Active repression of IFN regulatory factor-1-mediated transactivation by IFN regulatory factor-4

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Abstract

IFN regulatory factor-4 (IRF-4) is a transcription factor that is involved in the development and the functions of lymphocytes, macrophages and dendritic cells. Despite their critical roles in immune system regulation, the target genes controlled by IRF-4 are poorly understood. In this study, we determined the consensus DNA-binding sequences preferred for IRF-4 by in vitro binding site selections. IRF-4 preferentially bound to the sequences containing tandem repeats of 5'-GAAA-3', flanked by CpC, in most cases. IRF-4 repressed the promoter bearing tandem copies of the selected binding sequence, while IRF-1 activated the same constructs. Interestingly, the IRF-1-dependent transactivation is inhibited in the presence of IRF-4, but not IRF-2. A series of deletion mutants of IRF-4 revealed that its DNA-binding domain was necessary and sufficient to antagonize the IRF-1-dependent transactivation. This dominant negative action of IRF-4 over IRF-1 was also observed in a natural promoter context, such as the TRAIL gene. These results indicate that IRF-4 acts as a natural antagonist against IRF-1 in immune cells.

Introduction

IFN regulatory factors (IRFs) constitute a family of transcription factors that mediate IFN signaling, confer an anti-viral state and modulate the immune system. Ten cellular members and several virus-encoded IRF genes have been identified to date. Some IRFs are immune cell specific, while others are ubiquitously expressed (1–3). All IRFs share significant homology within the amino-terminal DNA-binding domain (DBD), which is characterized by a winged helix-turn-helix motif with five tryptophan repeats. Through this domain, activation by IFN-α/β typically induces IRF family members to bind to the IFN-stimulated response elements (ISRE) found within IFN-responsive genes (4). Some IRF proteins also contain the IRF association domain (IAD) within the carboxyl-terminus, through which protein–protein interactions with IRFs or non-IRF proteins are believed to occur. This domain is common to IRF-3, IRF-4, IRF-5, interferon consensus sequence binding protein/IRF-8 and ISGF3γ/IRF-9, but not to IRF-1, IRF-2, IRF-6 or IRF-7.

IRF-4 is a member of the IRF family, and its expression is restricted to immune cells, such as lymphocyte, macrophage and dendritic cells (5–9). It is not induced by IFNs, but rather by antigen-receptor-mediated stimuli, such as plant lectins, CD3 or IgM cross-linking (6). Consistent with its highly limited expression, the deficiency in IRF-4 was manifested in very specific manners in the immune system (10). As for B cell function, the serum immunoglobulin levels were dramatically reduced and the antibody responses were absent. IRF-4-deficient T lymphocytes showed a reduced proliferative response and lower cytokine production, and lacked cytotoxic and anti-tumor responses (10). IRF-4 was essential for the Th2
response (11–13). Interestingly, this response seems to be attributable not only to IRF-4-expressing Th2 cells but also to dendritic cells, as we recently discovered the importance of IRF-4 in the development of CD11b<sup>high</sup> CD8α<sup>+</sup> dendritic cells, which are important to polarize Th0 cells to Th2 (14). In contrast to the wealth of information about the physiological significance mentioned above, the downstream molecules of IRF-4 have remained essentially uncharacterized.

IRF-4 was initially identified as a transcriptional activator. This function was linked to a physical interaction with the hematopoietic cell-specific transactivator, PU.1, on a composite Ets/ISRE element within the Igα light chain enhancer in B cells (5). IRF-4 also associates with E47, Stat6, Bcl-6 and NFATc2 to synergistically activate particular genes (11, 15, 16). On the other hand, IRF-4 was reported to function as a transcriptional repressor when bound to the ISRE DNA motifs of some genes (7). Thus, IRF-4 may serve as either an activator or a repressor, depending on the context of the DNA-binding sequences and/or the protein-interaction partners. To clarify the former, we tried to determine the optimal DNA sequence recognized by IRF-4 in the absence of protein-interaction partners. Here we report that the selected binding sequence was a defined derivative of the consensus ISRE.

