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IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages

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Abstract IRF-8/ICSBP and IRF-1 are IRF family members whose expression is induced in response to IFN-γ in macrophages. IL-12 is a cytokine produced in macrophages that plays a critical role in host defense. IFN-γ and bacterial lipopolysaccharide (LPS) induce IL-12p40 transcription, which is necessary for the production of IL-12. We have previously shown that IL-12p40 expression is impaired in ICSBP-deficient mice and that transfection of ICSBP together with IRF-1 can activate IL-12p40 expression in mouse macrophage cells. To further study the role of ICSBP and IRF-1, we investigated murine IL-12p40 promoter activity in the macrophage cell line RAW 264.7. We show here that co-transfection of ICSBP and IRF-1 synergistically stimulates IL-12 promoter activity to a level comparable to that induced by IFN-γ/LPS. Mutation of the Ets or NFκB site previously shown to be important for IL-12p40 transcription did not abolish the activation by ICSBP and IRF-1. However, mutation of the ISRE-like site found downstream from the NFκB and C/EBP sites abrogated the activation by ICSBP and IRF-1. Together, these results indicate that ICSBP and IRF-1 cooperatively stimulate murine IL-12 transcription through a novel regulatory element in the murine promoter.

Key words: Interleukin-12; ICSBP; IRF-1; Mouse macrophage; ISRE; IFN-γ

1. Introduction

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa. While p35 is expressed constitutively in a broad array of cells, IL-12p40 is expressed specifically in macrophage/dendritic cells upon activation as well as B cells and neutrophiles [1]. IL-12 exerts an anti-microbial effect, primarily because it induces expression of interferon-γ (IFN-γ) in T cells or NK cells, thereby promoting the development of Th1 responses and inhibiting Th2 responses [2]. The finding that IL-12 p40 knockout mice have a severely depressed Th1 response supports the role of IL-12 in the Th1 response and resistance to infections [3–6]. Previously, treatment of macrophages with IFN-γ followed by LPS has been shown to potently induce IL-12p40 mRNA expression, where IFN-γ is thought to exert a priming effect [7].

Several laboratories have studied the transcriptional regulation of IL-12p40 [8,9]. Studying the mouse IL-12 promoter, Murphy et al. reported that the Rel/NFκB site is important for promoter activity stimulated by IFN-γ and LPS [10]. A later study by Plevy et al. demonstrated a role for the C/EBP site, located downstream from the Rel/NFκB site, which is shown to cooperate with the Rel/NFκB site [11]. On the other hand, Ma et al. studying the human promoter revealed that the Ets site, residing upstream from the Rel/NFκB site, conserved in the human and mouse genes, is required for stimulation by IFN-γ and LPS [12]. These authors showed that a sequence containing the Ets site binds to an inducible protein complex containing several factors. We have previously reported that IL-12 expression is impaired in IRF-8/interferon consensus sequence binding protein (ICSBP) knockout mice and that such animals are highly susceptible to infections of *Toxoplasma gondii* and *Leishmania major* because they do not produce IFN-γ [13,14]. Later we showed that IRF-8/ICSBP can enhance human and mouse IL-12p40 promoter activity when transfected into ICSBP−/− and +/+ mouse macrophages [15]. Further, ICSBP was assembled into a multi-protein complex containing interferon regulatory factor-1 (IRF-1) that bound to the Ets site in the promoter identified by Ma et al. [7]. In this study we further investigated the role of ICSBP in the activation of the murine IL-12 p40 promoter, which slightly differs from its human counterpart in nucleotide sequence. We show that ICSBP and IRF-1 synergistically stimulate murine IL-12p40 promoter activity through a novel site that resembles the interferon stimulated responsive element (ISRE), a target element for the IRF family that is located downstream from the NFκB site. Our results further support a critical role for ICSBP and IRF-1 in IL-12p40 expression.

