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Differential Expression and Distinct Functions of IFN Regulatory Factor 4 and IFN Consensus Sequence Binding Protein in Macrophages

Sylvia Marecki,* Michael L. Atchison,† and Matthew J. Fenton²*

IFN regulatory factor 4 (IRF4) and IFN consensus sequence binding protein (ICSBP) are highly homologous members of the growing family of IRF proteins. ICSBP expression is restricted to lymphoid and myeloid cells, whereas IRF4 expression has been reported to be lymphoid-restricted. We present evidence that primary murine and human macrophages express IRF4, thereby extending its range of expression to myeloid cells. Here, we provide a comparative analysis of IRF4 and ICSBP expression and function in distinct cell types. These IRF proteins can form specific complexes with the Ets-like protein PU.1, and can activate transcription via binding to PU.1/IRF composite sequences. EMSA analysis revealed that murine macrophages contained both IRF4/PU.1 and ICSBP/PU.1 complexes, analogous to B cells. Over-expression of ICSBP in these macrophages activated transcription of a PU.1/IRF-dependent promoter, whereas over-expression of IRF4 had no effect on this promoter. In contrast, over-expression of either IRF4 or ICSBP in both macrophages and NIH-3T3 fibroblasts suppressed transcription of the PU.1-independent H-2Ld MHC class I promoter. In NIH-3T3 fibroblasts, IRF4 and ICSBP also synergized with exogenous PU.1 to activate transcription of a PU.1/IRF-dependent promoter. Furthermore, both IRF4 and ICSBP activated transcription of the IL-1β promoter in both cell types. While this promoter is PU.1-dependent, it lacks any known PU.1/IRF composite binding sites. Synergistic activation of the IL-1β promoter by these IRF proteins and PU.1 was found to require PU.1 serine 148. Together, these data demonstrate that IRF4 and ICSBP are dichotomous regulators of transcription in macrophages.

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Several reports have described the phenotype of mice with a null mutation of the ICSBP gene. Holtschke et al. (12) reported that ICSBP-deficient mice display increased susceptibility to infections with either vaccinia or lymphocytic choriomeningitis viruses. These mice also spontaneously develop a syndrome similar to human chronic myelogenous leukemia. Fehr et al. (13) subsequently reported that ICSBP-deficient mice were highly susceptible to infection with *Listeria monocytogenes*, which correlated with impaired macrophage effector functions and IFN-γ responsiveness. Giese et al. (14) and Scharton-Kersten et al. (15) reported that ICSBP-deficient mice were unable to control infection with *Leishmania major* and *Toxoplasma gondii*, respectively. This impaired resistance to infection appears to be a consequence of the inability of these mice to express the IL-12 p40 subunit, and thereby to mount an effective Th1-type immune response (15). Impaired IL-12 p40 gene expression in the ICSBP-deficient mice implicated ICSBP as a transcription factor that directly or indirectly activates IL-12 p40 gene expression. This contrasts with earlier studies that concluded that ICSBP is a negative regulator of several IFN-responsive genes, including MHC class I, IFN-β, ISG-54, ISG-15, 2′-5′ oligoadenylate synthetase, and the Ig λ enhancer (5, 7).

The importance of IRF4 in vivo has been recently demonstrated in mice that lack a functional IRF4 gene. Lymphocyte development in IRF4-deficient mice was found to be blocked at a late stage, leading to a severe accumulation of immature lymphocytes and to defective T cell cytokine production (16). The effects of IRF4 deficiency on myeloid cell development and function have yet to be reported. Recent analyses of ICSBP- and IRF4-deficient mice have revealed both similar and distinct phenotypes. For example, ICSBP-deficient mice spontaneously develop a chronic myelogenous leukemia-like syndrome, whereas IRF4-deficient mice do not (16). In contrast, both IRF4- and ICSBP-deficient mice
were unable to mount an effective anti-viral response to lymphocytic choriomeningitis viruses, compared with wild-type mice (12, 16), demonstrating the requirement of these factors in antiviral immunity. A better understanding of the functional roles of IRF4 and ICsBP in vivo will depend upon the identification of genes that are uniquely regulated by each of these transcription factors.

IRF4 and ICsBP, by themselves, possess only weak DNA binding affinity (17, 18). However, the binding of ICsBP to DNA is dramatically increased following interaction with IRF1, IRF2, or the Ets-like transcription factor PU.1 (18, 19). Previous studies reported that IRF4 could bind to PU.1, and that these IRF4/PU.1 complexes were essential for Ig light chain expression (19–21). PU.1 stabilizes the binding of both IRF4 and ICsBP to a composite PU.1/IRF motif found in both the Ig λ and κ light chain enhancers in B cells. These interactions between PU.1 and IRF4 appear to be stabilized, at least in part, by interactions between DNA binding domains (22). Recent work by Brass et al. (23) has also demonstrated the importance of direct physical interaction between PU.1 and IRF4 in transcriptional regulation. The Ig enhancer composite motif consists of a 5′ PU.1 binding site and a 3′ sequence that resembles an ISRE half site. Most recently, a highly similar PU.1/IRF composite element was identified within the myeloid-specific gp91<sub>phox</sub> promoter (24). This gp91<sub>phox</sub> promoter element is bound by a complex comprised of PU.1, ICsBP, and IRF1.