We describe our functional characterizations of the selected binding site both in vitro and in vivo.

**Methods**

**Cell culture**

HeLa cells were grown in DMEM supplemented with 10% FCS and 100 U ml<sup>−1</sup> penicillin–streptomycin in a 37°C incubator with 5% CO<sub>2</sub> and 100% humidity. DMEM was purchased from Sigma (St Louis, MO, USA). FCS was purchased from Life Technologies (Rockville, MD, USA). 293 T cells were grown in αMEM supplemented with 10% FCS and 100 U ml<sup>−1</sup> penicillin–streptomycin in a 37°C incubator with 5% CO<sub>2</sub> and 100% humidity. αMEM was purchased from GIBCO BRL (Gaithersburg, MD, USA).

**Selected and amplified binding sites determination**

Selected and amplified binding site (SAAB) selection was done essentially as described by Blackwell and Weintraub (17), using affinity chromatography. The ‘random’ oligonucleotide contained 20 random nucleotides flanked by the known sequences ‘b’ and ‘a’, which could be recognized by the PCR primers ‘b’ (5′-AGACGGAATCCATGCA-3′) and ‘a’ (5′-TCCGGATTCTACAG-3′), respectively [sequences from Blackwell and Weintraub (17)]. Double-stranded random oligonucleotides were generated by annealing 1.5 μg of the single-stranded random oligonucleotide with 0.7 μg of the PCR primer ‘a’, followed by filling-in with Klenow DNA polymerase. The double-stranded oligonucleotides (0.4 μg) were incubated with the glutathione-S-transferase (GST)–IRF-4 fusion protein (400 ng) attached to glutathione-Sepharose beads in binding buffer [10 mM HEPES-KOH (pH 7.9), 0.2 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] with 200 μg ml<sup>−1</sup> poly(dG):poly(dC) at room temperature for 30 min. The beads were recovered by brief centrifugation, washed twice with binding buffer, suspended in 30 μl of H<sub>2</sub>O and incubated at 95°C for 5 min to release the oligonucleotides bound to the GST-IRF-4 affinity beads. A 10-μl aliquot was then used for PCR amplification in a 25-μl reaction for 30 cycles of 94°C (30 s), 38°C (30 s) and 72°C (30 s). For subsequent rounds, a 10-μl aliquot of the PCR was incubated with 40 ng of protein. After five rounds of selection, the recovered oligonucleotides were digested with BamHI and EcoRI, cloned into pBluescript KS+(+) and subjected to sequence analysis.

**Plasmid constructions**

To construct an expression plasmid for the GST–IRF-4 fusion protein, the human IFN regulatory factor-4 (hIRF-4) cDNA was inserted into the pGEX vector (Amersham Pharmacia Biotech, Uppsala, Sweden), via the BamHI and EcoRI sites. Flank-tagged hIRF-4 cDNA was excised from the plasmid pBlueScript Flag–hIRF-4 by HindIII and XbaI digestions and then ligated into the corresponding sites of the pcDNA3 vector (Invitrogen, San Diego, CA, USA). Detailed information about the construction of the plasmid pBlueScript Flag–hIRF-4 will be provided upon request.

To prepare the hIRF-4 carboxyl-terminal deletion protein 1–129, pcDNA3 Flag–hIRF-4 was digested with EcoRI and XbaI, blunt ended by Klenow polymerase and then self-ligated. To prepare the hIRF-4 carboxyl-terminal deletion proteins 1–200 and 1–336, pcDNA3 Flag–hIRF-4 was digested with ScaI or Apal, blunt ended by T4 DNA polymerase and then self-ligated. To prepare the hIRF-4 carboxyl-terminal deletion protein 1–432, pBlueScript Flag–hIRF-4 was digested with SpeI and BglII, blunt ended by Klenow polymerase and self-ligated to yield the plasmid pBlueScript Flag–hIRF-4 1–432. This plasmid was digested with HindIII and XbaI, and the DNA fragment containing the Flag–hIRF-4 1–432 sequence was recovered and ligated into the corresponding sites of pcDNA3. The Ndel–PvuII fragment of pBlueScript Flag–hIRF-4 1–432 was replaced by a linker DNA (5′-CGTTAACG-3′). Then, the HindIII–XbaI fragment containing Flag–hIRF-4 116–450 was recovered and ligated into the corresponding sites of pcDNA3.

