Interferon Regulatory Factor 8 (IRF8) Impairs Induction of Interferon Induced with Tetratricopeptide Repeat Motif (IFIT) Gene Family Members*

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The chromosomally clustered interferon-induced with tetratricopeptide repeat motif (IFIT) gene family members share structural features at the gene and protein levels. Despite these similarities, different IFIT genes have distinct inducer- and cell type-specific induction patterns. Here, we investigated the mechanism for the observed differential induction of the mouse Ifit1, Ifit2, and Ifit3 genes in B cells and demonstrated that the repressive effect of the transcription factor interferon regulatory factor 8 (IRF8), which is highly expressed in B cells, played an essential role in this regulation. Although IRF8 could impair induction of all three IFIT genes following stimulation of retinoic acid-inducible gene I (RIG-I), it could selectively impair the induction of the Ifit1 gene following IFN stimulation. The above properties could be imparted to IRF8-non-expressing cells by ectopic expression of the protein. Induction of reporter genes, driven by truncated Ifit1 promoters, identified the regions that mediate the repression, and a chromatin immunoprecipitation assay revealed that more IRF8 bound to the IFN-stimulated response element of the Ifit1 gene than to those of the Ifit2 and the Ifit3 genes. Mutational analyses of IRF8 showed that its ability to bind DNA, interact with other proteins, and undergo sumoylation were all necessary to selectively repress Ifit1 gene induction in response to IFN. Our study revealed a new role for IRFs in differentially regulating the induction patterns of closely related IFN-stimulated genes that are located adjacent to one another in the mouse genome.

Members of the interferon-induced with tetratricopeptide repeat motif (IFIT)†gene family are among the most highly induced IFN-stimulated genes (1). However, their contributions to physiology (2, 3) and antiviral defense (4, 5) are only starting to be elucidated. The mechanisms of action for IFIT family proteins are also active topics of research. To date, IFIT family members have been shown to inhibit protein translation initiation in vitro (6, 7) and sequester 5′-triphosphate RNA (8).

Initial investigations of the IFIT family revealed these genes to be located very close to each other on chromosomes in humans (9) and mice (10) and to be coordinately induced in response to IFN, poly(I:C) treatment, or virus infection by the interferon-stimulated response element (ISRE) in their promoters (9–11). Different IFIT family genes were later demonstrated to have cell type- and inducer-specific patterns of induction (12), suggesting that regulation of IFIT family genes is more complex than initially thought. In B cells but not T cells treated with IFN or IFN-inducing stimuli, poor Ifit1 gene induction is observed in vivo in mice (12). A similar phenomenon is observed in dendritic cells derived in vitro from mouse bone marrow; poor Ifit1 induction is seen in plasmacytoid dendritic cells (pDCs) but not conventional dendritic cells (13). Tissue- and inducer-specific induction of different IFIT family members raises the question of how these very similar genes may be independently regulated. Given the established role of interferon regulatory factor (IRF) family members in driving ISRE-mediated gene induction (14), members of this family are the most likely candidates for regulators of IFIT family gene induction.

As a member of the IRF family, IRF8 has a number of features that make it a potential regulator of IFIT family gene induction. The most striking of these is its tissue distribution. IRF8 expression is restricted to myeloid and lymphoid cells (15), with high levels observed in pDCs and B cells (16, 17), cell types in which impaired Ifit1 induction is observed in vivo (12, 13). Whereas many IRF family members are known to induce genes, IRF8 is able to repress gene transcription under some conditions (18) and forms complexes that activate gene transcription under other conditions (19–21). Exactly how IRF8 switches between activating and repressing gene transcription under different conditions is becoming understood. IRF8 generally requires a binding partner, such as IRF1 or IRF2 (22), IRF4, and/or PU.1 repeat motif; IRF, interferon regulatory factor; RIG-I, retinoic acid-inducible gene I; ISRE, interferon-stimulated response element; pDC, plasmacytoid dendritic cell; TSS, transcriptional start site.

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The abbreviations used are: IFIT, interferon-induced with tetratricopeptide repeat motif; IRF, interferon regulatory factor; RIG-I, retinoic acid-inducible gene I; ISRE, interferon-stimulated response element; pDC, plasmacytoid dendritic cell; TSS, transcriptional start site.
IRF8 Represses Ifit1 More than Ifit2 or Ifit3

(23, 24) to bind to different promoter elements and regulate gene transcription. More recently, post-translational modification has emerged as a method of regulating the ability of IRF8 to activate or repress gene transcription. Sumoylation of IRF8 has been shown to remove its ability to activate gene transcription, switching it from an activator to a repressor (25). As such, IRF8 may be capable of repressing Ifit1 gene induction in cells such as pDCs and B cells, where it is present at high endogenous levels.

Here we report that IRF8 regulates induction of different IFIT family members differentially. Through ectopic expression of IRF8 in cells that do not express IRF8, we were able to alter induction patterns of individual IFIT family members in a dose-dependent manner. IRF8 requires binding partners and post-translational modifications to effect its activation or repression of gene transcription, and we have shown that each of these properties of IRF8 is required for selective impairment of Ifit1 induction. Moreover, we show stimulus specificity of IRF8-mediated selective Ifit1 repression, suggesting that the ability of IRF8 to repress induction of a particular IFIT family member depends on the specific transcription factor driving gene induction in response to the stimulus used.