2. Materials and methods

2.1. Cell culture and reagents

The murine macrophage-like cells RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with glutamine, antibiotics and 10% FBS (endotoxin <1 ng/ml). Mouse IFN-γ was a gift from Toray. Lipopolysaccharide (LPS) was purchased from Difco Laboratories.

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Abbreviations: ICSBP, interferon consensus sequence binding protein; IRF-1, interferon regulatory factor-1; IFN-γ, interferon-γ; IL-12, interleukin-12; ISRE, interferon stimulated responsive element

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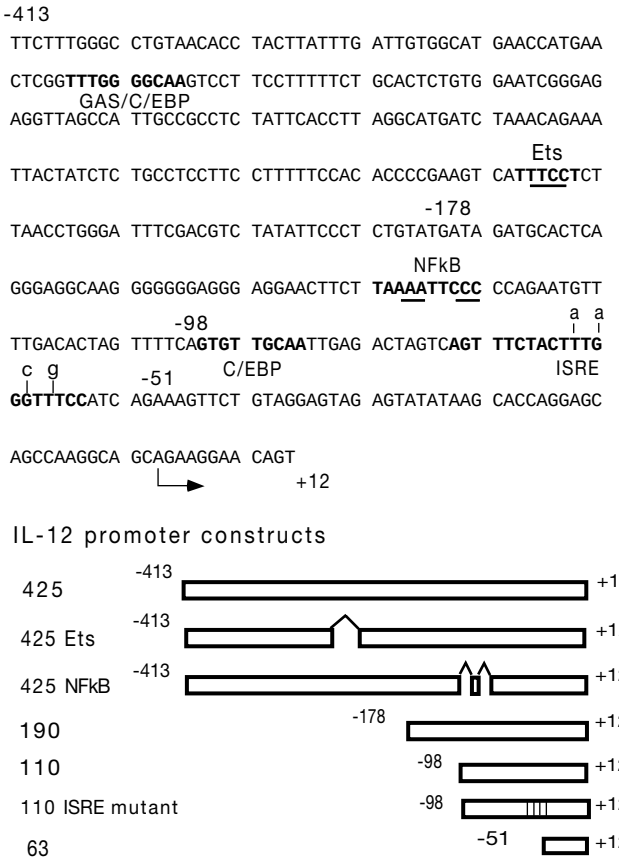


Fig. 1. A schematic representation of the truncated murine IL-12 promoter. Murine IL-12p40 promoter-luciferase plasmids were generated by PCR using murine genomic DNA. The mutations disrupt the Ets site (−220/−217) and the NFκB half-site (−133/−131, −128/126). Bold-faced type indicates GAS/C/EBP, Ets, NFκB, C/EBP and ISRE regions. Disrupted sequences of Ets and NFκB are underlined. Substition mutants were created in the context of an ISRE site (−75/−56) indicated by lower-case letters.

2.2. Plasmid construction

A PCR fragment generated from the promoter region of the murine IL-12 gene was cloned into the pGL2 luciferase vector (Promega, Madison, WI, USA) [10,11,16]. Deletion constructs were generated by PCR and sequenced by the dideoxy method. Expression vectors for ICSBPLK440 were generated under the control of the β-actin promoter as described [15], and IRF-1pcDNA3.1, under the control of CMV promoter. All plasmids used for transfection assays were prepared by CsCl double banding or with an EndoFree plasmid kit (QIAGEN) to minimize endotoxin contamination.

2.3. Transfection assay

RAW cells were transfected with 400 ng of luciferase reporter or given amounts of expression vectors (up to 200 ng DNA) using lipofectamine PLUS (Life Technologies). The amount of transfected DNA was adjusted with empty vectors LK440 or pcDNA3.1. Five hours after transfection, the cell culture medium was changed to complete medium containing 10% FBS and stimulated with IFN-γ (1000 u/ml) for 16–20 h, followed by LPS (100 ng/ml) for an additional 4–8 h. Cells were assayed for luciferase activity using the dual luciferase assay system (Promega). Reporter activity is shown as the avarage of three determinations and standard error.