Human IRF-1 and IRF-2 were amplified by PCR and inserted into the Ndel and NotI sites, respectively, of the plasmid pBlueScript Flag–PAF49. The resultant plasmids, pBlueScript Flag–hIRF-1 and pBlueScript Flag–hIRF-2, were digested with BamHI and NotI, and then the Flag–hIRF-1 and Flag–hIRF-2 fragments were ligated into the corresponding sites of pcDNA3, respectively. For the construction of reporter plasmids containing one, two or four copies of the selected binding site, the double-stranded oligonucleotides (5′-GCCCGGAAACCGAAACCATGC-3′) were tandemly ligated and cloned into the Smal site of the pGL2-Promoter vector (Promega, Madison, WI, USA). The region of DNA containing the DBD of IRF-4 was obtained by PCR with the following primer set: 5′-GGAATCCATATGGGCAACGGGAAGCTCCGC-3′ and 3′-CCGCTCGAGTCCTTTTTGGCTCCCTCAG-GAAC-3′. The PCR product was digested with Ndel and XhoI, and inserted into the corresponding sites of the expression vector pET21a (+).

The human TRAIL promoter constructs were kindly provided by B. Mark Evers, University of Texas Medical Branch (18). Point mutations were introduced in the putative ISRE sites on
the TRAIL promoter by the Quick Change Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA).

The SAAB<sub>DCIR</sub> luciferase vector was constructed in the pGL3 promoter vector with the concatemer consisting of four tandem repeats of a candidate SAAB motif, ACACGAAAAC-GAAACCT, found in human dendritic cell immunoreceptor (DCIR) gene promoter.

**Protein preparation**

*Escherichia coli* TG1 cells harboring pGEX hIRF-4 were grown in LB medium at 30°C to an optical density of 0.6 at 600 nm, and then isopropyl-β-D-thiogalactopyranoside was added to a 2 mM final concentration and the culture was further incubated for 3 h. Cells were re-suspended in PBS supplemented with 0.5 mM PMSF, and then lysed by sonication. Cellular debris was removed by ultracentrifugation. The supernatant was used for the SAAB assay. For EMSA, *E. coli* BL21 (DE3) cells harboring the plasmid pET-hIRF-4DBD were grown, and the lysate was prepared as described above. The lysate was applied to a HiTrap column (Amersham Pharmacia Biotech), and then the column was washed with PBS supplemented with 10 mM imidazole. Bound proteins were eluted by PBS supplemented with 200 mM imidazole. The eluted proteins were desalted by passage through a PD-10 column (Amersham Pharmacia Biotech) at 25°C. The eluted proteins were desalted and 20% glycerol. Protein concentrations were determined by passage through a PD-10 column (Amersham Pharmacia Biotech) at 25°C. The eluted proteins were desalted and 20% glycerol. Protein concentrations were determined by the column was washed with PBS supplemented with 10 mM imidazole. Bound proteins were eluted by PBS supplemented with 200 mM imidazole. The eluted proteins were desalted by passage through a PD-10 column (Amersham Pharmacia Biotech) and then the column was washed with PBS supplemented containing 10% fetal bovine serum with antibiotics for 24 h after Nucleofection, and then were harvested for the luciferase assay.

