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IFN Regulatory Factor Family Members Differentially Regulate the Expression of Type III IFN (IFN-λ) Genes

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Virus replication induces the expression of antiviral type I (IFN-α/β) and type III (IFN-λ1–3 or IL-28A/B and IL-29) IFN genes via TLR-dependent and -independent pathways. Although type III IFNs differ genetically from type I IFNs, their similar biological antiviral functions suggest that their expression is regulated in a similar fashion. Structural and functional characterization of the IFN-α1 and IFN-λ3 gene promoters revealed them to be similar to IFN-β and IFN-γ genes, respectively. Both of these promoters had functional IFN-stimulated response element and NF-κB binding sites. The binding of IFN regulatory factors (IRF) to type III IFN promoter IFN-stimulated response element sites was the most important event regulating the expression of these genes. Ectopic expression of the components of TLR7 (MyD88 plus IRF1/IRF7), TLR3 (Toll/IL-1R domain-containing adapter-inducing factor), or retinoic acid-inducible gene I (RIG-I) signal transduction pathways induced the activation of IFN-α1 promoter, whereas the IFN-λ3 promoter was efficiently activated only by overexpression of MyD88 and IRF7. The ectopic expression of Pin1, a recently identified suppressor for IRF3-dependent antiviral response, decreased the IFN promoter activation induced by any of these three signal transduction pathways, including the MyD88-dependent one. To conclude, the data suggest that the IFN-α1 gene is regulated by virus-activated IRF3 and IRF7, thus resembling that of the IFN-β gene, whereas IFN-λ2/3 gene expression is mainly controlled by IRF7, thus resembling those of IFN-α genes.

One of the major cellular responses to virus infection is the synthesis of antiviral cytokines, IFNs. Type I IFNs (IFN-α/β) are the classical antiviral IFNs, but recently, a novel class of antiviral cytokines was discovered and classified as type III IFNs (IFN-λ1/II-29, IFN-λ2/II-28A, IFN-λ3/II-28B) (1, 2). These two types of IFNs, which display similar antiviral activities (1–4), have evolved separately and are only distantly related to each other. IFNs induce the expression of several antiviral proteins, such as dsRNA-activated protein kinase R, oligoadenylate synthetases, and Mx proteins, which ultimately mediate the antiviral actions of IFNs (5). In addition to the direct antiviral action, IFNs have many immunoregulatory functions. They can induce the expression of several cytokine and chemokine genes and prime cells for enhanced virus-induced production of IFNs and other cytokines. However, the main function of IFNs is likely to be the ability to inhibit virus replication in virus-infected cells and to protect uninfected cells from virus infection. Type I IFN induction is mainly regulated by IFN regulatory factor (IRF)3 and IRF7 (6, 7). IRF3 is expressed constitutively, whereas IRF7 is an IFN-stimulated gene and often induced at later phases of virus infection. It has been shown that IRF3 regulates the expression of IFN-β gene rather than IFN-α genes, whereas IRF7 activates both the late-phase IFN-α and IFN-β gene expression (6, 8, 9). Thus, the induction of IRF7 in response to IFNs and its activation after viral infection provide a positive feedback for the production of IFNs (6). Very recently, it was reported by Honda et al. (10) that in IRF7-deficient mice IFN induction is more severely impaired as compared with that in IRF3-deficient mice. IRF7 was suggested to play a major role in IFN induction even in the absence of IRF3, whereas IRF3-mediated IFN induction remains minimal without the presence of even low amounts of IRF7. In the mouse, IRF8 has also been suggested to positively regulate the later phase of type I IFN induction (11).