PU.1 regulates the transcription of several myeloid-specific genes in the absence of interaction with either IRF4 or ICsBP. These include the Ig heavy and light chains (19, 20), M-CSF receptor (25), IL-1β (26), gp91<sub>phox</sub> (27), and macrophage scavenger receptor (28) genes. A protein kinase CK2 phosphorylation site at serine 148 has been shown to be functionally important (21). Mutation of this serine to an alanine resulted in a complete loss of LPS-induced transcriptional activity. These data demonstrate that serine 148 phosphorylation is critical for inducible transcriptional activation by PU.1. Consistent with these findings, physical and maximal functional interaction between PU.1 and the IRF members IRF4 and ICsBP also requires the presence of serine 148 (20, 21).

Unlike most other IRF family members, ICsBP expression is mainly restricted to cells of the immune system, including lymphocytes and macrophages (5). However, recent work by Li et al. (29, 30) demonstrated that ICsBP is expressed in the retinal epithelia and ocular lens. ICsBP is constitutively expressed in macrophages, although its expression can be further up-regulated by IFN-γ, but not significantly by IFNαβ (31–33). Moreover, LPS and IFN-γ synergistically enhance ICsBP steady-state mRNA and protein levels (34). IRF4 has been reported to only be expressed in lymphocytes (7, 10, 16, 35, 36), and its expression was not altered by IFN-γ treatment (10, 36). In contrast to these earlier reports, we demonstrate here that macrophages express both IRF4 mRNA and protein. The capacity of macrophages to express both IRF4 and ICsBP raises the possibility that they perform distinct functions in these cells. To assess the function of these IRF proteins in macrophages, we examined their ability to regulate the transcription of selected promoters. Here, we report that IRF4 and ICsBP can function as both transcriptional activators and repressors of different promoters in a cell type- and promoter-specific manner. Our data suggest a complex molecular mechanism that regulates the expression of genes by IRF4 and ICsBP in macrophages.

Materials and Methods

Reagents and Abs

Trypsin, proteinase K, antipain, aprotinin, chymostatin, and leupeptin were purchased from Sigma (St. Louis, MO). Recombinant murine IFN-γ was purchased from R&D Systems (Minneapolis, MN). LPS, <i>Escherichia coli</i> serotype 055:B5, was purchased from Sigma. Polyclonal rabbit antiserum that specifically recognize PU.1, IRF1, or IRF2, as well as polyclonal goat antiserum that specifically recognize IRF4 or ICsBP, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal, preimmune goat and rabbit sera were purchased from Pierce (Rockford, IL).

Cell lines and tissue culture conditions

The RAW264.7 murine macrophage, NIH-3T3 murine fibroblast, and Sup-T1 murine T cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). RAW264.7 and NIH-3T3 cells were maintained in DMEM culture medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone Laboratorys, Logan, UT), 10 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Sup-T1 cells were cultured in RPMI 1640 culture medium (BioWhittaker) supplemented as described above. Murine peritoneal exudate macrophages (PEC) were elicited by thioglycollate injection of BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Following elicitation (72 h), peritoneal cells were harvested, and macrophages were obtained by adherence purification. Macrophages were used within 5 days of harvest. Murine splenic B cells were obtained from BALB/c mice using complement-mediated lysis to remove T cells and adherence to depletion macrophages, as previously described (37). Human monocytes were obtained by adherence purification from total PBMC, as we described previously (38). All cells were cultured at 37°C, 5% CO₂ in a humidified incubator. Endotoxin levels in all medium components were <10 pg/ml final concentration, as indicated by BioWhittaker or measured by <i>Limulus</i> amebocyte lysate kit (BioWhittaker). Cells were stimulated with LPS or recombinant IFN-γ at a final concentration of 100 ng/ml for the times indicated in the text.

Nuclear extraction and cytosolic lysate preparation

Nuclear extracts were prepared essentially as described by Schreiber et al. (39). Approximately 1.0 × 10⁶ cells were harvested, washed with Ca²⁺ and Mg²⁺-free PBS (BioWhittaker), and pelleted by centrifugation at 800 × g for 10 min at 4°C. The resulting cell pellets were resuspended in 400 μl of buffer 1 (10 mM KCl, 10 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 0.5 mM DTT, 0.3 M sucrose, 10 mM β-glycerophosphate, 0.1 mM EDTA, 0.5 mM PMSF, and 5 μg/ml each of aprotinin, leupeptin, chymostatin, and antipain), then incubated on ice for 10 min. Subsequently, 25 μl of 10% Nonidet P-40 (Sigma) was added to each sample and vortexed. The nuclei were pelleted by centrifugation for 1 min at 5000 × g. Supernatants were collected and stored for later use as cytosolic lysates. Nuclear pellets were resuspended in a nuclear extraction buffer (buffer II) containing 320 mM KCl, 10 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 0.5 mM DTT, 10 mM β-glycerophosphate, 0.1 mM EDTA, 0.5 mM PMSF, and 5 μg/ml each of aprotinin, leupeptin, antipain, and chymostatin. Samples were extracted on ice for 15 min followed by centrifugation at 16,000 × g for 10 min at 4°C. Protein concentration was determined using the Bio-Rad assay kit (Hercules, CA) assay kit. All nuclear extracts were stored at −70°C, and multiple freeze-thawing cycles were avoided.