2.4. DNA affinity binding assay

Biotinylated DNA fragments encompassing the ISRE-like site of IL-12p40 were synthesized from the sequence (−56 to −75) using biotinylated primer as detailed in [15,17]. Three hundred nanograms of biotinylated DNA was conjugated to 20 μl of streptavidin-bound magnetic beads (Dynabeads, M280, Dynal, Lake Success, NY, USA) in TGEDN buffer containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl, 1 mM DTT, 0.1% NP-40 and 10% glycerol and then incubated with 500 μg of RAW cell nuclear extract and 20 μg of salmon sperm DNA (Sigma) at 4°C for 2 h. Beads were washed in TGEDN buffer, and bound materials were eluted in 20 μl of the same buffer and boiled. Eluted materials were separated by 10% SDS–PAGE and detected by immunoblot analysis using rabbit anti-ICSBP [15,17], anti-IRF-1 [15,17] and anti-YY1 antibodies (Santa Cruz) with the enhanced chemiluminescence kit (Amersham). Nuclear extracts were prepared from RAW cells treated with IFN-γ (1000 U/ml) for 20 h.

2.5. RT-PCR

Quantitative PCR was performed as previously described [15]. cDNAs were prepared from 1 μg of total RNA using Molony murine leukemia virus reverse transcriptase (Life Technologies). Dilluted cDNA was subscribed to PCR for IL-12 (35 cycles) and HPRT (28 cycles), using each primer as previously reported [15].

3. Results

3.1. IRF-8/ICSBP and IRF-1 activate the murine IL-12p40 promoter in RAW264.7 cells

It has been shown that the IL-12p40 gene is expressed in the macrophage cell line RAW264.7 (hereafter: RAW) following treatment with IFN-γ followed by LPS [7,15]. We tested the activity of a luciferase reporter fused to a 425-bp murine IL-12 p40 promoter in RAW cells. The 425 promoter contains the NFκB, C/EBP and Ets site, previously reported to contribute to the transcription of IL-12p40 (Fig. 1). As seen in Fig. 2, IFN-γ and LPS alone modestly stimulated reporter activity, while IFN-γ plus LPS gave ∼ a five-fold increase, indicating that this promoter can be activated by IFN-γ/LPS in RAW cells. Previous study with gene knockout mice showed that both ICSBP and IRF-1 play a role in IL-12p40 expression [15]. The two factors are synergistically induced by IFN-γ and LPS in macrophages [18]. The aim of this work has

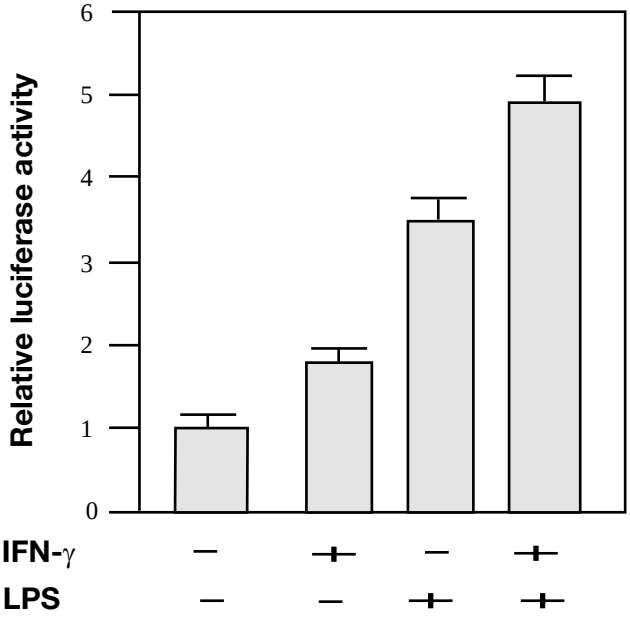


Fig. 2. Murine IL-12 promoter activated RAW 264.7 cells in the presence of IFN-γ/LPS. The IL-12 promoter (425 bp) was transfected into RAW cells and the cells were treated with IFN-γ and LPS as described in Materials and methods. Each result represents the mean ± S.D. of data from three to five experiments.