In viral infections, innate immune responses are initiated when viruses or their genetic material are recognized by cellular pattern recognition receptors such as TLRs. This leads to the activation of several transcription factor systems that are involved in the regulation of chemokine and cytokine gene expression. Recently, it was shown that the recognition of viral ssRNA, including that of influenza A virus, is mediated via TLR7. Thus, TLR7 seems to play an important role in the induction of antiviral responses against RNA viruses (12–14). TLR7 activation signal is transduced via MyD88 to the IL-1R-associated kinase 1/4 complex that activates IRF7 by a specific phosphorylation event. The TLR7 pathway is active, especially in plasmacytoid dendritic cells (DCs), which are responsible for the production of high levels of IFN-α (15). Viral dsRNA is also formed during the replication cycle of a virus and it is recognized by TLR3. TLR3 signaling takes place via the Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) adapter molecule, leading to the activation of IκB kinase (IKK) e.

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3 Abbreviations used in this paper: IRF, IFN regulatory factor; DC, dendritic cell; luc, luciferase; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN-β; IKKε, IκB kinase ε; TBK1, TANK-binding kinase 1; RIG-I, retinoic acid-inducible gene I; moDC, monocyte-derived DC; SV, Sendai virus; MOI, multiplicity of infection; ISRE, IFN-stimulated response element; PRDI, positive-regulatory domain I; HEK239, human embryonic kidney 239.

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and TANK-binding kinase 1 (TBK1), which are virus-specific kinasess that regulate the phosphorylation and activation of IRF3 and IRF7 and subsequent IFN-α expression (16, 17). The third signal transduction pathway activated in virus infection is a cytoplasmic RNA helicase, retinoic acid-inducible gene 1 (RIG-I) (18). This pathway seems to be the most crucial one in cellular response to influenza A viruses (19–22). Although TLR expression is more restricted to some specific cell types, RIG-I expression is found ubiquitously in all cell types, including lung epithelial cells (23).

Initially, the ligand for RIG-I was found to be dsRNA, but recently it was also shown that viral 5′-phosphorylated ssRNA can also activate RIG-I. Therefore, RIG-I is a unique molecule being able to distinguish between self- and non-self RNA (21, 24). RIG-I is transmitting the signal through a caspase recruitment domain to a mitochondrial protein Cardif (also known as IPS-1, VISA, and MAVS) (25–28). Although the precise mechanism of the signal transduction from RIG-I and Cardif to downstream is not known, this pathway leads to the activation of IRF3 via IKKe/TBK1 and NF-κB via the classical IKK complex.

The activation of transcription factors in virus infection is regulated by a complex network. So far, less attention has been paid on negative regulation of the activation signal, which is important in switching off the gene expression and avoiding excess inflammatory response. Pin1 is a prolyl isomerase that specifically recognizes phosphorylated serine or threonine residues on proteins and catalyzes the conformational change of the bound substrate (29). The ubiquitination and proteasome-dependent degradation of IRF3 has been reported to be regulated by Pin1 (29). The suppressor for IRF7 has not been reported, neither has the possible suppressing role of Pin1 on IRF7 functions.

Although IFN-As differ genetically from type I IFNs, their similar antiviral functions give reason to assume that their expression is regulated in a similar fashion. In the present study, we have analyzed the regulation of IFN-α and IFN-λ gene expression in human monocyte-derived dendritic cells (moDCs) in response to Sendai virus (SV) infection. We also identified the transcription factors and signaling pathways that control IFN-λ gene expression using cloned promoter elements of these genes. We found out that the IFN-λ1 gene is regulated by both IRF3 and IRF7 like IFN-β gene is, whereas the major regulator of IFN-λ2/3 genes is IRF7.

**Materials and Methods**

**Cell culture**

Monocyte-derived DCs (moDCs) were obtained as previously described (30). Briefly, monocytes were isolated from leukocyte-rich buffy coats of healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) by consecutive centrifugations in Ficoll-Paque and Percoll gradients (Amersham Biosciences) followed by depletion of the remaining T or B cells with anti-CD3 and anti-CD19 magnetic beads (Dynal Biotech). Monocytes were suspended in RPMI 1640 medium (Sigma-Aldrich) in the absence of serum, and 2.5 × 106 cells/well were allowed to adhere to plastic 6-well plates (Falcon; BD Biosciences). After 1 h of incubation at 37°C, nonadherent cells were removed, and the cells were washed with PBS. Monocytes were differentiated into immature DCs for 6 days in 2 ml of RPMI 1640 medium supplemented with 0.6 mg/ml penicillin, 60 mg/ml streptomycin, 2 mM l-glutamine, 20 mM HEPES, 10% FCS (Intergroco), 10 ng/ml recombinant human GM-CSF (BioSource International), and 20 ng/ml recombinant human IL-4 (R&D Systems). Fresh medium (1 ml/well) was added every 2 days.