Experimental Procedures

Mice—All experiments were conducted on C57Bl/6J mice between 8 and 12 weeks of age in accordance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Flow Cytometry—Multicolor flow cytometry analysis of IFIT family induction was conducted as described previously (12). Briefly, spleen cells were harvested from mice, red blood cells were lysed, and single cell suspensions of lymphocytes were cultured with or without stimulation, as indicated. Following stimulation, primary B cells or WEHI231 cells were stained with fluorochrome-conjugated antibodies against CD4, CD8, or B220 (clones RM4-5, 53-6.7, and RA3-6B2, respectively; BD Pharmingen) before paraformaldehyde fixation, saponin permeabilization, and incubation with antibodies against IFIT family members. Antibodies against murine Ifit1, Ifit2, and Ifit3 were rabbit polyclonal antibodies generated in the Lerner family members. Antibodies against murine IFIT1, IFIT2, and IFIT3 were rabbit polyclonal antibodies generated in the Lerner Research Institute hybridoma core (12, 13), and these were detected using a fluorochrome-conjugated goat anti-rabbit IgG (Molecular Probes). Flow cytometry was conducted on a FACScan flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar Inc.).

To sort L929 fibroblasts containing ZsGreen-containing lentivirus (described below), at least 5 × 10^6 cells were resuspended in divalent cation-free PBS (pH 7.1) containing 25 mM HEPES (pH 7.0), 1 mM EDTA, 1% FBS, and 2 μg/ml propidium iodide and filtered before sorting with a FACSAria II cell sorter (BD Biosciences). Viable cells were sorted based on ZsGreen levels with sorting gates set at 1-log increments. Sorted cells were cultured as described below.

B Cell Purification—B cells were isolated from splenocyte cell suspensions for RNA analysis of IFIT family gene induction by negative depletion using MACS beads (Miltenyi Biotech), according to the manufacturer’s instructions. Non-B cells were coated with biotinylated antibodies followed by binding of these cells to streptavidin-coated magnetic beads. Bead-bound cells were removed from culture with a magnet, whereas B cells remained in the culture. Culture purity was confirmed by flow cytometry using antibodies to B220 as described above and was routinely >95%. RNA isolation was conducted as described below. RNA was isolated from at least 10^6 cells treated with 1000 units/ml IFNβ (PBL) or 1000 units/ml IFNa (Calbiochem) for 4 h.

Cell Culture and Generation of Stable Cell Lines—L929 fibroblasts were maintained and all assays were conducted in DMEM supplemented with 10% FBS (Atlanta Biological), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco), unless otherwise indicated. WEHI231 B cells were cultured in DMEM + 10% FBS + 100 units/ml penicillin (Gibco) + 100 μg/ml streptomycin (Gibco) + 50 μM β-mercaptoethanol (Sigma). All cell culture incubations were at 37°C in 5% CO2 in a humidified incubator.

To generate L929 fibroblasts stably expressing ectopic IRF8, L929 cells were transduced with lentivirus (vector or murine IRF8; production described below) as follows. 7.5 × 10^5 cells were infected with 4 ml of virus inoculum containing 4 μg/ml Polybrene (Sigma). Following overnight incubation, virus was removed and replaced with standard culture medium as above. 72 h postinfection, the presence of virus was confirmed microscopically by ZsGreen expression (Leica DM IRB), and cells were passaged four more times before flow cytometric sorting for different levels of ZsGreen expression (described above). Ectopic IRF8 expression was confirmed by Western blot as described below. Three independently generated pools of cells ectopically expressing IRF8 were used in experiments.

Cloning of Lentiviral and Luciferase Vectors and Packaging of Lentivirus—Wild-type or mutant IRF8 lentiviral vectors were cloned as described below. These vectors were then used to generate lentivirus particles for infection. Replication-deficient, VSV-pseudotyped recombinant lentivirus was produced by cotransfection of pLVX IRES ZsGreen1-derived plasmids with the packaging plasmid pCMV-dR8.74 (Addgene) and the pseudotyping plasmid pVSV-G (Clontech) using calcium phosphate into HEK293T cells (26). Three lentivirus-containing supernatants were harvested at successive 12–16-h intervals, the collections were pooled and filter-sterilized, and the lentivirus was titered on HT1080 cells.

pCMV Sport8 containing full-length cDNA for murine IRF8 was purchased from Thermo Scientific (catalog no. MMB1013-63877). This cDNA served as a template for all subsequent IRF8 clonings unless otherwise indicated. Full-length IRF8 coding sequence was amplified by PCR using primers with XhoI and XbaI sites (gctctagagtttagacggtgatctggattttc). Following digestion with XhoI and XbaI (New England Biolabs), this PCR product was ligated into pLVX IRES ZsGreen1 (Clontech) using a Rapid DNA Ligation kit (Roche Applied Science) as per the manufacturer’s instructions to generate pLVX mIRF8 IRES ZsGreen1.

To generate pLVX mIRF8 K310R IRES ZsGreen1, a plasmid containing the K310R mutant of murine IRF8, a pcDNA3.1IRF8 K310R construct was used as a PCR template. A cloning procedure identical to that used to generate pLVX mIRF8 IRES
ZsGreen1 (above) was used to generate pLVX mIRF8 K310R IRES ZsGreen1.

pLVX mIRF8 K79E IRES ZsGreen1, a plasmid containing the K79E mutation of murine IRF8, and pLVX mIRF8 R289E IRES ZsGreen1, which contains the R289E mutant of murine IRF8, were generated by direct subcloning. IRF8 K79E and IRF8 R289E inserts were excised from pcDNA3.1 (+) backbones using EcoRI. The released products were ligated into pLVX IRES ZsGreen1 (Clontech) using a Rapid DNA ligation kit (Roche Applied Science) as per the manufacturer’s instructions.