**Reporter assays**

HeLa or 293T cells (1.5 × 10<sup>5</sup>) were seeded into six-well plates and transfected with 1 μg reporter plasmid and 1 ng pRL-β-actin as an internal control (19), together with up to 1 μg of pcDNA3, pcDNA3Flag-hIRF1 or pcDNA3Flag-hIRF4. Twenty-four hours after transfection, extracts were prepared from the transfected cells and the luciferase activity was determined using the Dual Luciferase Assay kit, according to the manufacturer's protocol (Promega). The luciferase activity was normalized with the *Renilla* luciferase activity from the internal control, and is represented as the relative luciferase activity.

PBMCs were prepared from blood according to the standard Ficol Paque protocol, and were subjected to gene transfer using the Nucleofector equipment, according to the manufacturer's recommendations (Amaxa Biosystems, Allemagne, Germany). Typically, 2 × 10<sup>6</sup> PBMCs were used for the Nucleofection with 1 μg reporter plasmid and 1 ng pRL-β-actin as an internal control, together with 1 μg of pcDNA3, pcDNA3Flag-hIRF1 or pcDNA3Flag-hIRF4, to keep the total amount of plasmid at 3 μg. Cells were cultured in DMEM containing 10% fetal bovine serum with antibiotics for 24 h after Nucleofection, and then were harvested for the luciferase assay.

**Nuclear extract preparation**

Nuclear extracts were prepared according to the method of Schreiber et al. (20). Briefly, 5 × 10<sup>5</sup> cells were treated with 250 U ml<sup>-1</sup> IFN-γ. After 12 h, the cells were washed with ice-cold PBS, suspended in 200 μl of buffer A [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF] and incubated on ice for 15 min. The cells were then lysed in the presence of 0.6% Nonidet P-40 by vortexing for 10 s. The nuclei were collected by centrifugation for 15 s and were resuspended in 50 μl of buffer C [200 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF] at 4°C for 15 min. The nuclear extracts were recovered after centrifugation at 15 000 r.p.m. for 5 min and were stored at −80°C.

**Reverse transcription–PCR**

Splenic T cells were enriched from a spleen cell suspension by density-gradient centrifugation on Lymphocyte-M (Cedarlane Laboratories, Ontario, Canada), and then were negatively selected with a Pan T Cell Isolation Kit (Miltenyi Biotec, Germany). Total RNA was isolated from splenic T cells of wild-type, IRF-1<sup>−/−</sup>, IRF-4<sup>−/−</sup> and IRF-1<sup>−/−</sup>,IRF-4<sup>−/−</sup> mice, which were either unstimulated or stimulated by Con A for 6, 12 and 24 h, using the ISOGEN reagent according to the manufacturer's protocol (Nippon Gene, Tokyo, Japan). The first-strand cDNA was synthesized using the ProSTAR first-strand RT–PCR kit (Stratagene) with oligo(dt) primers. One microlitre of the first-strand cDNA reaction was used in a 20-μl PCR amplification with the specific primers for murine DCIR (5’-GTGAT-CCAGAGCCAGGAAGA-3’/5’-TCATCTGAGTGGCAGGATGT-3’) and β-actin (5’-TGGAAATCTTGGCAGCATGAAAC-3’/5’-TAAACGCGCTCAGTACAGTCCG-3’), respectively. Cycling conditions were as follows: denaturing at 94°C for 60 s, annealing at 56°C for 60 s for DCIR and at 60°C for 60 s for β-actin and extension at 72°C for 60 s.

**EMSA**

The oligonucleotides used in this study had the following sequences: SAAB1, 5’-GCCCCGAACCGAAACCATGC-GC3’; and GBP-IRF4, 5’-GAATCGAAGATCTTACCT3’. The double-stranded DNAs were labeled by a filling-in reaction at the 5’-G overhangs with Klenow enzyme and [α<sup>32P</sup>]dCTP. Binding reactions were conducted with 2 μl of nuclear extracts in a 10-μl reaction containing 10 mM HEPES-KOH (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT and 10% PMSF at 4°C for 30 min. In some cases the reactions were further incubated with anti-IRF-1 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-His antibody (His-probe; Santa Cruz Biotechnology) at 25°C for 30 min. The nuclei were collected by centrifugation for 15 s and were resuspended in 50 μl of buffer C [200 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF] at 4°C for 15 min. The nuclear extracts were recovered after centrifugation at 15 000 r.p.m. for 5 min and were stored at −80°C.