For human embryonic kidney (HEK) 293 cell line (American Type Culture Collection; CLRL573) was maintained in Eagle’s MEM (Sigma-Aldrich) with antibiotics, l-glutamine, HEPES, and 10% FCS.

**Virus infections**

Murine SV (strain Cantell) originates from the National Public Health Institute (Helsinki, Finland) and it was cultured in embryonated chicken eggs and stored at −70°C (31). The hemagglutination titer of SV was 4096 and the infectivity of the virus stock in DCs was 6 × 106 PFU/ml (4). Cells were infected with a multiplicity of infection (MOI) of 5 (1/500 dilution of virus stock) to reach maximal infectivity without significant cytopathic effects in 18 h of infection. For RNA analysis, DCs were infected with SV for 1, 2, 4, 6, 9, and 12 h, and total cellular RNA was isolated with the RNeasy Midi kit (Qiagen). For DNA-binding experiments, virus-infected DCs were harvested at 2, 4, and 8 h after infection and nuclear protein extracts were prepared (4). The experiments were performed with cells obtained from three to four different blood donors. In transfection assays, HEK293 cells were infected with SV, since IRF3 and IRF7 molecules need to be C-terminally phosphorylated by virus-activated IKKe/TBK1 kinase complexes to induce their nuclear translocation and transcriptional activity.

**Plasmids**

The pGL3-luciferase (luc) reporter constructs for IFN-β and IFN-α4 promoters were previously described (32). Promoter sequences for IFN-α1, IFN-α1, and IFN-λ3 were cloned from pEF2-luc reporter plasmids (33) into MfeI and XhoI restriction sites of the pGL3-basic vector (Promega). Expression vectors for IRF1, IRF5 (variant 2, 498 aa) (34), IRF3 (35), IRF7 (36), p50, p65 (37), RIG-I, ∆RIG-I (18), Cardif (33), IKKe, and TBK1 (17) were described elsewhere. IRF8 cDNA was provided by Dr. B.-Z. Levi (Department of Biotechnology and Food Engineering, Technion, Haifa, Israel). The sequence of IRF8 was amplified and cloned into the BamHI site of pcDNA3.1–FLAG-tagged expression vector. The expression constructs for TRIF and MyD88 were gifts from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The cDNA encoding Pin1 was amplified from total cellular RNA isolated from human macrophages and cloned into the BamHI site of the pcDNA3.1–FLAG-tagged expression vector using primers (upstream) 5′-GACCTGGATCATCACCATGGGACGACGAGGAGAAGCT and (downstream) 5′-CCTGGCGGATCCTCAGTTTCTCTTTCCCTCCAGC (encoding a 130-amino acid fragment).

**Northern blot analysis**

For mRNA expression analyses, total cellular RNA was isolated by a RNeasy Midi RNA isolation kit (Qiagen). RNA was quantified photometrically, and samples containing equal amounts (10 μg) of total cellular RNA were size-fractionated on 1% formaldehyde-agarose gels and transferred onto Hybond-N nylon membranes (Amersham Biosciences). The membranes were hybridized with human IFN-α (α1), IFN-β (bovine or human) IFN-α1, IFN-α2, IFN-α7, and IFN-λ1-2-3 cDNA probes. The probes for Northern blot analysis were labeled with [α-32P]dATP (3000 Ci/mmol; Amersham Biosciences) using a Klenow random primed DNA labeling kit (Boehringer Mannheim). Membranes were hybridized (Ultrahyb buffer; Ambion), washed in 1× SSC/0.01% SDS and exposed to Kodak X-Omat AR films (Eastman Kodak) at −70°C with intensifying screens.