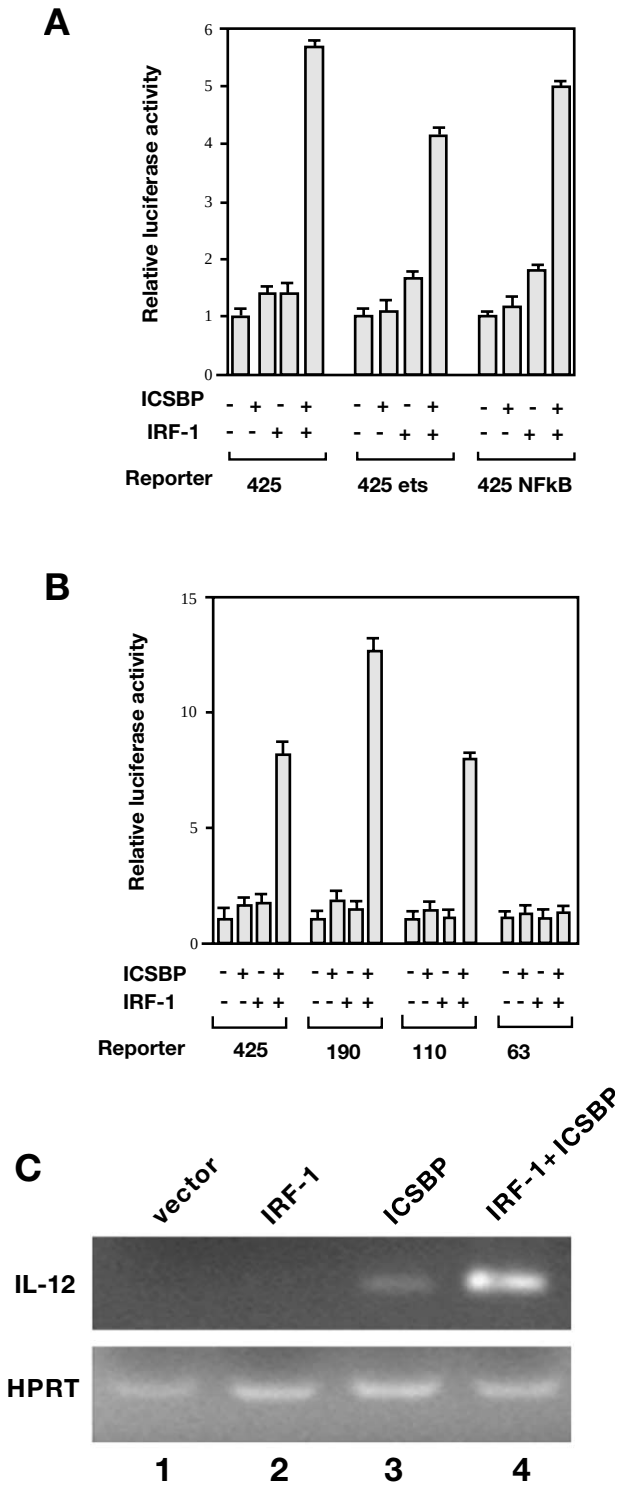


Fig. 3. Stimulation of murine IL-12 promoter activity by ICSBP and IRF-1. A: RAW cells were transfected with the murine IL-12 promoter (400 ng) and its mutant luciferase reporters (400 ng) with ICSBP (100 ng), IRF-1 (100 ng) and control vector (100 ng). B: ICSBP and IRF-1 were transfected into RAW cells with IL-12 deletion reporters. Luciferase assay was performed 24 h post-transfection. Each result represents the mean ± S.D. of data from three to five experiments. C: Stimulation of endogenous IL-12 transcription by co-transfection of ICSBP and IRF-1. RAW cells were transfected with ICSBP (200 ng), IRF-2 (200 ng) and empty vector. Total RNA was isolated and RT-PCR was performed by using oligonucleiotids specific for murine IL-12 or HPRT as described in Materials and methods.