EMSA and DNA probes

A double-stranded oligonucleotide containing a single copy of the Ig κ3 enhancer (Ig κ) composite PU.1/IRF sequence (5′-CTTGGAGGACT-GAAACAGAAGCCT-3′; (40)) was utilized as an EMSA probe. Italicized sequences represent PU.1 (5′-CTTGGAGGACT-GAAACAGAAGCCT-3′; (40)) was utilized as an EMSA probe. Italicized sequences represent PU.1 (5′-CTTGGAGGACT-GAAACAGAAGCCT-3′; (40)). All other DNA probes were labeled with [γ-³²P] deoxynucleotide triphosphates (DuPont-NEN, Boston, MA) using <i>E. coli</i> DNA polymerase Klenow fragment (United States Biochemicals, Cleveland, OH), as recommended by the manufacturer. Unincorporated nucleotides were removed using Sephadex G-25 columns (5 Prime → 3 Prime, Boulder, CO). Nuclear extracts (typically 3 μg) were incubated with radiolabeled probe DNA (0.1 ng, typically 10,000 cpm) in the presence of 2 μg poly dI·dC (Pharmacia, Piscataway, NJ), 1.0 mM EDTA, 10 mM Tris-HCl (pH 7.9), 25 mM glycerol, and 0.5 mM DTT in a final volume of 20 μl, as previously described (41, 42). Binding reactions were incubated at room temperature for 30 min. In competition experiments, unlabeled DNA was added at a 100-fold molar excess in addition to the binding reaction. For supershift experiments, 2 μg of antisem was added as indicated in the text in addition to the binding reaction. Following incubation, a portion of each binding reaction (7 μl) was electrophoresed through a 7% nondenaturing low ionic strength polyacrylamide gel, dried, and visualized by autoradiography.
Western blot analysis

Nuclear extracts and cytosolic lysates were prepared from cells as described above. Samples (50–150 µg total protein) were electrophoresed through either a 15 or 10% SDS-polyacrylamide gel (noted in figure legend), transferred to a nitrocellulose membrane (Bio-Rad), and blocked with 5% nonfat dry milk (Carnation) in TBST containing 0.1% Tween 20 (Sigma). Membranes were subsequently probed, using a 1:10 dilution of antibody and developed using a 1:4500 dilution of a protein A conjugated to HRP (Amersham, Arlington Heights, IL). IRF4 and ICSBP proteins were visualized using a 1:3000 dilution of specific antisera and developed with a 1:1500 dilution of protein G-HRP (Pierce). Membranes were visualized using an enhanced chemiluminescence (ECL) reagent (CL-HRP substrate system; Pierce), according to the manufacturer’s instructions.

RNA isolation and RT-PCR

Total RNA from macrophages, fibroblasts, and murine B cells was purified using RNA STAT-60 (Leedo Medical Laboratories, Houston, TX), as recommended by the manufacturer. RNA was converted to cDNA using avian myeloblastosis virus RT (Promega, Madison, WI). PCR were performed using between 100 ng and 2 µg of cDNA, 0.5 µM oligonucleotide primers (each), 1.5 mM MgCl₂, 150 µM dNTPs and 2.5U Taq polymerase in a final reaction volume of 75 µl. Thirty amplification cycles were performed (95°C denaturation, 30 s; 55°C annealing, 1 min; 72°C extension, 1.5 min). Introns-spanning IRF4, CD19, and β-actin PCR primers used in this study are listed below. The IRF4 primers correspond to sequences that do not share sequence similarity with ICSBP. Following amplification, a portion of the PCR reactions were electrophoresed through a 1.2% agarose gel. The 554-bp IRF4, 747-bp CD19, and 285-bp β-actin products were visualized using ethidium bromide. Sense-strand IRF4 primer: 5′-GCC GTA TAT CTG CCT GTA TTA CCG-3′; anti-sense strand IRF4 primer: 5′-GTG GTA ACG TGT CTA GGT AAC TCG TAG-3′; sense-strand CD19 primer: 5′-CCC CAC AAG TCC TTA CTG-3′; anti-sense CD19 primer: 5′-GTC GCC TTC CAA TGA CCA GC-3′; sense-strand β-actin primer: 5′-TCA TGA AGT GTG AGC TTC ACA GAT GAC-3′; anti-sense strand β-actin primer: 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′.