**Results**

**Determination of the optimal DNA-binding sequence of IRF-4**

To determine the optimal DNA-binding sequence of IRF-4, the SAAB selection assay was performed with recombiant
GST–IRF-4 fusion protein (17). Oligonucleotides containing 20 random nucleotides, flanked by known sequences that could be recognized by PCR primers, were annealed with one of the PCR primers, and then converted to the double-stranded form by the Klenow enzyme. The double-stranded oligonucleotides were incubated with GST–IRF-4 attached to glutathione-Sepharose beads. The beads were collected, and the bound oligonucleotides were eluted and amplified by PCR. For subsequent rounds, the PCR products were incubated with GST–IRF-4 and the bound oligonucleotides were further selected. Repeating the binding-selection cycle several times should concentrate the oligonucleotides that have higher binding affinity to IRF-4. The sequences of the oligonucleotides selected after five sequential SAAB rounds are shown in Fig. 1. In general, all the selected oligonucleotides contained one or two copies of the ISRE core sequence, 5'-GAAA-3'. It is interesting to note that CpC dinucleotides are preferred for the sequences flanking the core sequence.

To examine whether IRF-4 can bind to the selected sequence, we performed an EMSA. As shown in Fig. 2, IRF-4 can bind to one of the representative selected sequences (SAAB1, 5'-GCCCCGAAACCAGAACCATGC-3'). The DNA–protein complex was challenged by a competition with excess amounts of unlabelled oligonucleotides. The SAAB1 sequence competed well with the probe DNA (lanes 3–5), similar to the ISRE of the guanine-binding protein gene (GBP), a well-known sequence used as the target for IRF family proteins (7) (lanes 6–8). These results clearly demonstrate that the selected sequence actually binds to IRF-4 with sufficiently high affinity.

**Transcriptional repression by IRF-4**

To examine whether the selected sequences function in vivo, we constructed luciferase reporters containing one, two or four copies of SAAB1, and transfected them into HeLa cells (Fig. 3). The promoter activity was increased, depending on the SAAB1 copy number (Fig. 3A), probably due to the activities of the intrinsic IRFs. Actually, IRF-1 could activate the reporter activity, in an SAAB1 sequence-dependent manner (Fig. 3B). In contrast, IRF-4 actively repressed the spontaneous activation of the SAAB1-Luc reporter gene (Fig. 3A). We also examined the effect of IRF-2, but it showed only a marginal repression of the SAAB1-Luc reporter gene (Fig. 3B). Next, we compared the effect of IRF-2 or IRF-4 on IRF-1 transactivation (Fig. 3C). IRF-2 is known to repress the IRF-1-mediated induction of several genes, such as IFN-β and major histocompatibility complex class I genes (1–3, 7). However, IRF-2 did not repress the IRF-1-mediated transactivation of the SAAB1-containing promoter. In contrast, IRF-4 reduced the IRF-1-mediated activation of the promoter almost to the basal level, even with only a small amount of the IRF-4 expression vector DNA. These results suggest that the selected sequence can serve as the target for the activation by IRF-1 and for the repression by IRF-4.

**The DBD of IRF-4 is necessary and sufficient for the antagonistic effect against IRF-1**

To examine the mechanism by which IRF-4 represses IRF-1-mediated transactivation, we constructed a series of deletion mutants of IRF-4 (Fig. 4). Structure–function analyses revealed...
Luciferase activity was normalized to the Renilla luciferase activity from the internal control plasmid, which was transfected simultaneously. Fold activations are indicated as relative values to the normalized luciferase activity of the parental reporter plasmid pGL2-Promoter.