been to study whether exogenous ICSBP and IRF-1 stimulate IL-12p40 reporter activity, and if so, to determine the regulatory element responsible for the activity of these factors. As shown in Fig. 3A, transfection of ICSBP and IRF-1 alone stimulated IL-12 promoter activity only slightly. However, co-transfection of both factors led to a stronger reporter activity comparable to that seen by IFN-γ and LPS (Fig. 3A).

To determine the element through which ICSBP and IRF-1 activate the IL-12p40 reporter, mutant reporters in which the Ets or NFκB sites were mutated were tested (see Fig. 1 for mutated nucleotides). Neither mutation affected the ability of ICSBP and IRF-1 to stimulate reporter activity, indicating that these factors act through a site distinct from the Ets or NFκB site (Fig. 3A). To further delineate the region necessary for activation by ICSBP and IRF-1, reporters with progressive 5′ deletions (see Fig. 1) were tested. The data in Fig. 3B show that while reporters with 190-bp and 110-bp IL-12p40 promoters could be stimulated by ICSBP and IRF-1, the reporter containing only a 63-bp promoter could not, indicating that the critical element resides downstream of position ~−98 and upstream of position ~−51. We tested the effect of ICSBP and IRF-1 on endogenous IL-12 gene expression in RAW cells. Transfection of ICSBP induced IL-12 gene modestly; however, co-transfection of both factors IRF-1/ICSBP led to a much greater level of stimulation of IL-12 gene expression (Fig. 3C).

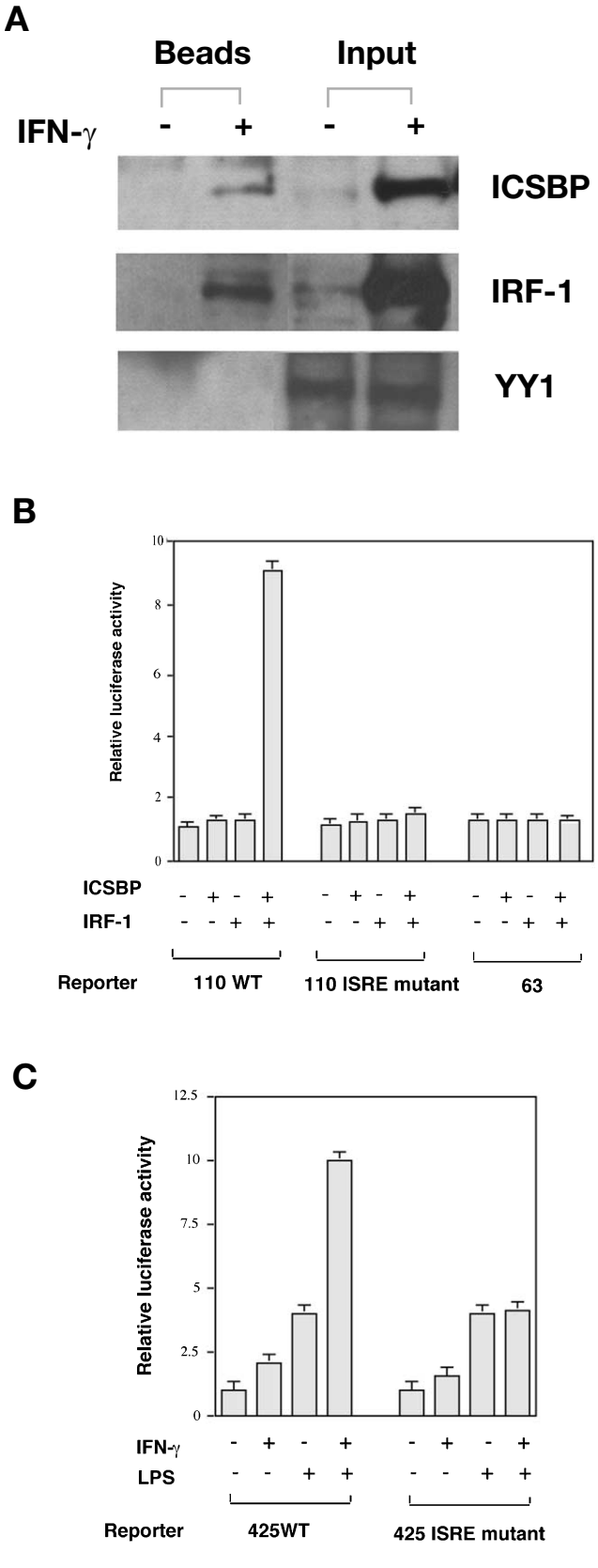
3.2. *ICSBP and IRF-1 bind to the ISRE-like site in the IL-12 promoter*

