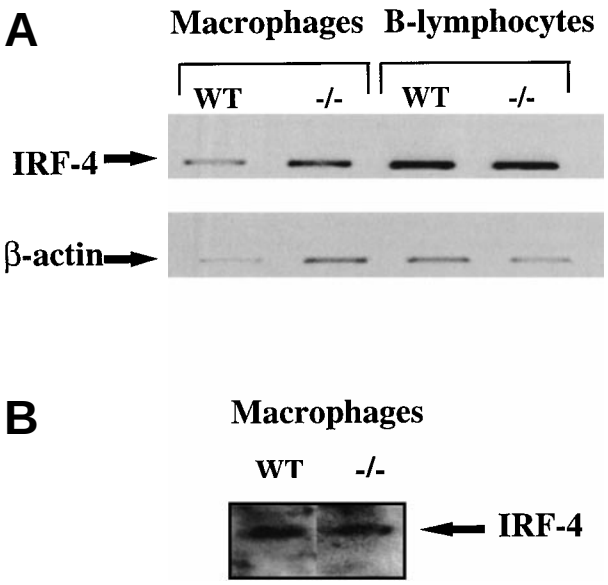

Fig 3. IRF-4/Pip protein and mRNA are expressed in mouse primary macrophages. (A) IRF-4/Pip mRNA is expressed in mouse primary macrophages and B lymphocytes. The expression levels of the IRF-4/Pip gene was determined in primary macrophages and B lymphocytes from wild-type and ICSBP-deficient mice. The arrows show the band for IRF-4/Pip and for the β-actin control. (B) The IRF-4/Pip protein is present in mouse primary macrophages from wild-type and ICSBP-deficient mice. The presence of the IRF-4/Pip protein in mouse macrophages in wild-type and ICSBP−/− mice was determined by a Western blotting assay.

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.[9] 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, (Table 1). From these results, one may conclude 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 oligonucleotide, 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.

ICSBP and IRF-4/Pip synergistically repress the activity of the ISG15 promoter. Our results to this point show that ICSBP and IRF-4/Pip form a complex and bind to the ISRE region of the ISG15 promoter in both macrophage and B-cell nuclear extracts. Previous results have shown that both ICSBP and IRF-4/Pip individually act as negative regulators of ISRE-dependent transcription.[9,20] To determine the function of the ICSBP-IRF-4/Pip complex, we constructed a luciferase vector that contained approximately 150 bp of the ISG15 promoter incuding the ISRE-region. This construct was transfected into K562 cells, which express endogenous IRF-2, but no IRF-4/Pip or ICSBP.[8,24] The K562 cells were then transfected with an IRF-4/Pip or an ICSBP expression vector or with both vectors. Mobility shift assays confirmed the presence of the ICSBP and IRF-4/Pip proteins binding to the ISG15 ISRE in transfected K562 cells and its absence in control cells (data not shown). The results of the luciferase assay are shown in Fig 5. K562 cells transfected with the ISG15 reporter plasmid showed high levels of luciferase activity. Transfection of the ISG15 luciferase vector into K562 cells cotransfected with the IRF-4/Pip or the ICSBP vector reduced the expression approximatelly 15-fold. However, when the ISG15 luciferase vector was transfected into K562 cells expressing both ICSBP and IRF-4/Pip, the luciferase expression was reduced over 150-fold to background levels. These results confirm that a complex of IRF-4/Pip and ICSBP synergistically repress the expression of the ISG15 gene promoter.

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.[6-8]

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.[6] In contrast, the other IRF proteins, IRF-1, IRF-2, and ISGF3γ, bind to the same site independently from PU.1.[20] The binding of ICSBP to the ISG15 ISRE in vitro is enhanced by IRF-1 or IRF-2.[11] Although ICSBP and IRF-4/Pip are closely related by sequence homology, cooperative binding between IRF-4/Pip