Fig. 4. The DBD of IRF-4 is necessary and sufficient for the repression of IRF-1-mediated transactivation. The deletion mutants of IRF-4 are schematically represented on the left. The functional domains of IRF-4 are defined as follows: DBD, DNA-binding domain (aa 1–150); Pro, a proline-rich region (aa 151–237); IAD, an IRF association domain (aa 238–410); I, a C-terminal autoinhibition domain (aa 411–450). HeLa cells were transfected with the 4 × SAAB1 reporter construct, with (represented by solid bars) or without (represented by hatched bars) 0.5 µg of the IRF-1 expression plasmid, together with 0.5 µg of each IRF-4 deletion construct or an empty vector. The luciferase assay was performed as described in Fig. 3.

that IRF-4 contains the N-terminal DBD [located between amino acids (aa) 1 and 150], the proline-rich region (aa 150–237), the IAD (aa 237–410) and the C-terminal autoinhibition domain (aa 410–450) (1–3). Deletion of up to 321 aa from the C-terminus of IRF-4 had no effect on the repression activity for the IRF-1-mediated transactivation. In contrast, the removal of 115 residues from the N-terminus caused a complete loss of the repression activity. These results clearly indicate that the IRF-4 DBD is necessary and sufficient for the antagonizing effect against IRF-1-mediated transactivation.

**IRF-1 transactivates the TRAIL promoter**

We next examined the effects of IRF-4 and IRF-1 on the SAAB sequences in a natural promoter context in vivo. For this purpose, we searched the human genome database for sequences similar to the selected sequences in the known gene promoter, and found that the human TRAIL gene promoter contains two sequences, one (–129 to –140) that exactly matches and another (+4 to –8) that is highly homologous to one of the selected sequences (Fig. 5A). The luciferase reporter construct containing a 1.6-kb fragment of the human TRAIL promoter region (–1523) was dramatically activated by IRF-1 (Fig. 5B). Deletion of the 5′-upstream region up to –165 had no effect on the response to IRF-1, indicating that this region contains the elements that can confer the IRF-1 responsiveness to the promoter. We then mutated the two SAAB-like sequences found in this region, individually or simultaneously. Mutations in the distal site
(-140 TCTTTTCAGACGC -129) resulted in more than a 50% reduction in the transactivation by IRF-1. When we introduced mutations in the proximal site (-8 TCATTCGACGC +4), the response of the promoter to IRF-1 was reduced to one-tenth of that of the wild-type promoter. It should be noted that this region does not include the consensus initiator sequence, which functions as a core promoter element for some particular genes (22), suggesting that the mutated sequence did not violate the basal promoter activity. When the distal and proximal sites were mutated simultaneously, the promoter completely lost the ability to respond to IRF-1, indicating a synergistic activity of these sites. These results clearly demonstrate that the SAAB-like sequences found in the human TRAIL promoter actually serve as the target of IRF-1, and suggest that TRAIL gene expression is regulated by IRF family transcription factors.

**Fig. 5.** The SAAB-like sequences in the human TRAIL promoter respond to IRF-1. (A) Schematic representation of luciferase reporter constructs containing the human TRAIL promoter. The SAAB-like sequences and the corresponding mutations are indicated. (B) 293T cells were co-transfected with the TRAIL promoter–luciferase reporter plasmid with an internal control luciferase vector, together with pcDNA3Flag-hIRF-1 or an empty pcDNA3 vector. After 24 h, the luciferase activity was analyzed. The results shown are the averages of three independent experiments with standard deviation.

**Fig. 6.** The repression of IRF-1-mediated transactivation by IRF-4 in the human TRAIL promoter. (A) 293T cells containing the human TRAIL–luciferase constructs were co-transfected with the IRF-1 and/or IRF-4 expression vector. After a 24-h incubation, the luciferase activity was analyzed. The results shown are the averages of three independent experiments with standard deviation. (B) Experiments were done as in (A), with the exception that IRF-2, but not IRF-4, expression vector was used. (C) Experiments were done as in (A), with the exception that PBMCs were used.