Western Blotting—Unless otherwise indicated, cells were treated with 1000 units/ml IFNβ (PBL), Sendai virus infection, or poly(I:C) transfection for 8 h before lysis. For Sendai virus infection, cells were infected with Cantell strain Sendai virus (Charles River SPAFAS) at a multiplicity of infection of 10. For 10-cm dishes, 2 ml of virus inoculum in DMEM + 2% FBS was applied for 1 h, with regular plate agitation. Following adhesion, inoculum was removed and replaced with medium as for culture. For poly(I:C) transfection, cells were transfected with poly(I:C) (Amersham Biosciences) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Protein was isolated from cultured cells by washing in PBS followed by lysis in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, supplemented with a Complete protease inhibitor tablet and PhoSTOP tablet (Roche Applied Science). These firefly luciferase constructs were cotransfected in a 10:1 ratio with pGL3 Basic vector (Promega), as described below. An empty pGL3 Basic vector or the pGL3 Basic vector encoding pGL3 Basic vector (Promega). All assay plates also contained cells transfected with the empty pGL3 Basic vector or the pGL3 Control vector to provide negative and positive controls, respectively, for the subsequent luciferase assay. 6 h after transfection with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions, cells were trypsinized and replated (2.5 × 10⁵ cells/ml, 100 µl/well) in white-walled 96-well plates (Costar). Cells were left overnight to adhere before medium was replaced with 75 µl of fresh medium supplemented with 1000 units/ml murine IFNβ (PBL) where indicated. Following 8 h of stimulation, luciferase reporter activity was measured using the Dual Glo luciferase assay system (Promega) and a Wallac2 plate reader according to the manufacturer’s instructions. Firefly luciferase activity (i.e., promoter activity) was normalized to Renilla luciferase activity for each well. For each treated IFN sample, -fold induction relative to untreated wells of the same cells was calculated for triplicate wells. Unstimulated samples from different cell lines showed comparable low levels of normalized firefly luciferase activity. Because IFNβ-treated cells lacking ectopic IRF8 showed the highest normalized signal and -fold induction of promoter activity, this sample was defined as 100% induction. Induction in other samples was then calculated as a percentage of this value.

Cloning of IFIT Gene Promoter Constructs for Reporter Assays—The full-length Ifit1 promoter luciferase construct was generated by amplifying a 1 kb upstream of the Ifit1 transcriptional start site from C57BL/6 mouse genomic DNA using the primers 5’ MluI-Ifit1 1-kb promoter (CAGGTTACCGAGCTTCTACCGGTTCGATTCGCTTACGAGCCTTCTCCTCCTCA) and 3’ Xhol-Ifit1 1-kb promoter (CCATCGGACACCAACTGGAAAGCTGTCAG). The PCR product was digested with MluI and Xhol (New England Biolabs) and ligated into MluI-Xhol-digested pGL3 Basic (Promega). The full-length Ifit2 promoter luciferase construct was similarly generated using the PCR primers 5’ MluI-Ifit2 1-kb promoter (CCACCGTGTCGCACTTTCCATTTGAGACTG) and (3’ Xhol-Ifit2 1-kb promoter (CCATCGAGCTGACCCCAAGAAGAGAATGAAACC). In order to generate 5’-truncated reporter constructs of Ifit1, MluI-Ifit3 1 kb was used as a template for PCR using the following 5’ primers: for Ifit1 900 bp, 5’ MluI Ifit1 900 bp (CAGGTTACCGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 800 bp, 5’ MluI-Ifit2 800 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 700 bp, 5’ Ifit1 700 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 600 bp, 5’ Ifit1 600 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 500 bp, 5’ Ifit1 500 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 400 bp, 5’ Ifit1 400 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 300 bp, 5’ Ifit1 300 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC); for Ifit1 200 bp, 5’ MluI-Ifit1 200 bp (CAGCGATTACCGGAGCTTACCGGTATAATCTCTTGATATAGAGAAGAAGGAGAATGAAACC). For each truncated Ifit1 promoter construct, a PCR product was digested using MluI and Xhol and ligated into pGL3-Basic digested with MluI and Xhol.

Internal deletion reporter constructs were generated by pairing 5’ MluI-Ifit1 1 kb with a 3’ internal mutagenesis primer and pairing 3’ Xhol-Ifit1 1 kb with a 5’ internal mutagenesis primer in two separate reactions. Pairs of PCR products were gel-extracted using a QIAquick gel extraction kit (Qiagen) and used as templates for a second round of PCR using 5’ MluI-Ifit1 1 kb and 3’ Xhol-Ifit1 1 kb. Second round PCR products were purified using a PCR purification kit (Qiagen), digested using MluI.
and XhoI, gel-purified using a QIAquick gel extraction kit (Qiagen), and ligated into pGL3-Basic digested with MluI and XhoI using a Rapid DNA Ligation kit (Roche Applied Science) as per the manufacturer’s instructions.

**Real-time PCR** — For real-time PCR analysis, RNA was isolated from 1.5 × 10^6 L929 cells (or lentivirus-infected derivatives), or 1–5 × 10^6 B cells or WEHI231 B cells stimulated for 4 h with 1000 units/ml IFNα (Calbiochem) or 1000 units/ml IFNβ (PBL) as indicated using a High Pure RNA isolation kit (Roche Applied Science) according to the manufacturer’s instructions. 2 μg of RNA was reverse transcribed in a 20-μl volume using the random hexamer primers and Superscript III reverse transcription kit (Invitrogen) according to the manufacturer’s instructions. Lack of genomic DNA contamination was confirmed in samples by the lack of a band in PCR for the 18S rRNA in all RNA samples. To quantify IFIT family member and IRF8 transcript levels, 2 ng of cDNA/reaction was used as a template in real-time PCR. Real-time PCR was conducted using the SYBR Green PCR system (Applied Biosystems) according to the manufacturer’s instructions for two-step RT-PCR, except that the final reaction volume used was 12.5 μl. Accumulated PCR product for each sample was quantified by comparison with a standard curve and normalized to an 18S rRNA loading control reaction.

