

# Interferon Regulatory Factor (IRF)-1 and IRF-2 Regulate Interferon $\gamma$ -dependent Cyclooxygenase 2 Expression

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## Abstract

Cyclooxygenases (Cox) are rate-limiting enzymes that initiate the conversion of arachidonic acid to prostanoids. Cox-2 is the inducible isoform that is upregulated by proinflammatory agents, initiating many prostanoid-mediated pathological aspects of inflammation. In this study, we demonstrate that interferon (IFN)- $\gamma$  alone or in synergy with lipopolysaccharide (LPS) or interleukin 1 $\alpha$  induces Cox-2 expression in mouse peritoneal macrophages, which is paralleled by changes in Cox-2 protein levels and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release. Induction of Cox-2 was abrogated in macrophages that lack IFN regulatory factor (IRF)-1, consistent with an attenuated hepatic mRNA response in IRF-1<sup>-/-</sup> mice injected with LPS. Conversely, the absence of IRF-2 in macrophages resulted in a significant increase in both basal and inducible Cox-2 gene and protein expression as well as IFN- $\gamma$ -stimulated PGE<sub>2</sub> release, identifying IRF-2 as negative regulator of this promoter. Two IFN stimulation response elements were identified in the mouse Cox-2 promoter that were highly conserved in the human Cox-2 gene. Both bind endogenous IRF-1 and IRF-2 and regulate transcription in an IRF-1/2-dependent manner. Our data demonstrate conclusively the importance of IFN- $\gamma$  as a direct activator and coactivator of the Cox-2 gene, and the central role of IRF-1/2 family members in this process.

Key words: inflammation • prostaglandin • macrophage • cytokine • septic shock

## Introduction

PGs are involved in homeostatic, developmental, and inflammatory processes (1). PG endoperoxidase H synthases (PGHSs),<sup>1</sup> also called cyclooxygenases (Cox), are the rate-limiting enzymes that initiate conversion of arachidonic acid to all PGs, prostacyclins, and thromboxanes (2). Two distinct Cox genes have been cloned and characterized. They encode similar proteins, sharing 60% amino acid se-

quence identity, although they are expressed after different developmental and temporal patterns (3). Cox-1, also known as the "constitutive" cyclooxygenase, is detected in almost all tissues. This enzyme is believed to be responsible for the production of PGs involved in the maintenance of homeostasis. Cox-2, the "inducible" cyclooxygenase, is not generally expressed basally, but is rapidly and strongly activated in many cells upon induction with a variety of proinflammatory agents, cytokines, hormones, and tumor promoters (2).

IFN- $\gamma$  is considered to be a potent regulator of immunological and inflammatory responses (4). A homodimer of IFN- $\gamma$  initially binds to the  $\alpha$  chain of a heterodimeric receptor that carries Janus kinase (JAK)1. Dimerization with IFN- $\gamma$  receptor  $\beta$  chains that carry JAK2, transphosphorylation of JAK kinases, and ultimately phosphorylation of preformed cytosolic signal transducer and activator of transcription (STAT)1 $\alpha$  subunits, follow. Phosphorylated STAT1 $\alpha$  homodimers translocate to the nucleus and acti-

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<sup>1</sup>Abbreviations used in this paper: Cox, cyclooxygenase(s); EMSA, electrophoretic mobility shift assay; GBP, guanylate binding protein; HPRT, hypoxanthine guanine phosphoribosyl transferase; ICSBP, IFN consensus sequence binding protein; iNOS, inducible nitric oxide synthase; IRF, IFN regulatory factor; ISG15, IFN- $\gamma$ -stimulated protein 15; ISRE, IFN-stimulated responsive element; JAK, Janus kinase; NF, nuclear factor; NO, nitric oxide; PGHS, PG endoperoxidase H synthase; RT, reverse transcription.