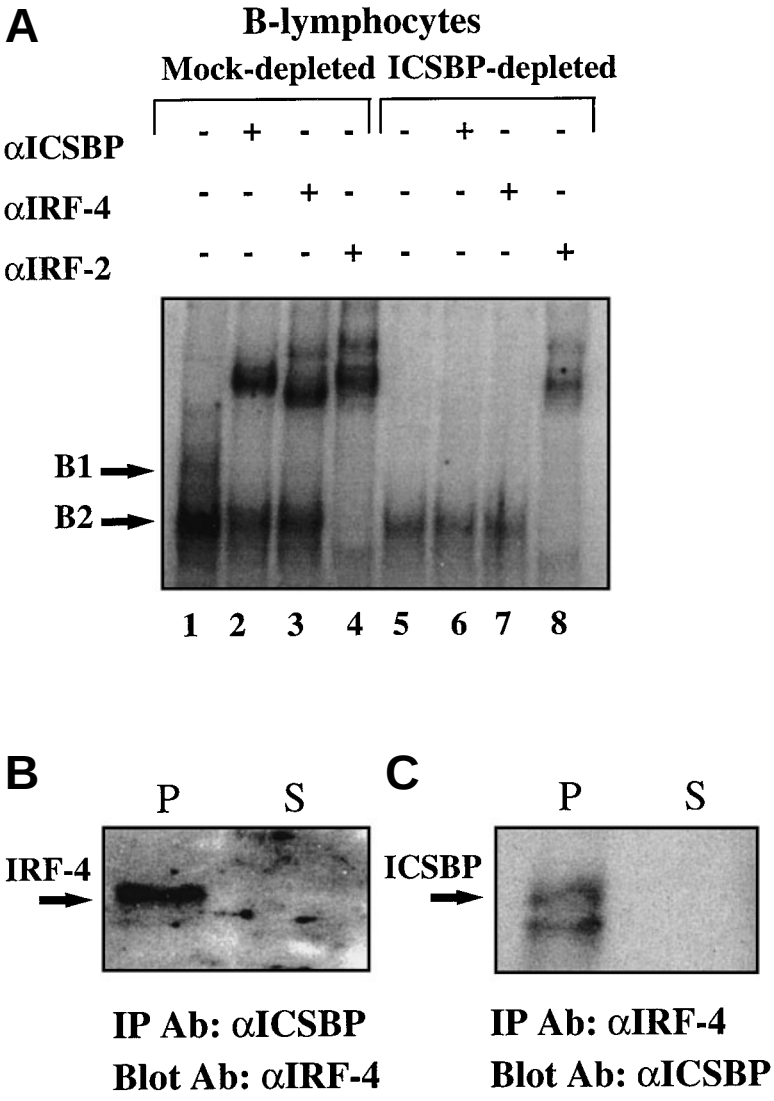

Fig 4. ICSBP and IRF-4/Pip form a complex in B cells and macrophages. (A) IRF-4/Pip is precipitated out of mouse B-lymphocyte extracts with an antibody against ICSBP. (A) Shows a mobility shift assay incubating the ISG15 ISRE oligonucleotide with wild-type mouse B-lymphocyte extracts, either mock-depleted (lanes 1 to 4) or depleted for the ICSBP protein (lanes 5 to 8). In lanes 2 and 6, the extracts are incubated with antibody against ICSBP, lanes 3 and 7 are with anti-IRF-4/Pip, and lanes 4 and 8 are with anti-IRF–2. (B and C) Evidence for ICSBP and IRF-4/Pip complex by coimmunoprecipitation in B-cell line, A20.2j (B) and in primary macrophages (C). The IRF-4/Pip protein was precipitated with an antibody against ICSBP and vice versa as described in Materials and Methods. Fluorographs of the immunoprecipitated fractions (P) and the supernatants (S) are shown.

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

		Binding*				
		B Lymphocyte			Macrophage	
Oligo	Sequence	ICSBP	IRF-2	IRF-4	ICSBP	IRF-4
ISRE	GATCCTCGGGAAAGGGAAACCGAAACTGAAGCC	++	++	++	++	++
M1	GATCCTCGGGAAAGGGcgtCCGAAACTGAAGCC	−	−	−	−	−
M2	GATCCTCGGGcgtGGGcgtCCGAAACTGAAGCC	−	−	−	−	−
M3	GATCCTCGGGcgtGGGcgtCCGcgtCTGAAGCC	−	−	−	−	−
M4	GATCCTCGGGcgtGGGAAACCGAAACTGAAGCC	+	+	+	+	+
M5	GATCCTCGGGAAAGGGAAACCGcgtCTGAAGCC	−	−	−	−	−
GG1	GATCCTCGGGAAAGGggGAAACCGAAACTGAAGCC	+	+	−	+	+
GG2	GATCCTCGGGAAAGGGAAACCggGAAACTGAAGCC	−	−	−	−	−

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.

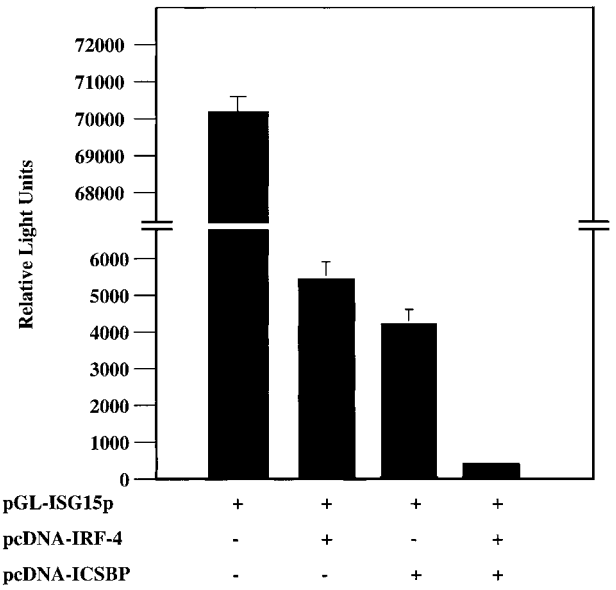

Fig 5. ICSBP and IRF-4 synergistically downregulate the expression of the ISG15 promoter. K562 cells were transfected with the luciferase expression vector pGL-ISG15p and pcDNA-IRF-4 and/or pcDNA-ICSBP. The graph shows the results of 3 independent experiments. The values were standardized relative to the internal control pRL-TK (see Materials and Methods).

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.[9] IRF-4/Pip may function in controlling both the transcriptional activity and the recombinational specificity of immunoglobulin light-chain genes in B cells.[20] 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.[12,25] 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.[1,2,26] 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.[10] γ-Interferon is a key regulator of inflammatory responses, and its uncontrolled activity may lead to deleterious pathological changes.[26] 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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