**Real-time PCR Cycling** — Real-time PCR cycling was conducted on a Roche Light Cycler480 II thermocycler as follows: 3 min at 95 °C, followed by 45 cycles of 30 s at 94 °C, 1 min at 52 °C, and 30 s at 72 °C. The primers used are shown in Table 1.

**Microarray Analysis** — For microarray experiments, RNA was prepared and quality-assessed as for real-time PCR from 5 × 10^6 lentivirus-infected L929 cells stably expressing low level ectopic IRF8 (or vector control), generated as described elsewhere. Ectopic IRF8 expression (Fig. 2A) was induced by a 30-min treatment with 1000 units/ml IFNβ (PBL). Samples were prepared and PCR was conducted as described previously (28).

**ChIP PCR Cycling** — Immunoprecipitated chromatin was used in PCRs with the following primers and cycling conditions: 2 min at 94 °C followed by 15 s at 94 °C, 30 s at Tm, 30 s at 72 °C for the indicated number of cycles followed by 7 min at 72 °C using ChoiceTaq and the supplied buffer (Denville). For real-time PCR on ChIP samples, cycling was conducted as for real-time PCR above, with a Tm of 50 °C using the primers shown in Table 2.

**Statistics** — p values from two-tail Student’s t tests and two-way analysis of variance with Tukey’s multiple-comparison test were calculated using GraphPad Prism statistical software (GraphPad).

**Results**

Different Patterns of IFIT Induction Are Observed When B and T Cells Are Stimulated in Vitro — We had previously observed impaired Ifit1 induction in B cells but not T cells in spleens isolated from mice treated with IFNα, IFNβ, or type 1 IFN-inducing stimuli (12). To confirm a cell-intrinsic basis for this observation and to start analyzing the mechanistic basis for impaired Ifit1 gene induction, IFIT family member induction was analyzed at the protein and RNA levels in cultures of splenocytes and purified B and T cells stimulated with IFN in vitro. Impaired Ifit1 induction in B cells was observed in splenocyte cultures stimulated with IFNβ (Fig. 1A) or IFNα (Fig. 1B), with a smaller percentage of B cells showing expression of Ifit1 compared with Ifit2 or Ifit3 in response to these stimuli. When IFIT transcript levels were determined by real-time PCR in purified B cells treated with IFNβ (Fig. 1C) or IFNα (Fig. 1D), Ifit1 transcript levels were much lower than Ifit2 or Ifit3 transcript levels. This suggested that the impaired Ifit1 induction seen in B cells was cell-intrinsic.

Ectopic Expression of IRF8 Impairs IFIT Family Gene Induction — In addition to the impaired Ifit1 gene induction observed in B cells stimulated in vivo (12) and in vitro (Fig. 1), impaired Ifit1 gene induction has also been observed in pDCs but not conventional dendritic cells (13). One common feature of B cells and pDCs is high levels of endogenous IRF8 relative to other cell types (16, 17). To determine whether IRF8 levels alter patterns of IFIT family gene induction, IRF8 was expressed ectopically in mouse L929 cells, which do not express IRF8. Stable L929-derived cell lines ectopically expressing different levels of IRF8 were generated by sorting lentivirus-infected cell lines, by flow cytometry, for different levels of IRF8 (Fig. 2A). IFIT family gene induction in different cell lines was determined by Western blotting (Fig. 2B). As expected, following IFNβ treatment of cells with undetectable protein levels of IRF8, all IFIT family members were induced (Fig. 2B). However, in cells containing low levels of ectopic IRF8, Ifit1 induction was impaired to a much greater degree than Ifit2 or Ifit3 levels (Fig. 2B). A cell line containing higher levels of ectopic IRF8 showed impaired induction of all IFIT family members (Fig. 2B). No IFIT family members were expressed in the absence of IFNβ stimulation irrespective of the level of ectopic IRF8 expression (Fig. 2B). Similar trends were observed at the RNA level (Fig. 2C) by real-time PCR with impaired induction

### Table 1: Primers for real-time PCR cycling

| Gene  | 5' Sequence | 3' Sequence |
|-------|-------------|-------------|
| Ifit1   | CAGAAGCACAGCATGGATGAGACCCAGGCCTTGAAGATGGAAGCA | GGCCTCATCTGGAAGAATGGAAGCA |
| Ifit2   | GGGCTGATCTGGGAAAATGATGAGACCAGGCCTTGAAGATGGAAGCA | GGCCTCATCTGGAAGAATGGAAGCA |
| Ifit3   | GGGCTGATCTGGGAAAATGATGAGACCCAGGCCTTGAAGATGGAAGCA | GGCCTCATCTGGAAGAATGGAAGCA |
| IRF8    | GGGCTGATCTGGGAAAATGATGAGACCCAGGCCTTGAAGATGGAAGCA | GGCCTCATCTGGAAGAATGGAAGCA |

**Chromatin Immunoprecipitation** — Chromatin was harvested and immunoprecipitated from 4.5 × 10^7 WEHI231 cells following a 30-min treatment with 1000 units/ml IFNβ (PBL) using the ChIP-IT express chromatin extraction kit (Active Motif). 15–20 μg of chromatin was immunoprecipitated with 3 μg of anti-IRF8 (Santa Cruz Biotechnology clone C19X) or 3 μg of anti-mouse IgG (Active Motif). PCR for regions of the IFIT gene family promoters was conducted as described below. For real-time PCR following ChIP, chromatin was harvested and immunoprecipitated from 4 × 10^7 cells following a 1-h treatment with 1000 units/ml IFNβ (PBL). Samples were prepared and PCR was conducted as described previously (28).
of all IFIT family transcripts seen in the cell lines with high levels of IRF8. At low levels of ectopic IRF8, less Ifit1 transcript was detected than Ifit2 or Ifit3 transcript (p < 0.0001 for Ifit1 versus Ifit2 and for Ifit1 versus Ifit3). Impairment of Ifit transcript suggested that IRF8 acted to regulate IFIT family gene induction at the RNA level.