**Quantitative RT-PCR**

Before cDNA synthesis, 2 μg of purified total cellular RNA was treated with DNAse I (Roche Diagnostics) to remove any contaminating DNA. Generation of cDNA was done by using a TaqMan Reverse Transcription kit (Applied Biosystems) with random hexamers as primers. The cDNA was amplified by PCR using a TaqMan Universal PCR Mastermix and the commercial Gene Expression Assay with primers and probes for IFN-α1, IFN-β, IFN-α1, IFN-α2/3, IFN-3, and IFN-7 from Applied Biosystems. The data were normalized to 18S rRNA (TaqMan Endogenous Control kit) and presented as the relative gene expression in relation to 18S rRNA.
Transfections

HEK293 cells were seeded into 24-well plates at 75,000 cells/well or into 96-well plates at 20,000 cells/well and the cells were allowed to attach overnight. For luc assays, the cells were transfected with 100 ng for a 24-well format and 20 ng for a 96-well format of indicated expression plasmids, unless otherwise stated, along with 15 ng for a 24-well format and 3 ng for a 96-well format of pRL-RSV plasmid (Renilla luc) and 100 ng for a 24-well format and 20 ng for a 96-well format of firefly luciferase reporter under the control of different IFN promoters using TransIT-LT1 transfection reagent (Mirus Bio). The DNA:TransIT ratio was 1:4. Total amount of DNA was adjusted with the empty expression vector. At 4 h after transfection, the cells were infected with SV for 18 h when indicated. The luciferase activities were detected by using the Dual-Luciferase Reporter Assay System (Promega) and Victor multilabel reader (Wallac). The firefly luciferase activities were normalized to Renilla luc activities and the results are presented as the fold induction in relation to the unstimulated promoter and Renilla transfections in the absence of SV infection.

Results

SV-induced expression of IFN genes in human moDCs

Because human DCs produce large quantities of IFNs and they can readily be infected with RNA viruses such as influenza A and SV (4), they form a good system to study the regulation of type I and type III IFN gene expression. To reveal the kinetics and magnitude of IFN gene expression, we infected moDCs with SV and isolated total cellular RNA at different time points after infection. The expression of IFN-α, IFN-β, IFN-α1, and IFN-α2/3 genes was analyzed with Northern blotting. For a more quantitative approach, we used RT-PCR. Because human IFN-λ2 and IFN-λ3 genes are almost identical (96% amino acid identity) (2), the probe used in Northern blot analysis and the primers used in RT-PCR could not distinguish between the latter two IFN-A types. RIG-I and IRF7 mRNAs were also analyzed in Northern blots to verify the expression kinetics of these two IFN-stimulated genes. Using RT-PCR, we quantified the expression levels of IRF3 and IRF7 during virus infection. We observed that the gene expression of IFN-α1 and IFN-β was induced already 2 h after infection of moDCs with SV, whereas the expression of IFN-α and IFN-α2/3 genes appeared to take place somewhat later (Fig. 1A). Based on RT-PCR analysis, the kinetics of IFN-β and IFN-α1 mRNA expression was also a few hours faster as compared with IFN-α and IFN-α2/3 genes. Quantitative analysis revealed that all studied IFNs showed ~1000-fold induction after SV infection (Fig. 1B). In Northern blot analysis, the expression of RIG-I and IRF7 became visible at the 4-h time point, showing an expression kinetic that coincided or preceded those of IFN-α and IFN-α2/3 genes (Fig. 1A). Quantification of IRF3 and IRF7 mRNA showed that IRF3 is expressed constantly whereas the maximal expression level of IRF7 observed at 6 h after SV infection (100-fold increase, Fig. 1B).