Plasmids

Expression plasmids encoding the full-length, wild-type and S148A mutant murine PU.1 proteins were previously described (21). An expression plasmid encoding the full-length murine ICSBP protein was provided by Dr. Keiko Ozato (National Institutes of Health, Bethesda, MD) and was previously described (43–45). Expression plasmids encoding the murine IRF4 proteins were previously described (35). The control vector pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA), and was used to maintain a constant quantity of plasmid DNA in each transfection. The PU.1, c-fos, c-jun promoters, and the chicken β-actin promoter (CAT; wild type), (m)pu.1/IRF4 CAT (PU.1 binding site mutant), and (PU.1/mIRF4 CAT (IRF binding site mutant) reporter constructs containing four copies of the composite PU.1/IRF binding site derived from the Ig κ enhancer were previously described (20). The IL-1β luciferase reporter construct was generated from the previously described 7 mXT-CAT reporter plasmid (26). Brieﬂy, the xIκB to Tαβ fragment (positions −3759 to +11) from the human IL-1β promoter was subcloned into the promoterless pGL3 luciferase reporter plasmid (Promega). The H-2Ld MHC class I reporter plasmid was provided by Dr. Keiko Ozato, and was previously reported (5).

Transient transfections

Transient transfections were performed using SuperFect reagent (Promega) as per the manufacturer’s instructions. Briefly, cells were plated on 6-well dishes and transfected when cells reached 80% confluence. Plasmid DNA was added to 100 µl of Opti-Mem reduced serum media (Life Technologies, Rockville, MD). All transfections utilized a total of 4 µg of plasmid DNA consisting of 2 µg of reporter plasmid, 1 µg of each expression vector, and the balance was made up with empty vector described above unless otherwise noted in the text. A total of 10 µl of SuperFect was added to the DNA-media mixture, incubated for 10 min, diluted with 600 µl of serum-containing media, and added to individual wells. Each reaction was prepared separately and in triplicate. Fresh media containing serum was added 2–3 h after transfection. All conditions were incubated for an additional 16–24 h. CAT and luciferase assays were performed as described below. All transfection experiments were repeated at least three times using different plasmid preparations, and a single representative experiment is shown. Each single experiment represents triplicate independent transfections, and data are expressed as average values ± SD.

Reporter gene assays

CAT reporter activity was assessed using a two-phase flow diffusion CAT assay as previously described (46). Equal concentrations of cell lysates (15–35 µg total protein per sample) were used in the assay. Protein concentration was determined utilizing the Bio-Rad protein assay kit, according to the manufacturer’s instructions. Data were calculated by plotting total cpm-counted unincubated cholamrophilic vs time, and the slopes for each reaction were calculated within a linear kinetic range. Luciferase activity was measured using the luciferase assay system (Promega), according to manufacturer’s instructions and performed as previously described (47). Lysates were assayed for total protein using the Bio-Rad protein assay. Luciferase activity was measured using 5–20 µg total protein as measured by light emissions in a scintillation counter.

Results

IRF4 and ICSBP can form specific complexes with PU.1 in macrophages

We performed a series of EMSA analyses to determine whether ICSBP/PU.1 complexes could be formed in RAW264.7 macrophages and primary B cells. The EMSA probe used in these experiments is a PU.1/IRF composite binding site (see Materials and Methods for sequence) that contains an ISRE half site located downstream and adjacent to a consensus PU.1 binding site. Such sites have been identified within the Ig light chain enhancers and the gp91phox promoter (19, 20, 24). IRF4 proteins alone do not bind to this composite site, although concomitant binding of PU.1 to the site stabilizes IRF binding to DNA as a consequence of its interaction with PU.1 (7). As shown in Fig. 1, PU.1 and IRF proteins that were present in unstimulated macrophage and B cell nuclear extracts specifically bound to the composite site. Abs against PU.1 (lane 7) and ICSBP (lane 3) could supershift the DNA-protein complexes, whereas Abs against IFR1 (lane 5) and IRF2 (lane 6) did not supershift the complexes. Abs that recognize PU.1 blocked the binding of both PU.1 and ICSBP to the DNA, suggesting that ICSBP could not bind to DNA in the absence of PU.1. Unexpectedly, specific Abs for IRF4 (Fig. 1A, lane 4) could also supershift the DNA-protein complexes generated using macrophage extracts. Like ICSBP, Abs that recognize PU.1 blocked the binding of both PU.1 and IRF4 to the DNA, indicating that IRF4 could not bind to DNA in the absence of PU.1. The IRF4 Ab was also able to supershift a complex when incubated with B cell nuclear extracts (Fig. 1B, lane 4). Furthermore, normal preimmune goat and rabbit sera used at similar concentrations as all other antisera did not produce any supershifted complexes when incubated with either macrophage or B cell nuclear extracts (lanes 8 and 9). In addition, when EMSA analysis was performed upon primary murine and human macrophage nuclear extracts, similar results were obtained (data not shown). Mutation of the IRF binding site abolished binding of the PU.1/IRF complexes, but did not affect the binding of PU.1 alone (data not shown), while mutation of the PU.1 site blocked the binding of both PU.1 and IRF proteins to the oligonucleotide (data not shown). Together, our data suggest that macrophages express IRF4, or a related protein that is also recognized by the Ab, and that this protein can bind to the PU.1/IRF composite sites in the presence of PU.1.