**Dominant action of IRF-4 over IRF-1 on human TRAIL gene expression**

Next, we examined the effect of IRF-4 on the transactivation of the human TRAIL promoter by IRF-1. To do this, the TRAIL–luciferase construct was introduced into 293T, together with the expression vectors of IRF-1 and/or IRF-4. As shown in Fig. 6A, IRF-1 activated the TRAIL promoter, while IRF-4 alone showed marginal transactivation of the promoter. Co-transfection of IRF-4 and IRF-1 reduced the promoter activity significantly. As expected, IRF-2 failed to reduce the promoter activity of the TRAIL gene activated by IRF-1 (Fig. 6B). We also examined whether a similar finding would be obtained with PBMC, in the natural context. To do this, the TRAIL–luciferase construct was introduced into PBMCs, together with the expression vectors of IRF-1 and/or IRF-4. As shown in Fig. 6C, IRF-1 activated the TRAIL promoter in PBMCs, while IRF-4 alone showed marginal transactivation of the promoter. Co-transfection of IRF-4 and IRF-1 reduced the promoter activity to the same extent, as observed for IRF-4 only. These results
indicate that IRF-4 works as a dominant negative effector to IRF-1 in the natural promoter context in vivo.

Expression of the DCIR gene is regulated by IRF-1 and IRF-4

We also searched the mouse genome database, and found that the 3′-UTR of the murine DCIR gene contains a sequence highly homologous to the selected consensus sequence (5′-ACACGAAACCGAAACCT-3′). We thus analyzed DCIR mRNA induction following Con A stimulation in mouse splenic T cells. As shown in Fig. 7A, real-time reverse transcription–PCR revealed that the DCIR mRNA was induced by the Con A treatment in the wild-type splenic T cells. The Con A-dependent induction of the DCIR mRNA was augmented in the IRF-4-deficient cells. In contrast, the DCIR mRNA was not induced in IRF-1-deficient cells. Consistently, we demonstrated that IRF-1 transactivates the SAAB-like motif of DCIR, and this was inhibited by the presence of IRF-4 (Fig. 7B). These results suggest that the expression of DCIR strictly depends on the concerted actions of IRF-1 and IRF-4.

Discussion

In the present study, we tried to establish the range of IRF-4 activity by determining the DNA sequences recognized by IRF-4. The in vitro binding site selection revealed several features specific to IRF-4. IRF-4 preferentially bound to the sequences containing tandem repeats of 5′-GAAA-3′, which in most cases are flanked by CpC. Although IRF-4 exhibited similar preferences to the SAAB1 and GBP-derived ISRE in the EMSA, our recent fluorescent anisotropy measurements and calorimetric studies clearly demonstrated the IRF-4 preference for the former; the Ke value for the CGGAAA was ~0.3 μM, whereas the GBP-derived GGGAAA was ~4 μM (23, C. Kojima et al., unpublished results).

A similar binding-selection study was reported previously for IRF-1 and IRF-2, and the selected common sequence was 5′-G(A)/AAAG/C T/G GAAA G/C T/C-3′ (24). This sequence contains two copies of the core sequence 5′-GAAA-3′, with varied spacer sequence lengths. Although the tandem repeat of the 5′-GAAA-3′ core sequence is the same, IRF-4 requires a more stringent spacer length as well as the sequence. In this regard, IRF-1 is quite tolerant to variations in the recognition sequences, which partly explains its wide variety of biological functions. Fine-tuning of the target gene responses by recognition sequence variation was also reported for other members of the IRF family. For example, IRF-3 binds to 5′-GAAA(C/G)(C/G)GAAAN(T/C)-3′, whereas IRF-7 binds to 5′-GAAA(A/T)N(C/T)GAAAN(T/C)-3′ (25). IRF-3 is sensitive to the replacement of a single nucleotide within the GAAA core sequence, whereas IRF-7 has a wider recognition capacity. Such differences in the target sequence preference may lead to the exclusive primary induction of IFN-β by IRF-3 and the subsequent continuous expression of IFN-α gene family members by IRF-7, in the host defense system against viral infection (26, 27). It thus may be possible to consider that a particular set of genes bearing IRF-4-SAAB or related sequences are regulated by the concerted actions of IRF-1 and IRF-4 in immune cells.