Structure of human IFN-α1 and IFN-α3 promoters

To characterize the transcription factors regulating the expression of IFN-α genes, we performed a computer sequence analysis of the human IFN-α1 and IFN-α3 promoter regions with the MatInspector (Genomatix software) and TESS (Transcription Element Search software (http://www.cbil.upenn.edu/tess), programs. Because the promoter sequences of IFN-α2 and IFN-α3 genes are identical, except for five nucleotides (Fig. 2B) and a 10 nt TATA repeat within the first 1000 nt upstream of the transcription start site, we concentrated on analyzing the IFN-α3 gene promoter in more detail. The sequences of IFN-α1 and IFN-α3 promoters are shown in Fig. 2. In the IFN-α1 promoter, we found a cluster of IRF-binding ISRE sites between nt −216 to −192 relative to the transcription start site (41). In addition, we identified a PRDI site at −65 to −79 which, according to the computer programs, was expected to be an IRF1 binding site. The promoter of IFN-α1 had two putative NF-κB-binding sites, one between nt −98 to −89 referred to as NF-κB1 and another one between nt −264 to −253 referred to as NF-κB2. The IFN-α3 promoter contained a putative ISRE site at −288 to −301 (relative to the transcription start site stated by GenBank) and a PRDI site at −182 to −195. In addition, a putative NF-κB binding site was located inside the transcribed sequence between nt +115 to +125 (Fig. 2).
To analyze the functionality of the putative IFN-\(\lambda\)1 and IFN-\(\lambda\)3 promoter transcription factor binding sites identified by the computer search, we conducted DNA-binding experiments. At 2, 4, and 8 h after SV infection, moDCs were collected and nuclear protein extracts were prepared. An oligonucleotide precipitation assay using ISRE, PRDI, or NF-\(\kappa\)B elements was conducted and factors binding to these elements were identified by Western blotting. Both IFN-\(\lambda\)1 and IFN-\(\lambda\)3 promoter elements were able to bind IRF and NF-\(\kappa\)B transcription factors (Fig. 3). Virus infection increased the binding of IRF1, IRF3, and IRF7 on ISRE and PRDI elements of these promoters, and moreover the binding of phosphorylated IRF3 was seen already at the 2- and 4-h time points. The binding of IRF1 and IRF7 was observed to take place in cell extracts collected at later time points. The binding of IRF8 on ISRE and PRDI elements was constitutive and not dependent on virus infection. The components of the classical NF-\(\kappa\)B pathway, p65 and p50, bound to the NF-\(\kappa\)B elements on both IFN-\(\lambda\)1 and IFN-\(\lambda\)3 promoters in response to SV infection. In addition, the binding of the component of the alternative NF-\(\kappa\)B pathway RelB,
but not p52, was also weakly increased in virus-infected cell extracts.

**Role of IRF and NF-κB transcription factors in type I and type III IFN gene expression**

Next, we wanted to analyze which of the IRF and NF-κB family members activate type I and type III IFN promoters. HEK293 cells were transfected with IFN-α1, IFN-α4, IFN-β, IFN-λ1, and IFN-λ3 promoter-reporter constructs in combination with expression constructs for IRF1, IRF3, IRF5, IRF7, IRF8, and p50 plus p65. Because IRF3 and IRF7 appeared to differentially regulate these promoters, they can be classified as IFN-α-type (IFN-α1, IFN-α4, and IFN-λ3) and IFN-β type (IFN-β and IFN-λ1) promoters (Fig. 4A). SV infection increased IFN-β- and IFN-λ1 promoter activation significantly and in IRF1, IRF3, or IRF7 gene-transfected cells even more (Fig. 4A). IRF7 induced the activation of all tested IFN promoters already without SV infection (Fig. 4A). IFN-α and IFN-λ3 promoters were not significantly induced by SV infection. Ectopic expression of IRF1 and IRF3 followed by SV infection led to increased IFN-α1, IFN-α4, and IFN-λ3 promoter activation. However, this activation was much greater in IRF7 gene-transfected cells, indicating that IFN-α and IFN-λ3 promoters were predominantly regulated by IRF7 (Fig. 4A).