IRF4 mRNA and protein are expressed in macrophages

To confirm that IRF4 is expressed by macrophages, we used sequence-specific, intron-spanning oligonucleotide primers to amplify cDNA synthesized from both primary and cell line murine mRNA. These primers were selected from regions of the IRF4 mRNA that lack sequence similarity with ICSBP transcripts. Using RT-PCR, these primers generated a 554-bp product using cDNA synthesized from RAW264.7 cells and primary murine PEC (Fig. 2, lanes 2 and 4). A PCR product of identical size was generated
using cDNA synthesized from primary murine B cells (lane 3). Meanwhile, no PCR product was generated from NIH-3T3 fibroblast cDNA (lane 1), cells that do not express IRF4, ICSBP, or PU.1. To confirm that the PCR product generated from primary murine macrophages was not due to B cell contamination, we performed PCR using primers to detect expression of the B cell-specific gene CD19. A PCR product of the expected size (747 bp) was observed in PCR reactions containing cDNA from the macrophages and B cells, but not the NIH-3T3 fibroblasts. The expected 747-bp product for CD19 was observed only in B cells. The expected 285-bp β-actin product was observed in all PCR reactions. No PCR products were generated using RNA that had not been reverse transcribed.

These analyses were performed using semiquantitative methods, where logarithmic dilutions of cDNA were used as templates for PCR and then analyzed by densitometry to assure that amplification was performed in the linear range. The amount of IRF4 PCR products generated using cDNA generated from the RAW264.7 cells was consistently and substantially less that that generated from the primary B cells or primary macrophages. When the levels of IRF4 message were normalized to β-actin, both RAW264.7 macrophages and primary macrophages were found to contain similar amounts of IRF4 mRNA. These levels were 25–35% of that seen in primary B cells (data not shown). From these data, we conclude that macrophages do express IRF4 transcripts, although at levels lower than those found in B cells.

To determine whether this IRF4 mRNA was translated into protein, Western blot analysis was performed. Nuclear extracts were prepared from primary murine macrophages, RAW264.7 macrophages, and primary human monocytes. These samples were fractionated by SDS-PAGE and probed using non-cross-reactive Abs against either IRF4 or ICSBP. Nuclear extracts from primary murine B cells were also included in the figure, because they express both IRF4 and ICSBP. As shown in Fig. 3A, immunoreactive IRF4 and ICSBP protein were present in both the macrophage and B cell lysates. To confirm that the Abs were non-cross-reactive and recognized proteins of the predicted molecular size, three duplicate lanes were transferred to nitrocellulose membranes and probed with the Abs singly, or in combination. As shown in Fig. 3B, the IRF4 and ICSBP Abs each identified a single protein with the expected molecular size (42 and 45 kDa, respectively). Together, these data demonstrate that macrophages express both IRF proteins.

**Differential expression of IRF4 and ICSBP in macrophages**

Yamagata et al. (10) reported that IRF4 expression and function in lymphocytes were not activated by IFN treatment. In contrast, ICSBP is expressed constitutively at low levels in macrophages, and this expression can be further up-regulated by IFN-γ treatment (32–34). We sought to determine whether IRF4 expression could be regulated by IFN-γ in macrophages. The capacity of IFN-γ to up-regulate ICSBP gene expression in the RAW264.7 macrophages was confirmed by Western blot analysis of nuclear extracts.
Fig. 4), and densitometric quantification of the Western blots revealed that 6 h of IFN-γ stimulation increased the nuclear levels of ICSBP by ~26-fold. In contrast to ICSBP, levels of nuclear IRF4 protein in the macrophages was not altered by IFN-γ treatment, consistent with previous findings. Cytosolic levels of IRF4 and ICSBP mirrored that observed in the nucleus (data not shown), consistent with previous findings in lymphocytes (13, 36). In addition, the levels of nuclear PU.1 protein were not altered by IFN-γ treatment (Fig. 4).

LPS induces nuclear translocation of IRF4

IRF4 activity has been reported to be induced in activated lymphocytes (10, 36). Therefore, we sought to determine whether LPS stimulation of the RAW264.7 cells could similarly activate IRF4. As shown in Fig. 5A, levels of nuclear IRF4 protein increased within 2 h following LPS stimulation, with a concomitant decrease in the cytosolic levels of this protein. In contrast, ICSBP protein levels in both the nucleus and cytosol were not measurably altered following LPS stimulation within the time period examined (data not shown). As a control for cross-contamination of the samples, we found that these nuclear extracts did not contain a protein found solely in the cytosol (p105 NF-κB; data not shown). Densitometric quantification of the Western blots revealed that 6 h of LPS stimulation increased the nuclear levels of IRF4 by ~3-fold (Fig. 5B).
As the mean rate of radiolabeled chloramphenicol accumulation over time. Transfections were performed in triplicate. Data are expressed as the mean rate of radiolabeled chloramphenolic accumulation over time (slope) ± SD.

While our data are only suggestive of nuclear translocation, they are consistent with a previous study by Lin et al. (8). These investigators reported that stimulation of various fibroblast cell lines with Sendai virus resulted in the rapid nuclear translocation of IRF3, a ubiquitously expressed IRF family member.