Differential transcriptional regulation of IFIT family members raised the question of whether additional IFN-stimulated genes were regulated by IRF8 in the same way as Ifit1. Identifying common regulatory features of such genes may provide insight into how IRF8 might control their expression. To iden-

TABLE 2
Primers for ChIP PCR cycling

| Target | 5’ | 3’ | Chromatin | Tm | Cycles |
|--------|----|----|-----------|----|--------|
| Ifit1 ISRE | GATTTCAAGTAGGAACTTGACATTG | CCTGCTCTCTTTTCACTGAGAGT | 50 | ng | 36 |
| Ifit2 ISRE | CCTGACCTGTAAGCTTGCTGC | CCAAGGCAACTCTCTCTCCTGCTT | 100 | 60 | 36 |
| Ifit3 ISRE | GGAGGAAATGCTCTACACCTC | ATCCATGCTGCTGCTGCTCTCCTT | 50 | 60 | 36 |
| Ifit1 1 kb--900 bp | ACCAGTTAACCAGACACAGA | GCTGCAATGTATCTGGAAGAT | 75 | 55 | 30 |

FIGURE 2. Ectopic expression of IRF8 in L929 cells impairs Ifit1 induction. A, stable pools of L929 cells ectopically expressing different levels of IRF8 were generated. Three pools were selected for analysis: no IRF8 (No), low IRF8 (L), and high IRF8 (H). B, induction of IFIT family members in these pools was compared by Western blot following 8-h 1000-unit/ml IFNα treatment or in untreated controls. Note that two untreated no IRF8 controls are included on the gel, and both were retained to provide a complete gel image. C, RNA levels of IFIT family members were determined by real-time PCR following 4 h of 1000-unit/ml IFNβ treatment in the cell lines used in A. IFIT family transcript levels were normalized to 18S rRNA. All ratios were multiplied by 100 for clarity. Bars, mean ± S.D. (error bars) of three independent experiments. Student’s t test was used to compare no IRF8 samples with low or high IRF8 samples for each IFIT family gene. ****, p < 0.0001; ***, p < 0.01.

FIGURE 1. Ifit1 induction is impaired in B cells stimulated in vitro. IFIT family gene induction was measured in a preparation of splenocytes stimulated for 16 h in vitro with 1000 units/ml IFNβ (A) or 1000 units/ml IFNα (B). Following stimulation, flow cytometry was used to determine B and T cell populations in the splenocyte preparation and measure IFIT family protein expression. C, Ifit mRNA induction was measured in purified B cells stimulated in vitro with 1000 units/ml IFNβ for 4 h. D, Ifit mRNA induction was measured in purified B cells stimulated in vitro with 1000 units/ml IFNα for 4 h. Data are shown as mean ± S.D. (error bars) of three independent experiments. Student’s t test was used to compare unstimulated and stimulated samples for each IFIT family gene. *, p ≤ 0.05; NS, not significant.

IRF8 Represses Ifit1 More than Ifit2 or Ifit3
TABLE 3
Other genes that behave like Ifit1 in L929 cells

Microarray analysis was used to compare transcript induction in L929 cells or L929 cells stably expressing low levels of ectopic IRF8 (as in Fig. 2A) following 4 h of 1000-unit/ml IFNβ treatment. Shown is the average signal from two independent experiments for genes that, like Ifit1, were more strongly induced in control cells than in those containing ectopic IRF8.

| Gene symbol | L929 unstimulated | L929 IFNβ | L929IRF8 unstimulated | L929IRF8 IFNβ |
|-------------|------------------|-----------|------------------------|--------------|
| Ifit1-like replicate 1 |                |           |                        |              |
| Ccl2        | 14.07            | 571.8     | 34.03                  | 295.4        |
| Ccl5        | 20.13            | 175.7     | 37.12                  | 95.68        |
| Cutn3       | 15.61            | 25.26     | 16.04                  | 10.07        |
| Dusp28      | 37.44            | 203.9     | 27.49                  | 62.80        |
| Igtp        | 20.48            | 9895      | 34.62                  | 77.07        |
| Il7         | 44.90            | 223.5     | 85.43                  | 135.9        |
| LOC100044190| 476.4            | 2606      | 867.4                  | 1962         |
| Plekha4     | 6.745            | 276.9     | 6772                   | 202.5        |
| Tmem169     | 17.96            | 107.9     | 25.17                  | 74.64        |
| Trim21      | 21.78            | 544.1     | 25.12                  | 228.3        |
| Ifit1-like replicate 2 |                |           |                        |              |
| Ccl2        | 14.07            | 571.8     | 34.03                  | 295.4        |
| Ccl5        | 20.13            | 175.7     | 37.12                  | 95.68        |
| Cutn3       | 15.61            | 25.26     | 16.04                  | 10.07        |
| Dusp28      | 37.44            | 203.9     | 27.49                  | 62.80        |
| Igtp        | 20.48            | 9895      | 34.62                  | 77.07        |
| Il7         | 44.90            | 223.5     | 85.43                  | 135.9        |
| LOC100044190| 476.4            | 2606      | 867.4                  | 1962         |
| Plekha4     | 6.745            | 276.9     | 6772                   | 202.5        |
| Tmem169     | 17.96            | 107.9     | 25.17                  | 74.64        |
| Trim21      | 21.78            | 544.1     | 25.12                  | 228.3        |