In another set of experiments, we conducted transfection with p50- and p65-protein-expressing constructs to analyze the role of NF-κB in type I and type III IFN gene expression. Because IkBa is not present, p50 plus p65 heterodimer is transcriptionally active. Although IFN-α1 and IFN-α4 promoters were very weakly activated by p50 plus p65, both IFN-λ1 promoters as well as the IFN-β promoter were readily induced by transfected NF-κB components (Fig. 4B). It was of great interest that IFN-λ3 promoter activation was induced by p50 plus p65 heterodimers to the level similar to or even greater than that induced by SV infection (Fig. 4B).
IRF5 has been reported to play a crucial role in the expression of type I IFNs (42) and IRF8 has been suggested to be involved in the late-phase type I IFN gene expression in the mouse (11). In our HEK293 cell transfection/SV infection system, IRF5 induced only a minimum increase in the IFN promoter activations, except IFN-β promoter that was clearly activated by IRF5 (Fig. 4A). This observation is consistent with some recent publications (11, 43). We did not see any increasing effect on IFN promoter activation by IRF8, rather there was an inhibitory effect (Fig. 4A). To further characterize the role of IRF8 in IFN gene expression, we cotransfected the IRF8 expression plasmid with IRF3 or IRF7 and infected the cells with SV. There was a remarkable IRF8 dose-dependent decrease in IRF7-mediated IFN promoter activation (Fig. 5). This phenomenon was not so clear with IRF3, although some decrease in IRF3-mediated activation of IFN-α1 and IFN-β promoters was seen. Since IRF8 was found to bind to the ISRE element on IFN promoters (Fig. 3), it is possible that IRF8 could compete with other IRFs in their DNA-binding activity. Although the expression and stability of IRF3 was not altered by overexpression of IRF8, IRF7 stability seemed to be impaired, because the amount of full-length IRF7 was reduced and correspondingly another form of IRF7 protein appeared (Fig. 5B). This may indicate that IRF8 interferes with IRF7 DNA binding, rendering it unstable and susceptible for proteolytic degradation.

**RIG-I- and TLR-dependent signaling pathways regulate type III IFN promoters**

Based on current knowledge, RNA virus infection can trigger three separate signal transduction pathways (TLR7, TLR3, and RIG-I), leading to the activation of transcription factors that regulate the expression of antiviral genes. To identify the signaling pathways regulating type III IFN gene expression, we overexpressed RIG-I, the constitutively active form of RIG-I (ΔRIG-I), Cardif, IKKα, TBK1, TRIF, and MyD88 alone or in combination with IRF1, IRF5, or IRF7 and analyzed IFN promoter activation in HEK293 cells. The data revealed that overexpression of upstream signaling molecules ΔRIG-I, Cardif, IKKα, TBK1, and TRIF induced IFN promoter activation independently of SV infection (Fig. 6). Full-length RIG-I, instead, required SV infection for full transcriptional activation. Also in this experiment, there were similarities between IFN-β and IFN-α1 as well as between IFN-α and IFN-λ3 promoters. All tested signaling molecules activated IFN-β and IFN-λ3 promoters very well, whereas the activation of IFN-α1 and IFN-α4 promoters remained at a moderate level (Fig. 6).

Interestingly, activating the MyD88-dependent signaling cascade (mimicking TLR7 signaling) and, most importantly, MyD88 plus IRF7 combination led to a significant activation of IFN-α and IFN-λ3 promoters (Fig. 6B). The activation induced by the combination of MyD88 and IRF7 appeared to be independent of SV infection. Expression of MyD88 alone induced a moderate activation of IFN-α1 and IFN-α4 promoters. However, the IFN-α4 promoter was only weakly activated with the other signaling cascades. In addition, the IFN-α4 promoter was activated with the combination of MyD88 and IRF5, while IFN-α1 and IFN-α3 promoters were not responding significantly to this activation stimulus. The expression of MyD88 with IRF1 enhanced virus-dependent activation of IFN promoters, especially those of IFN-β and IFN-λ1.