A composite PU.1/IRF site is functional in macrophages

One objective of these studies was to determine whether IRF proteins could form functional associations with PU.1 in macrophages. This physical and functional interaction has been previously demonstrated for Ig light chain enhancer expression in B cells (19, 20). We sought to determine whether such composite sites were also functional in macrophages, cells that express PU.1 constitutively. We transfected RAW264.7 macrophage cells transiently with a reporter plasmid containing the CAT reporter gene, was transiently transfected into RAW264.7 macrophages, as described in the text. RAW264.7 cells were also transfected with reporter plasmids in which the IRF binding site (PU.1/mIRF)4) or the Ets binding site (mPU.1/IRF)4) was mutated. In some cases, these cells were cotransfected with either an IRF4 expression vector, an ICSBP expression vector, or the empty expression vector. CAT activity was determined as described in the text. Transfections were performed in triplicate. Data are expressed as the mean rate of radiolabeled chloramphenolic accumulation over time (slope) ± SD.

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Functional synergy between PU.1 and ICSBP proteins

Interactions between PU.1 and ICSBP, or between PU.1 and ICSBP, cannot be easily examined in macrophages because these cells constitutively express all three transcription factors. To study these interactions in isolation, we expressed these factors in fibroblast lines that do not intrinsically express PU.1, IRF4, or ICSBP. We cotransfected NIH-3T3 cells with the (PU.1/IRF)4 reporter plasmid and an expression plasmid encoding IRF4 or ICSBP. As shown in Fig. 6, over-expression of ICSBP resulted in a 4-fold enhancement in basal promoter activity. Meanwhile, over-expression of IRF4 had no effect upon the basal expression of either the Rous sarcoma virus long terminal repeat, or the NF-kB-dependent E-selectin (ELAM-1) reporters. Reporter plasmids, in which either the IRF or PU.1 binding sites were mutated, lacked basal activity and could not be activated by over-expression of ICSBP. In contrast, over-expression of IRF4 did not result in enhanced transcription of the promoter, as was seen with ICSBP. We also found that the cells with increasing amounts of the ICSBP expression plasmid (100–2000 ng) resulted in dose-dependent activation of the reporter, whereas increasing amounts of IRF4 had no effect on reporter gene expression (data not shown). Moreover, these data also demonstrate that IRF4 over-expression in macrophages does not activate the (PU.1/IRF)4 reporter plasmid, which contrasts with its ability to activate this reporter in fibroblast cell lines (19, 20).

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FIGURE 8. Both IRF4 and ICSBP repress transcription of an MHC class I promoter in distinct cell types. An H-2Ld MHC class I reporter plasmid was cotransfected with either an IRF4 or ICSBP expression plasmid, or with empty vector alone, into RAW264.7 macrophages (A) or NIH-3T3 fibroblasts (B). CAT activity was determined as described in the text. Transfections were performed in triplicate. Data are expressed as the mean rate of radiolabeled chloramphenicol accumulation over time (slope) ± SD.

**IRF4 and ICSBP can either activate or repress transcription in a promoter-specific manner**

Our data demonstrate that IRF4 and ICSBP can activate transcription of a promoter controlled by PU.1/IRF composite elements. Previous studies have reported that IRF4 and ICSBP function as a transcriptional represor in various cell types (5, 10, 18). Therefore, it was important to confirm that IRF4 and ICSBP could also suppress transcription in our model system. The PU.1-independent H-2Ld MHC class I promoter had been previously reported to be negatively regulated by both IRF4 and ICSBP (5). We cotransfected RAW264.7 cells with a CAT reporter plasmid under the control of the H-2Ld promoter and an expression plasmid encoding either IRF4 or ICSBP. As shown in Fig. 8, over-expression of either IRF4 or ICSBP resulted in reduced basal promoter activity, compared with cells transfected with the reporter alone. Similar results were obtained using transfected RAW264.7 cells (Fig. 8A) and NIH-3T3 cells (Fig. 8B). Together, these findings demonstrate that IRF4 and ICSBP can repress transcription of the PU.1-independent H-2Ld promoter in both cell types, whereas only ICSBP can activate transcription of the PU.1-dependent (PU.1/IRF)4 reporter in both cell lines. Furthermore, repression of the H-2Ld promoter in RAW264.7 cells (that constitutively express PU.1) and NIH-3T3 cells (that lack PU.1) by both IRF4 and ICSBP suggest that repression by these two IRF family members is independent of their ability to interact with PU.1.

**Effects of IRF proteins on a natural PU.1-dependent promoter**

The studies described above assessed the ability of IRF4 and ICSBP to interact with PU.1 and to activate a synthetic promoter containing tandem copies of a PU.1/IRF composite site. We subsequently sought to determine whether IRF4 and ICSBP could activate transcription of a natural PU.1-dependent promoter. We selected IL-1β as a potential gene that could be regulated by both PU.1 and IRF proteins in macrophages. The IL-1β promoter has been previously shown to be regulated by both LPS and IFN-γ, and to require a functional PU.1 binding site within the cap site-proximal promoter (46). We cotransfected RAW264.7 cells with an IL-1β reporter plasmid and increasing amounts of expression plasmids encoding either IRF4 or ICSBP (100–2000 ng). As shown in Fig. 9, we found that over-expression of ICSBP resulted in a consistent 40–50% increase in basal transcriptional activity, which was not further enhanced by increasing the quantities of ICSBP expression plasmid used. In contrast, over-expression of IRF4 activated the IL-1β reporter in a dose-dependent fashion, with more than 7-fold activation at the highest amount of IRF4 expression plasmid used. These results are qualitatively distinct from those obtained using the (PU.1/IRF)4 reporter plasmid (Fig. 6), suggesting that the two promoters are regulated differently by these IRF proteins.