**FIGURE 3. Impaired Ifit1 but not Ifit2 promoter activity in cells ectopically expressing IRF8.** L929 cells stably expressing low levels of IRF8 (or lentiviral expression vector control) were transiently transfected with a firefly luciferase reporter plasmid containing 1 kb upstream of the Ifit1 or Ifit2 transcriptional start site and a Renilla luciferase plasmid to control for transfection efficiency and cell viability. Cells were treated with IFNβ (1000 units/ml) for 8 h, and firefly and Renilla luciferase activities were measured. Bars, mean ± S.D. (error bars) of three independent experiments. Student’s t test was used to compare L929 and L929IRF8 cells for each IFIT family gene. ***, p ≤ 0.01; NS, not significant.

RNA level results and suggested that these constructs could be used to analyze the features of the Ifit1 promoter required for impairment by IRF8. To this end, truncation mutants of the Ifit1 promoter lacking successive 100-bp increments at the 5′-end were generated, and promoter activity was determined as for Fig. 3. All truncation mutants showed reduced promoter activity in control cells compared with the Ifit1 1-kb promoter (Fig. 4A), and promoter activity was not further impaired by ectopic expression of IRF8 (p > 0.001 for L929 versus L929IRF8 for 1 kb, p = 0.15 for 900 bp). This suggests that the region between 1 kb and 900 bp upstream of the Ifit1 transcriptional start site (TSS) is necessary for IRF8 impairment of IFIT gene induction. This was an unexpected observation, given the original description of the IFIT genes showing the ISREs located within 109 bp of the TSS (10). To further understand the importance of this ISRE region, promoter activity of internal deletion constructs lacking regions between 600 and 109 bp upstream of the Ifit1 TSS was determined. Whereas constructs with deletion between 600 bp and up to 200 bp upstream of the Ifit1 TSS showed impaired induction in cells containing ectopic IRF8 (Fig. 4B, p = 0.002), selective impairment of Ifit1 induction was not seen in cells with a promoter lacking the region between 600 and 109 bp upstream of the Ifit1 TSS. Both control and IRF8-expressing cells showed comparable promoter activity from this construct (Fig. 4B, p = 0.5166), suggesting that the −200 to −109 bp region adjacent to the ISREs also contributes to IRF8 repression of IFIT gene induction. To investigate the importance of both regions implicated in IRF8 repression of Ifit1, promoter activity was measured in a series of internal deletion mutants of the Ifit1 promoter lacking portions of the promoter between 900 and 200 bp upstream of the TSS. These promoter constructs, including one containing only the −1000 to −900 bp and the −200 bp to TSS regions of the Ifit1 promoter, retained the IRF8 repression of promoter activity seen in the intact Ifit1 1-kb promoter (Fig. 4C, L929 versus L929IRF8, p = 0.0110). In contrast, a promoter construct containing only 200 bp upstream of the Ifit1 TSS did not show impaired Ifit1 induction in cells containing ectopic IRF8 (L929 versus...
L929IRF8, \( p = 0.4436 \), suggesting that the region between 200 and 109 bp upstream of the TSS is not sufficient for IRF8 impairment of \( \text{Ifit1} \) gene induction.

**IRF8 Is Bound to the \( \text{Ifit1} \) ISRE in a B Cell Line**—To confirm IRF8 regulation of \( \text{Ifit1} \) in the context of an endogenous promoter, we investigated \( \text{IFIT} \) gene family induction in the WEHI231 B cell line. In this line, like in primary B cells and cultured pDCs treated with IFN\( \beta \), we observed poor induction of \( \text{Ifit1} \), but not \( \text{Ifit2} \), as measured at the protein level (Fig. 5A) and RNA level (Fig. 5B). We next tried to identify whether IRF8 interacted with the endogenous promoter at either the ISRE or the \( -1000 \) to \(-900 \) bp region identified through the promoter-reporter assays described above. ChIP assays were conducted on WEHI231 cells, with PCR primers used to amplify either the ISRE or the \( -1000 \) to \(-900 \) bp region of the \( \text{Ifit1} \) promoter (Fig. 5C). In both IFN\( \beta \)-treated and untreated cells, we detected IRF8 binding to the \( \text{Ifit1} \) ISRE but not the \(-1000 \) to \(-900 \) bp region; moreover, IRF8 was not bound to the ISREs of the \( \text{Ifit2} \) and \( \text{Ifit3} \) promoters.
IRF8 Represses Ifit1 More than Ifit2 or Ifit3

or Ifit3 genes. To obtain more quantitative estimates, IRF8 binding to IFIT ISREs was quantified after ChIP by real-time PCR (Fig. 5D). IRF8 bound to the Ifit1 ISRE preferentially, and binding was enhanced upon IFNβ stimulation. These results indicate that the extent of IRF8 binding to the ISREs of different IFIT family genes correlates inversely with their levels of expression.