**FIGURE 6.** Activation of type I and type III IFN promoters by signaling components of the TLR or RIG-I pathways. HEK293 cells were transfected with expression constructs for the components of the RIG-I (A), TLR3, or TLR7 (B) signal transduction pathways along with the IFN promoter luc reporter constructs. Four hours after transfection, cells were infected with SV for 18 h and luc activity was measured. The luc activities were normalized with Renilla activities and data are presented as fold induction from basal promoter activation without transfected expression constructs or virus infection. Bars, The means ± SD of triplicate determinations. One representative experiment of three is shown.
Interestingly, also the MyD88-dependent activation of IFN promoters was down-regulated by Pin1. The decreased promoter activation induced by Pin1 was seen in samples activated by MyD88 combined with IRF1 or IRF7. Transfected Pin1 had no inhibitory effect on the expression of transfected IRFs (Fig. 7B). inhibitory effect of Pin1 was likely IRF specific, since NF-κB-induced promoter activation was not reduced by ectopic expression of Pin1 (data not shown).

Discussion
IFNs have a crucial role in host resistance against viral infections. To ensure efficient IFN-mediated antiviral responses, type I IFNs have evolved to a large family of molecules including IFN-β, IFN-α, and multiple copies of IFN-α genes. The regulation of type I IFN gene expression is relatively well characterized, although the nature of virus-specific signals leading to the activation of antiviral gene expression is still somewhat unclear. Quite recently a new class of antiviral cytokines, type III IFNs (IFN-α1–3 or IL-28/29), which are genetically and structurally different from type I IFNs, were identified (1, 2). In the present study, we conducted a detailed analysis of the regulation of type III IFNs using human primary DCs and transfection analyses in HEK293 cells with different IRF sequences of IFN-3 genes were almost identical, whereas the promoter of IFN-1A was somewhat different from the IFN-1A/3 promoters. However, all IFN-α gene promoters had several putative ISRE and NF-κB binding sites (Fig. 2), suggesting that these genes are under a complex regulation by multiple IRF and NF-κB family transcription factors. When we compared the IRF-regulated activation of the five different IFN gene promoters, i.e., IFN-α1, IFN-α4, IFN-β, IFN-1A, and IFN-3 promoters, we found a clear similarity between IFN-β and IFN-1A promoters which were activated by several different IRF isotypes. The regulation of the IFN-3 promoter, like IFN-α promoters, was more restricted to the positive regulatory functions of IRF7 (Fig. 4A). This was also evidenced by analyses on TLR- or RIG-I-dependent signal transduction cascades that activated these IFN promoters. All three analyzed pathways, i.e., RIG-I and Cardif-mediated pathway, TLR3-mediated signaling through TRIF and TLR7/8-mediated MyD88-dependent pathway, were able to activate IFN-β and IFN-1A promoters. In contrast, IFN-α and IFN-3 promoters were activated mainly by the MyD88-dependent signal transduction pathway via IRF7 and to a lesser extent via IRF1 (Fig. 6). Consistent with our findings, Onoguchi et al. (41) recently reported that the expression of IFN-α genes is regulated in a similar fashion as type I IFN genes involving RIG-I, Cardif, TBK1, and IRF3 molecules. Similar to us, they observed that IRF3 regulates IFN-1A gene expression, but it can induce the IFN-1A/3 gene only weakly. In addition, in EMSA analysis, Onoguchi and coworkers demonstrated the binding of p50/p65 heterodimer to a putative NF-κB
site on the IFN-α1 promoter (NF-κB1 in the present work). Based on the computer analysis, the IFN-α2 promoter was suggested to have two putative binding sites for NF-κB, although these sites were not very well in line with the consensus NF-κB site (44). We, instead, found another putative NF-κB binding site on the IFN-α3 (thus on IFN-α2 promoter as well) promoter, and in functional analyses we also showed that the IFN-α3 promoter was activated by the p50/p65 heterodimer (Fig. 4B). Such an activation was not seen for IFN-α1 and IFN-α4 promoters, which lack a clearly identifiable NF-κB site. In conclusion, we can say that the IFN-α1 gene can be induced in a TLR- and RIG-I-dependent fashion and that NF-κB and multiple IRF family members induce its expression. IFN-α2 and α3 genes were also regulated by NF-κB, but out of the IRF family members IRF7 appeared to be the major factor regulating the expression of these genes. This places IFN-α2/3 genes functionally closer to IFN-β and IFN-γ, which also in the mouse act independently via their specific receptors. Thus, the disruption of the expression of these genes. This places IFN-α2/3 genes functionally closer to IFN-β and IFN-γ, which also in the mouse act independently via their specific receptors. Thus, the disruption of the expression of these genes. It was of interest that IFR8, which appears to play a positive regulatory role in the IFN-γ expression in mice (45, 46), rather seems to be an inhibitory factor for the human type I and type III IFN genes.