IRF-dependent activation of PU.1/IRF composite elements depends on phosphorylation of PU.1 at serine 148 (7, 19–21). We subsequently sought to determine whether synergistic activation of the PU.1-dependent IL-1β promoter by PU.1 and IRF proteins also required serine 148. We cotransfected NIH-3T3 cells with the IL-1β reporter plasmid, the IRF4 or ICSBP expression plasmids, and the wild-type or S148A mutant PU.1 expression plasmids, as described above. As shown in Fig. 10A, IRF4, PU.1, and S148A alone were incapable of activating expression of the reporter plasmid. Synergistic activation of the IL-1β promoter was observed when cells were cotransfected with IRF4 and PU.1 expression plasmids. Unlike wild-type PU.1, the S148A mutant failed to synergize with IRF4 to activate the reporter plasmid. Similar results were obtained using this promoter in cells that over-expressed ICSBP (Fig. 10B). However, cotransfection of ICSBP with the
S148A mutant resulted in a complete loss of synergy, as compared with the incomplete loss of activity seen with coexpression of IRF4 with S148A. These data demonstrate that a natural PU.1-dependent promoter can be synergistically activated by these IRF proteins and PU.1. This synergistic activation with ICSBP requires a critical serine residue on PU.1, whereas IRF4 may be less dependent upon this serine.

Discussion

ICSBP is expressed in both lymphoid and myeloid cells, and has been reported to repress the expression of several IFN-responsive genes, although the contribution of ICSBP to macrophage functions remains largely unknown. Our finding that IRF4 is expressed in macrophages raises the possibility that these two IRF proteins differentially regulate the same target genes. Thus, we compared the capacity of IRF4 and ICSBP to regulate transcription in RAW264.7 murine macrophages. We also tested the hypothesis that these two IRF proteins could form functional complexes with the Ets-like protein PU.1. The function of these IRF proteins in different cell lines, both with and without PU.1, are summarized in Table I. Together, these data suggest a complex molecular mechanism that regulates the expression of IFN-responsive genes by IRF4 and ICSBP.

Previous studies had failed to detect IRF4 mRNA in macrophage cell lines using Northern blot analysis (7, 10). Our success in detecting these transcripts presumably resulted from the use of the more sensitive semiquantitative RT-PCR approach. Consistent with this possibility is our finding that the RAW264.7 cell line expressed markedly lower levels of IRF4 transcripts than primary B cells (Fig. 2). When normalized to β-actin expression, it appears that macrophages express 25–35% of the IRF4 message expressed in B cells. The lack of CD19 transcripts in RNA prepared from primary thioglycollate-elicited macrophages demonstrates the lack of B cell contamination (Fig. 2). These data support the conclusion that macrophages produce IRF4 message. In summary, confirmation that macrophages express IRF4 came from: 1) the sequence of the RT-PCR product (data not shown), 2) specific binding of a 45-kDa protein by non-cross-reactive anti-IRF4 Abs (Fig. 3B), and 3) supershifting of a specific DNA-protein complex by both anti-PU.1 and anti-IRF4 Abs (Fig. 1). Therefore, we cannot fully explain why previous studies failed to identify macrophages as cells that can produce IRF4, although the paucity of IRF4 message in macrophages may have been below the level of detection of detection techniques used in previous studies.

Several features indicate that IRF4 and ICSBP are differentially regulated in macrophages. First, only ICSBP expression was increased following IFN-γ treatment. This is consistent with data obtained using lymphocytes where ICSBP, but not IRF4, expression was up-regulated by IFN-γ (10, 33). Second, LPS stimulation appeared to induce nuclear translocation of IRF4, but not of ICSBP (Fig. 5). Third, over-expression of ICSBP activated transcription of the (PU.1/IRF)4 reporter plasmid (Fig. 6) and weakly activated transcription of the IL-1β reporter plasmid (Fig. 9). In contrast, over-expression of IRF4 only enhanced transcription of the IL-1β reporter plasmid. However, both IRF4 and ICSBP were capable of repressing the PU.1-independent H-2Ld promoter (Fig. 8). Together, these data suggest that the expression and function of IRF4 and ICSBP are regulated by different mechanisms in macrophages. Furthermore, such differences suggest that these IRF proteins serve nonredundant functions in macrophages which may, in part, be related to their ability to interact with PU.1.

![Figure 10](http://www.jimmunol.org/)

**FIGURE 10.** The IL-1β promoter can be synergistically activated by PU.1 and IRF proteins. NIH-3T3 fibroblasts were transiently cotransfected with the IL-1β reporter plasmid, wild-type, or S148A mutant PU.1 expression plasmid, and either the IRF4 (A) or ICSBP expression plasmid (B). Luciferase activity was determined as described in the text. Transfections were performed in triplicate. Data are expressed as mean fold activity over background ± SD.