IRF8 Must Bind DNA, Interact with Protein Partners, and Be Sumoylated to Impair Ifit1 Induction—In addition to their ISRE-binding property, to be fully active, many IRFs require post-translational modifications and the ability to bind partner proteins. Three major properties of IRF8 have been identified as contributors to its transcription-regulatory activities: DNA binding, interaction with other proteins through the IRF association domain, and sumoylation (21, 25, 29). Each of these properties can be independently disrupted by point mutations of key residues of the relevant domain. To this end, point mutants of IRF8 lacking either the ability to bind DNA (29, 30), interact with partner proteins (21, 31), or be sumoylated (25) were used to determine whether they could still impair Ifit1 gene induction. IRF8 (wild-type or mutant constructs) was transiently transfected into L929 cells, and two-color flow cytometry was used to investigate Ifit1 induction in cells expressing different levels of IRF8. As seen in cell lines stably expressing ectopic IRF8 (Fig. 2), transient expression of IRF8 impaired Ifit1 induction, with higher levels of IRF8 required to impair Ifit2 gene induction than Ifit1 induction (Fig. 6A). Impairment of Ifit1 gene induction was then compared between wild-type and mutant forms of IRF8. At expression levels where wild-type IRF8 impaired Ifit1 (p = 0.0070), but not Ifit2 gene induction (p = 0.0829), the mutants defective in DNA binding, protein interaction, or sumoylation were all ineffective to impair Ifit1 gene induction (Fig. 6B, control versus IRF8; p = 0.0819, p = 0.5072 and p = 0.1854, respectively). This suggests that effective impairment of Ifit1 gene induction requires that IRF8 is sumoylated and can bind DNA and interact with binding partners.

The Patterns of IFIT Induction Impairment by IRF8 Vary with Signaling Pathways—The role of IRF8 in impairing IFIT gene induction has been studied to date in IFNβ-treated cells in which gene induction is predominantly driven by the IRF9-containing ISGF3 complex (32). However, IFIT genes can also be induced by IRF3 following stimulation through RIG-I (33). To investigate the nature of IRF8 regulation of IFIT gene induction by the RIG-I pathway (34, 35), L929 cell lines stably expressing ectopic IRF8 were infected with Sendai virus. In Sendai virus-infected cells, low levels of ectopic IRF8 expression impaired induction of all IFIT family members (Fig. 7A), in contrast to the selective sensitivity of Ifit1 induction in IFNβ-treated cells (Fig. 7A). Transfected poly(I:C) is another activator of the cytosolic nucleic acid receptors, and total impairment of IFIT gene induction by low level ectopic IRF8 was also observed in cells stimulated by poly(I:C) transfection (Fig. 7B). This suggests that IRF8 can more effectively impair IFIT gene induction by IRF3, activated by the RIG-I pathway, than ISGF3, activated by the JAK-STAT pathway.

Discussion

The results described above provide the first insights into a mechanism for mediating cell type- and stimulus-specific induction patterns observed in different members of the IFIT gene family (12). We show that high levels of IRF8, either endogenous or through stable or transient ectopic expression, can impair induction of IFIT genes. We had previously observed impaired Ifit1 induction in B cells and pDCs, which express high levels of endogenous IRF8. Despite poor Ifit1 induction in these cell types, other IFIT family members were induced in these cells, whereas strong induction of all IFIT family members was observed in T cells or conventional DCs,
IRF8 represses Ifit1 more than Ifit2 or Ifit3

FIGURE 7. IRF8 impairs induction of all IFIT family members when cells are stimulated with RIG-I stimuli. L929 cells stably expressing low levels of ectopic IRF8 (or vector control) were treated with indicated stimuli, and induction of IFIT family members was detected by Western blot 8 h after stimulation. A, L929 cells were treated with 1000 units/ml IFNβ or Sendai virus infection (SeV; multiplicity of infection 10). B, cells as in A were transfected with poly(I:C) using Lipofectamine 2000 as indicated under “Experimental Procedures.” Data are representative of two independent experiments.

respectively, which express low levels of endogenous IRF8 and were obtained from the same animals or cell cultures (12, 13). IFIT gene transcription has been shown to be driven by the ISREs present in their promoters (10), making members of the IRF family the most likely regulators of IFIT gene induction. The observations that these poor Ifit1-inducing cell types contain particularly high endogenous levels of IRF8 (16, 17) suggested a potential role for IRF8 in the cell type- and inducer-specific IFIT family gene patterns we observed. IRF8 can act to either enhance or repress gene transcription, depending on the binding partner it interacts with (23), making it an attractive candidate for differential regulation of IFIT family gene induction (36). Testing this hypothesis through ectopic expression of IRF8 in L929 fibroblasts, which do not express IRF8, revealed two phenomena. Following IFN treatment, at low levels of ectopic IRF8 expression, Ifit1 induction was selectively impaired in these cells, as was observed in B cells and pDCs. In cells expressing low levels of ectopic IRF8, a greater impairment of Ifit3 induction was seen at the RNA level than at the protein level (Fig. 2, compare B and C). This may reflect lesser stability of Ifit3 mRNA relative to protein, compared with Ifit1 and Ifit2 mRNAs. However, at higher levels of ectopic IRF8 expression, induction of all IFIT family members was impaired (Fig. 2B).