Mice have no ortholog gene for human IFN-α1, and they have the genes corresponding to the human IFN-α2 and IFN-α3 genes (47). In the case the regulation of murine IFN-α genes is controlled by a similar mechanism it is in humans, the murine IFN-α system is greatly dependent on IRF7 expression, IRF7 expression is induced by the IFN-stimulated gene factor 3 complex, which can be activated by both IFN-αβ and IFN-αs, which also in the mouse act independently via their specific receptors. Thus, the disruption of type I IFN receptor is likely to lead to an impaired IFN-α system in mice, since the early phase of IFN-α induction from a functional murine IFN-α1 gene is missing.

The prolyl isomerase Pin1 has been reported to be able to modulate virus-induced gene expression by interacting with phosphorylated IRF3. Pin1 triggers polyubiquitination and proteasome-dependent degradation of IRF3, which leads to termination of IFN promoter activation (29). In elucidating the role of Pin1 in type I and type III IFN gene expression, we found out that, in addition to IRF3-dependent IFN gene transcription, the MyD88-dependent signal transduction pathway resulting in the activation of IRF7 and IFR1 was also sensitive to the inhibitory effects of Pin1. Ectopic expression of Pin1 clearly led to dose-dependent down-regulation of IFN promoter activation (Fig. 7). Consistent with the observation by Saitoh et al. (29), we observed that both RIG-I and TRIF-mediated (TLR3 pathway) IFN promoter activation was reduced by Pin1, indicating the negative regulation of at least IRF3. We did, however, also observe that MyD88/IRF7-dependent IFN gene expression was drastically reduced. This observation does, however, not rule out the possibility that IRF3 and IRF7 pathways cross-talk with each other and that the down-regulation induced by Pin1 could be mediated via inhibition of endogenous IRF3. There is increasing evidence that different IRF family members form heterodimers that bind to the ISRE sites on IFN promoters (48). Further studies are in progress to reveal the mechanism of action of Pin1 on other IRF family members apart from IRF3.

In conclusion, although type III IFNs show only limited sequence conservation with type I IFNs and they use their own specific receptors, both type I and type III IFNs are expressed in a similar fashion in virus-infected cells exhibiting both early and late phases of IFN induction. Yet, type III IFN genes show a more complex regulation than type I IFNs, because they have a higher number of regulatory elements on their promoters. In addition, type III IFN genes have multiple exons, which adds another post-transcriptional regulatory event into the biology of these IFNs. Further work with type I and type III IFNs or their receptor knock-out mice is clearly warranted to reveal the role of these IFN systems in innate and adaptive immune responses in viral infections. In addition, further understanding of the fine-tuning of gene regulatory events involved in the expression of both type I and type III IFN genes is of great interest. With these gene promoter model systems, we are likely to learn a lot of the cooperativity of the host cell signaling pathways and different transcriptional systems including those of IRFs and NF-κB.

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Disclosures

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References