With the knowledge accumulated to date, it is possible to predict the genome-wide distribution of transcription factor-binding sites in silico. We could actually find sequences related to IRF-4-SAAB within the promoter regions of several genes (data not shown). Among them, we demonstrated that two IRF-4-SAAB-related sequences, found in the promoter region of the human TRAIL gene, were necessary for the transactivation by IRF-1. Importantly, IRF-4 dominantly controlled the TRAIL promoter activity over the action of IRF-1. Sequential deletion analyses of IRF-4 revealed that aa 1–129, corresponding to the DBD, were sufficient to repress the IRF-1-dependent transactivation (Fig. 4). This result is consistent with the previous study demonstrating that an IRF-4 mutant, consisting of only the DBD, blocked IFN-α/β- and IRF-1-mediated activation (28). Interestingly, we could not detect the dominant action of IRF-4 over IRF-1 in embryonic carcinoma cells, such as P19 (data not shown), suggesting that the action of IRF-4 may be cell-type specific, even though it can be successfully expressed by transfection. It is also interesting to note that IRF-2, a well-known transcriptional repressor on ISRE, does not show this activity on either the SAAB1 or TRAIL promoter. Although the repressive activity of IRF-2 and IRF-4 was previously demonstrated on the ISREs of the IFN-β and H-2Ld promoters (7), we consider SAAB to be the first ISRE to be negatively regulated by IRF-4, but not IRF-2.

IRF-4 also seems to regulate the expression of the DCIR gene (29). DCIR, also called C-type lectin superfamily 6 protein.
IRF-4 is a natural antagonist against IRF-1 (CLECSF6) (30) or lectin-like immunoreceptor (31), is a transmembrane protein containing an extracellular lectin-like domain and an intracellular immunoreceptor tyrosine-based inhibitory motif (32). DCIR mRNA is expressed strongly in peripheral blood leukocytes, with moderate quantities in the spleen, lymph nodes and bone marrow, and at very low levels in the thymus (29). Although purified blood T cells did not express DCIR mRNA, we observed the weak but reproducible induction of DCIR in splenic T cells by Con A treatment. This induction was dependent on IRF-1, since no induction was observed in IRF-1-deficient T cells. In IRF-1-deficient splenocytes, it rather seems that DCIR was up-regulated at basal level, but repressed after the Con A treatment. It is possible that IRF-4 induced by Con A is involved in this repression (6). Indeed, IRF-4 seemed to repress the induced expression of DCIR, because the knockout of IRF-4 resulted in the augmentation of DCIR induction by Con A treatment. It is also possible that IRF-4 competitively represses IRF-1 and potentially other activators, including IRFs under certain physiological conditions. Since IRF-4 is exclusively expressed in the CD11b<sup>high</sup> CD8<sup>−</sup> dendritic subset, but not in the CD11b<sup>low</sup> CD8<sup>+</sup> dendritic subset (14), the active repression of DCIR by IRF-4 in the former subset would be involved in the development of CD11b<sup>high</sup> CD8<sup>−</sup> dendritic cells.

IRF-4 is unique among the IRF family members because it is not induced by IFN, but by antigen stimuli. Further research to investigate the role of IRF-4 in immune cells, particularly in terms of its ability to bind the IRF-4-SAAB sequence, may reveal the regulatory nature of IRF-4 in the IFN system.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DCIR</td>
<td>dendritic cell immunoreceptor</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>GBP</td>
<td>guanine-binding protein gene</td>
</tr>
<tr>
<td>hIRF</td>
<td>human IFN regulatory factor</td>
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<td>IAD</td>
<td>IRF association domain</td>
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<tr>
<td>IFN</td>
<td>IFN regulatory factor</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<tr>
<td>SAAB</td>
<td>selected and amplified binding site</td>
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References