Inspection of the sequence between −98 and −51 reveals the presence of an element resembling the ISRE (see Fig. 1A TTTCTSCTTTGGGTTTC). A typical ISRE contains a direct repeat of the GAAA motif with a spacer of several nucleotides [19]. To test the possibility that ICSBP and IRF-1 bind to this element, we performed DNA affinity binding assays. The biotinylated ISRE-like element was immobilized on magnetic beads and incubated with nuclear extracts from RAW cells treated with or without IFN-γ for 16 h. As shown in the input lanes in Fig. 4A, IFN-γ enhanced the expression of IRF-1 and ICSBP. Both proteins in IFN-γ-treated samples were bound to the ISRE-conjugated beads (Fig. 4A). In contrast, untreated samples showed no binding. YY1, expressed in RAW cells with and without IFN-γ, did not bind to the ISRE beads, indicating the specificity of the binding.

To confirm that the ISRE-like element is important for the activity of ICSBP/IRF-1, a mutant IL-12 reporter containing 4-bp mutations in the ISRE was tested in co-transfection assays. As shown in Fig. 4B, the wild-type 110-bp IL-12p40 reporter was activated by co-transfection of ICSBP and IRF-1. In contrast, the mutant reporter was not activated by ICSBP and IRF-1. These results indicate that ICSBP and IRF-1 act through the ISRE-like element to stimulate IL-12p40 promoter activity in macrophages. To test whether the IFN-γ/LPS treatment induce IL-12 promoter activity through ISRE-like site, RAW cells were transfected with 425 ISRE mutant reporter and treated with IFN-γ/LPS. IFN-γ/LPS synergistically stimulated 425 wild-type promoter (Figs. 4C and 2), whereas synergistical effect of IFN-γ/LPS was not observed in RAW cells transfected with 425 ISRE mutant reporter (Fig. 4C). These results indicated that cooperative regulation of ICSBP and IRF-1 on an ISRE-like site are important for IL-12 stimulation by IFN-γ/LPS.

3.3. The role of coactivator p300 in activating the IL-12p40 promoter

IRF-1 has been shown to interact with the coactivator p300/CBP [20]. With their histone acetylase activity, the coactivators regulate the transcription of many genes. Further-