vate several primary IFN- $\gamma$  response genes by binding to IFN- $\gamma$ -activated sites within their promoters. Among these IFN- $\gamma$  primary response genes are several IFN regulatory factors (IRFs [5, 6]), a growing family of transcription factors that contains, among others, IRF-1 and IRF-2. IRF-1 is strongly inducible by IFN- $\gamma$  and binds IFN-stimulated responsive elements (ISREs) within promoters, activating transcription. In contrast, IRF-2 is constitutively expressed and acts mostly as a repressor by competing with IRF-1 for the same cis-element (the ISRE has also been called IRF-1/IRF-2 binding sequence motif, or IRF-E), and possibly by repressing activators positioned nearby in the promoter (6–8). These ISREs, usually defined by palindromic TTTC sequences separated by two or three nucleotides (9, 10), are located in the promoter region of many IFN-inducible genes. It has been shown that IRF-1-deficient cells exhibit impaired induction of several genes involved in the innate immune response, including the 2-5A synthetase (11), inducible nitric oxide synthase (iNOS [12]), and IL-12 p40 and p35 genes (13). Similarly, dysregulated nitric oxide (NO) release (14) and IL-12 gene induction (13) and secretion have been reported for IRF-2-deficient macrophages.

In this report, we show that IFN- $\gamma$  works as a pivotal regulator of the *Cox-2* gene, activating its expression or coactivating LPS- and IL-1 $\alpha$ -dependent *Cox-2* expression in primary murine macrophages. This regulation is dependent on the expression of IRF-1, and requires the presence of two novel ISREs that are localized in the promoter of the murine *Cox-2* gene and are conserved in the human *Cox-2* gene. Importantly, our data reveal a mechanism for negative regulation of the *Cox-2* gene through IRF-2 action.

## Materials and Methods

**Reagents and Antibodies.** Purified recombinant murine IFN- $\gamma$  ( $1.3 \times 10^7$  U/ $\mu$ g) was provided by Genentech, Inc. Protein-free (<0.008%, phenol/water-extracted) *Escherichia coli* K235 LPS was prepared by the method of McIntire et al. (15). Mouse rIL-1 $\alpha$  was provided by Hoffmann-LaRoche. Anti-Cox-1 (no. 160110) and anti-Cox-2 (no. 160116) antibodies were purchased from Cayman Chemical. Rabbit polyclonal antibodies to IRF-1, IRF-2, and IFN consensus sequence binding protein (ICSBP) were as described previously (16, 17).

**Mice.** All animals were housed in cages with filter tops in a laminar-flow hood, fed food and acid water ad libitum, and bred in a virus antibody-free facility. Mice with a targeted mutation in the IRF-1 or IRF-2 gene (homozygous [–/–] mice and their heterozygous [+/-] littermates) were originally obtained from Dr. Tak Mak (Amgen Institute, Toronto, Canada) and had been backcrossed to C57BL/6 mice three to five times. The IRF-1<sup>-/-</sup> and IRF-2<sup>-/-</sup> colonies have been maintained at Uniformed Services University of the Health Sciences as detailed elsewhere (14). Whenever possible, IRF-1<sup>+/+</sup> and IRF-2<sup>+/+</sup> mice derived from heterozygous littermates were used as controls; otherwise, C57BL/6J<sup>+/+</sup> mice (The Jackson Laboratory) were used. All mice bred for use in this study were genotyped. Male and female mice were used and were typically between 6 and 12 wk of age. For temporal analysis of *Cox-2* expression in the liver, mice were injected intraperitoneally with 25  $\mu$ g (~1–1.4 mg/kg) of LPS. In

these experiments, groups of four mice were used for each time point. Experiments were carried out according to the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

**Tissue Culture.** Peritoneal macrophages from all mice were isolated 4 d after intraperitoneal injection of 3 ml of sterile 3% thioglycollate broth. Cells were washed with pyrogen-free saline and resuspended in RPMI 1640 supplemented with 2 mM l-glutamine, 100 IU/ml of penicillin, 100  $\mu$ g/ml streptomycin, 10 mM Hepes, 0.3% sodium bicarbonate, and 2% FCS, then were plated in six-well plates ( $4 \times 10^6$  cells/well) and incubated overnight at 37°C and 6% CO<sub>2</sub>. Cells were washed twice with PBS to remove nonadherent cells, and were incubated with different stimuli or vehicle for the indicated time periods. The RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection and was cultured in RPMI medium supplemented with 10 mM Hepes, 0.3% sodium bicarbonate, 2 mM l-glutamine, 100 IU/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and 10% FCS, and was incubated as described above.