### Table I. Summary of transfection data

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Reporter</th>
<th>Overexpression of IRF4/Pu</th>
<th>Overexpression of ICSBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW264.7</td>
<td>(PU.1/IRF)4</td>
<td>No activation</td>
<td>Activation</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>(PU.1/IRF)4</td>
<td>Synergy with PU.1</td>
<td>Synergy with PU.1</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>(PU.1/IRF)4</td>
<td>No synergy with S148A</td>
<td>No synergy with S148A</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>IL-1β</td>
<td>Activation</td>
<td>Weak activation</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>IL-1β</td>
<td>Synergy with PU.1</td>
<td>Synergy with PU.1</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>IL-1β</td>
<td>Partial synergy with S148A</td>
<td>No synergy with S148A</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>H-2Ld</td>
<td>Repression</td>
<td>Repression</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>H-2Ld</td>
<td>Repression</td>
<td>Repression</td>
</tr>
</tbody>
</table>
Our findings also extend several previous reports on the functions of both ICSBP and PU.1/IRF complexes. Nelson et al. (5) demonstrated that ICSBP repressed several ISRE-containing promoters in macrophages. In contrast, Eklund et al. (24) recently reported that the PU.1/IRF composite site within the gp91phox promoter was positively regulated by ICSBP in U937 cells. These investigators found that PU.1, ICSBP, and IRF1 functioned together to activate transcription of a reporter plasmid containing tandem copies of this composite element. Our data agree with these results, and support a model in which ICSBP functions to repress transcription when bound to ISRE sequences, but instead activates transcription when bound to PU.1. Thus, the capacity of ICSBP to activate or repress transcription appears to be determined in a promoter-specific context, defined by the proteins with which it interacts.

These previous studies used reporter plasmids containing tandem copies of the Ig light chain enhancer or gp91phox PU.1/IRF composite elements to drive transcription (19, 20, 24). Here, we showed that a natural promoter (i.e., IL-1β) could be activated by IRF4, and weakly by ICSBP, in RAW264.7 macrophages (Fig. 9). Furthermore, this promoter is activated synergistically by both IRF4 and ICSBP with PU.1 in NIH-3T3 fibroblasts (Fig. 10). The IL-1β promoter is not known to contain functional PU.1/IRF composite sites, and future studies will be needed to identify the elements within this 3.7-kb IL-1β promoter fragment that mediates this synergy. The function of ICSBP/PU.1 complexes has been previously examined in NIH-3T3 fibroblasts (35). One study reported that ICSBP/PU.1 complexes failed to activate the same (PU.1/IRF)4 reporter plasmid used also in our study. This finding contrasts with our data in these same cells, but the differences may be due to the IRF expression plasmids used in each study. Brass et al. (35) used an epitope-tagged ICSBP expression plasmid, whereas the experiments reported here were performed using a wild-type ICSBP cDNA that lacked an epitope tag.

Phosphorylation of ICSBP has been reported to affect its ability to associate with other transcription factors (18). While the regulation of IRF4 function by phosphorylation has not been reported, it is likely to share this regulatory mechanism with ICSBP. PU.1 that is phosphorylated at serine 148 by protein kinase CK2 has a 9- to 10-fold higher binding affinity for IRF4, compared with unphosphorylated PU.1 (21, 35). Similarly, Eklund et al. (24) showed that the ability of ICSBP to activate transcription from a synthetic promoter containing four copies of the gp91phox composite element also required PU.1 phosphorylation at serine 148. Thus, both IRF4 and ICSBP appear to require PU.1 phosphorylation at the same site for maximal protein-protein interaction. Like Eklund et al. (24), we also found that ICSBP and PU.1 could synergistically activate transcription from a PU.1/IRF composite element. These data are consistent with earlier studies showing that IRF4 shares this capacity in NIH-3T3 cells, and that serine 148 on PU.1 is required for both IRF proteins to activate transcription (48). We have extended these findings by showing that the natural IL-1β promoter could be synergistically activated by these IRF proteins in conjunction with PU.1. In macrophages, over-expression of ICSBP modestly enhanced basal transcription of the IL-1β reporter plasmid, and IRF4 was able to activate basal transcription in a dose-dependent manner (Fig. 9). In NIH-3T3 fibroblasts, serine 148 of PU.1 was required for maximal synergy with these IRF proteins in the activation of transcription of the IL-1β promoter. However, we propose that the interaction between PU.1 and ICSBP is more dependent upon serine 148 phosphorylation than the interaction between PU.1 and IRF4. This is based on our findings: 1) that only IRF4 could increase IL-1β reporter activity in a dose-dependent manner in unstimulated RAW264.7 macrophages (i.e., cells that contain little phosphorylated PU.1), and 2) that only IRF4 was capable of synergizing with the SI48A PU.1 mutant (albeit at lower levels than wild-type PU.1). A role for ICSBP in the regulation of the gp91phox and IL-12 p40 promoters has been reported, whereas the specific role of IRF4 in macrophages remains to be determined. However, mice lacking either a functional IRF4 or ICSBP gene do exhibit impaired resistance to intracellular infection (13–16). Mice deficient in IRF4 do not generate mature, functional lymphocytes (16), although the effects of IRF4 deficiency on macrophage function have not yet been reported. Future studies will be needed to determine precisely how these transcription factors contribute to the phenotype observed in these knockout mice.

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References