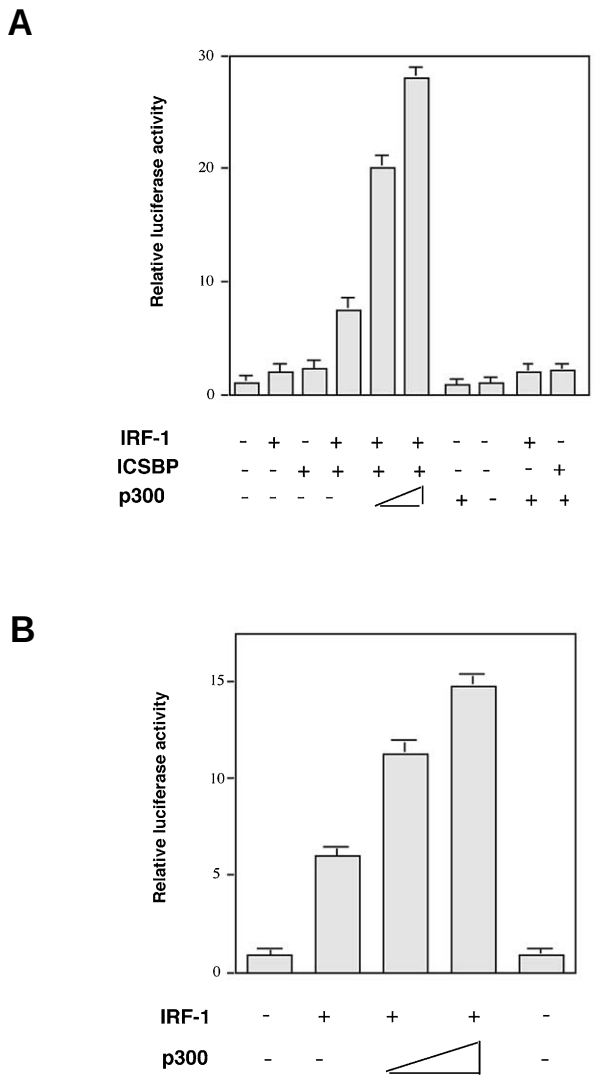


Fig. 5. Effect of p300 on IRF-1 and ICSBP-stimulated IL-12 promoter activity. A: p300 (100 ng and 200 ng) was co-transfected with ICSBP (100 ng), IRF-1 (100 ng) and a 110-bp IL-12 reporter (400 ng) into RAW cells. B: p300 (100 ng and 200 ng) was transfected with IRF-1 (100 ng) and ISG15-ISRE reporter (400 ng) into RAW cells. Luciferase assay was performed 24 h post-transfection. Each result represents the mean ± S.D. of data from three to five experiments.

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Fig. 4. ICSBP and IRF-1 bind to the ISRE-like site of the IL-12 promoter. A: Three hundred nanograms of biotinylated DNA containing the ISRE-like site (−56 to −75) was conjugated to 20 μl of magnetic beads and incubated with 500 μg of nuclear extract from RAW cells treated with or without IFN-γ. The beads were washed, and bound materials were eluted and detected by immunoblot assays. B: ICSBP, IRF-1 and the IL-12 reporter containing an ISRE-like site or mutant ISRE-like site were transfected into RAW cells. Luciferase assay was performed 24 h post-transfection. Each result represents the mean ± the standard deviation of data from three to five experiments. C: IL-12 reporter containing an ISRE-like site (425 WT) or mutant ISRE-like site (425 ISRE mutant) was transfected into RAW cells. Cells were treated with IFN-γ/LPS and luciferase assay was performed as described in Materials and methods. Each result represents the mean ± S.D. of data from three to five experiments.

more, we have previously shown that IRF-1 can be acetylated by p300 [21]. It was of interest to determine whether activation of the IL-12p40 promoter by ICSBP and IRF-1 involves p300. RAW cells were co-transfected with ICSBP and IRF-1 in the absence or presence of p300 (Fig. 5A). Co-transfection of p300 and IRF-1 without ICSBP did not stimulate IL-12p40 promoter activity. Similarly, co-transfection of p300 and ICSBP without IRF-1 failed to stimulate the promoter. However, when p300 was the co-transfected with ICSBP and IRF-1, promoter activity was markedly enhanced in a p300-dose-dependent manner. These results suggest that the coactivator p300 is recruited to the IL-12p40 promoter depending on both ICSBP and IRF-1. Unlike the IL-12p40 promoter, which required both factors, p300 enhanced the activity of a classic ISRE promoter from the ISG15 gene [17] when transfected with IRF-1 alone (without ICSBP) (Fig. 5B). The ISRE promoter is composed of GGAAACCGAAAC (Fig. 5B), which is related to, but distinct from, that of the IL-12p40 element.