We wanted to understand the mechanism leading to the former observation, given its biological relevance. We initially investigated gene induction, in cells stably expressing IRF8, by microarray analysis to identify a cohort of genes that behaved like Ifit1. Although we were able to identify a small cohort of these genes (Table 3), more detailed analysis is required to identify common features of these genes that may be regulated by IRF8. As an alternative approach to understanding IRF8 regulation of Ifit1, we used promoter reporter assays to identify regions of the Ifit1 promoter required for repression by IRF8. Although 1 kb upstream of the Ifit1 TSS was sufficient for repression by IRF8, truncation and deletion of portions of this promoter identified a key region, between 1 kb and 900 bp upstream of the TSS, required for selective impairment of Ifit1 gene induction (Fig. 4). This region does not contain any predicted transcription factor binding site that is absent from the Ifit2 and Ifit3 promoters, for which less repression by IRF8 was seen, and it does not contain a PRD1 consensus sequence (5'-TCACTT-3') where IRF8 has been shown to interact directly with DNA and repress gene transcription (18). As anticipated, ChIP assays showed that IRF8 does not bind to this region (Fig. 5). Currently, it is unclear how this region of the Ifit1 promoter regulates its transcription. It is possible that our observation reflects differences in regulation, by IRF8, between the transiently introduced promoter-reporter constructs and the chromosomal Ifit1 promoter. It is also possible that to mediate repression, IRF8, bound to the Ifit1 ISRE, needs to interact with...
IRF8 Represses Ifit1 More than Ifit2 or Ifit3

a protein that specifically binds to the upstream regulatory element.

IRFs require post-translational modification and interaction with binding partners (reviewed in Ref. 37) to regulate gene transcription. IRF8 has been shown to interact with IRF1 and IRF2, bind to ISREs, and repress gene transcription (38). However, IRF8 can also interact with PU.1 and drive activation of promoters containing an Ets-ISRE composite element (23, 24). Because DNA binding and interaction with other proteins are mediated by the DNA-binding domain and IRF-association domains of IRF8, respectively, these functions could be independently impaired using point mutants in each of these domains. Mutating Lys-79, a residue shown by homology to interact with the DNA phosphate backbone (30), to Glu (K79E) in the DNA-binding domain disrupts DNA binding (29). Arg-289 is a highly conserved residue among the IRF family and is present in the interaction domain of IRF8 (31). Mutation of this residue to Glu (R289E) removes the ability of IRF8 to bind to IRF2 or PU.1 and modulate gene transcription (21). These K79E and R289E mutants could not impair Ifit1 gene induction as effectively as wild-type IRF8 (Fig. 6B), demonstrating that IRF8 must be able to bind DNA and interact with binding partners for this activity.

Post-translational modification can also regulate activity of IRF8. Recent studies have shown sumoylation of IRF8 on Lys-310 converts IRF8 from an activator to a repressor of gene transcription in macrophage cell lines. Rather than altering binding partners for IRF8, sumoylation was shown to reduce mobility of IRF8 in the nucleus. Mutating Lys-310 to Arg (K310R) removes IRF8 sumoylation and leads to higher levels of Il12p40 and Ccl9 gene induction in stimulated RAW264.7 macrophages (25). To test the importance of this sumoylation, the ability of IRF8 K310R to impair Ifit1 induction was evaluated. The K310R IRF8 mutant could not impair Ifit1 induction as effectively as wild-type IRF8 (Fig. 6B). This is consistent with sumoylation being required for IRF8 to repress gene induction. Sumoylation is only one of many possible post-translational modifications of IRF8 that may regulate its ability to activate or repress gene transcription. Tyr-107 and Tyr-211 have both been implicated in controlling the ability of IRF8 to interact with binding partners and regulate gene transcription. Phosphorylation of both of these residues has been shown to be required for interaction with PU.1 and activation of gene transcription in mouse and human experimental systems (19, 39). However, unlike the sumoylation-deficient mutant used in the current study, IRF8 mutants lacking Tyr-107 or Tyr-211 phosphorylation showed reduced, rather than increased, target gene induction (19, 39), making them less attractive candidates to test for IRF8-mediated impairment of Ifit1 induction. Nonetheless, additional studies are required to determine whether these and other post-translational modifications also regulate the ability of IRF8 to repress Ifit1 induction.

Finally, we observed different patterns of IFIT gene induction in cells containing ectopic IRF8, depending on the stimulus used. Whereas selective impairment of Ifit1 induction was seen in IFNβ-treated cells containing low levels of ectopic IRF8 (Figs. 2 and 7), these same cells showed impaired induction of all IFIT family members following stimulation through RIG-I (Fig. 7). In cells not expressing IRF8, RIG-I signaling is known to induce the IFIT genes through the direct action of activated IRF3, without any involvement of IFN (5). IRF9, as part of the ISGF3 complex, is the primary driver of IFIT gene induction following stimulation through the type I IFN receptor (36, 40), whereas IRF3 drives ISRE-dependent gene induction following RIG-I stimulation (33). Because a low level of IRF8 expression was sufficient to block IRF3 action completely, whereas a higher level of IRF8 was required to block the action of ISGF3 on all IFIT family genes, it appears that IRF8 can impair gene induction driven by IRF3 more strongly than that driven by IRF9. One potential mechanism to explain this observation is that IRF8 can compete better for binding partners or other cofactors required for gene transcription with IRF3 than IRF9. The idea that IRF8 competes with activating transcription factors for cofactors or binding partners would also be supported by our observations that at high levels of ectopic IRF8 expression, induction of all IFIT family members was impaired, even when driven by IRF9 (Fig. 2B). Further investigation will be required to study the role of IRF8 in repressing IFIT gene induction by other IRFs, such as IRF7.

Author Contributions—C. L. W. and G. C. S. designed experiments and analyzed and interpreted data. C. L. W., P. M. K., and B. K. D. conducted experiments and analyzed and interpreted data. C. L. W. wrote the manuscript. G. C. S., K. O., P. M. K., and B. K. D. edited the manuscript.

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IRF8 Represses Ifit1 More than Ifit2 or Ifit3

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