4. Discussion

In this study, we have identified a novel cis-acting element in the murine IL-12p40 promoter, upon which ICSBP/IRF-8 and IRF-1 act to enhance transcription. The element, resembling the ISRE, resides downstream from the NFκB and C/EBP site. This element has not been implicated in the activation of IL-12p40 before. In our previous analysis of the human IL-12p40 promoter, ICSBP and IRF-1 were also found to synergistically stimulate transcription, but through another upstream regulatory element [7,15]. In the present study, a mutation of the Ets site in the murine promoter did not eliminate the activation by ICSBP and IRF-1, while a mutation in the ISRE-like element completely abrogated it. We also showed that both ICSBP and IRF-1 expressed in IFN-γ-stimulated RAW cells bind to the ISRE-like element, supporting the direct involvement of ICSBP and IRF-1 in IL-12p40 promoter activity. These results indicate that ICSBP and IRF-1 can activate both the human and murine IL-12p40 promoter, but through different elements. It is of note that although the Ets site and the ISRE-like element are similar between the human and murine IL-12p40 promoters, there are subtle nucleotide differences, mostly in the flanking sequences (the murine Ets TTTCCTCTTAACCT vs. human Ets TTTCCTCTTAGTT and murine ISRE TTTCTACTTTGGGTTTCC vs. human ISRE TTTCTAGTTTAAGTTTG). These small sequence differences may dictate the relative importance of the two elements for eliciting the activity of ICSBP and IRF-1. On the other hand, these elements may in fact be functional in both species, depending on the type of stimulation and cellular environment.

Previous reports on ICSBP−/− and IRF-1−/− mice are consistent with the importance of ICSBP and IRF-1 in regulating IL-12p40 transcription [22,13,14]. The impaired IL-12 expression in these mice is associated with a failure to express IFN-γ and to mount an effective host defense upon exposure to various infectious pathogens [23].

In this study and previous reports [7,15], ICSBP acted as an activator for both mouse and human IL-12p40 transcription. However, ICSBP was originally shown to repress the transcription of interferon-inducible promoters carrying the ISRE by repressing IRF-1 activity [24,25]. Several recent studies have indicated that this transcription factor has dual activity and can activate transcription from a subgroup of ISRE [26] and related GAS sequences [27].

It is worth noting that the ISRE-like element in the IL-12p40 promoter differs from the classic ISRE that tends to contain two GAAA motifs with a 2–3-bp spacer. The element in the IL-12p40 contains two GAAA motifs with a much longer spacer of 8 bp. Furthermore, the 3′ part of the GAAA motif overlaps with an Ets site of a GGAA motif. It is likely that the binding of ICSBP and IRF-1 on the IL-12p40 element is distinct from that on the classic ISRE. Although it is not clear how ICSBP and IRF-1 make contact with the element, the way in which ICSBP and IRF-1 bind the DNA may influence their functionality as a transcription factor. It may also influence how a coactivator such as p300 and PCAF is recruited to the element. Previously we noted that p300 can bind to IRF-1 and IRF-2 [21]. In the present study, p300 enhanced the IL-12 promoter activity when both ICSBP and IRF-1 were co-transfected, but not when only IRF-1 was transfected. In contrast, p300 enhanced ISG15 ISRE promoter activity with IRF-1 alone (Fig. 5B). In conclusion, the present study revealed that ICSBP/IRF-8 can activate the murine IL-12p40 promoter in cooperation with IRF-1 through a novel ISRE-like element. Further work is required to elucidate the mechanism by which this element recruits ICSBP, IRF-1 and other transcription factors and activates transcription.

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