**Analysis of mRNA.** Total RNA from cultured macrophages or from the livers of individual LPS-treated mice was isolated using RNA-Stat60 (Tel-Test) according to the manufacturer's instructions. The relative quantities of mRNA for *Cox-1*, *Cox-2*, and the "housekeeping gene," hypoxanthine guanine phosphoribosyl transferase (HPRT), were determined by coupled reverse transcription (RT)-PCR. The primers and probes used in each case were as follows: *Cox-1* sense primer 5'-CCCAGAGTCATGAGTCGAAGGAG-3', antisense 5'-CAGCGCATGAGTACTTCTCGG-3', and probe CCACTGTGATTGTACTCGCACG; *Cox-2* (18) sense primer 5'-GCAAATCCTTGCTGTTCCAATC-3', antisense primer 5'-GGAGAAGGCTTCCCAGCTTTG-3', and probe CCCACTCAAGGGAGTCTGGAACA; HPRT (19) sense primer 5'-GTTGGATACAGGCCAGACTTTGTTG-3', antisense primer 5'-GATTCAACTTGCCTCATCTTAGGC-3', and probe GTTGTGGATATGCCCTTGAC. Enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) were used for visualization of the amplified RNA transcripts after electrophoresis of the samples and Southern blot hybridization.

**Plasmid Construction and Transfection.** PGHS-2 "short" and "long" reporters were as described previously (20). PGHS-2 "medium" was constructed by cloning the 2.1-kb BglII-Hind III fragment from PGHS-2 long into the same sites in pGL2 basic luciferase vector. The PGHS-2 (-43) reporter, which contains the basic promoter, initiator sequence, and 88 bp of untranslated region from the murine *Cox-2* gene, was constructed by PCR amplification of PGHS-2 long plasmid with GL2 (Promega) and 5'-GCGCGCGCCTCGAGCCGCTTACAGACTTAAAAGCAAGGTTTC-3' primers, and the resulting fragment was inserted into pGL2 basic vector. ISRE I (-43) and ISRE II (-43) were constructed by cloning three copies of the ISRE I (5'-TAAAACAGAAAACAAGAAC-3') and ISRE II (5'-CCTTTCATTTTGTGTTTGTGTT-3') sequence into the SmaI-SacI site of PGHS-2 (-43) plasmid. Mutant versions of these ISREs (ISRE I mutant: 5'-TAAAACATTAACAATTAC-3', ISRE II mutant: 5'-CCTAACATTTTAAATTTTGTGTT-3') were cloned as described above. Guanylate binding protein (GBP)-Ld40 and AP1-Ld40 luciferase reporters were described previously (21, 22). All reporter constructs were confirmed by sequencing analysis. The IRF-1 expression vector was constructed by cloning its cDNA into the EcoRI site in pcDNA3.1 vector (Invitrogen). IRF-2 expression vector was described previously (22, 23). For transfection, RAW 264.7 cells were seeded the night before the experiment on 24-well

plates ( $2 \times 10^5$  cells per well). The next day, reporter plasmid (0.2  $\mu$ g) and expression vectors for IRF-1, IRF-2, or control plasmids were transfected along with 0.25  $\mu$ g of pBluescript II SK(+) and 0.125  $\mu$ g of  $\beta$ -galactosidase expression plasmid (pCH110; Amersham Pharmacia Biotech) using SuperFect transfection reagent (Qiagen) as described (23). After transfection (3 h), cells were incubated with fresh media, IFN- $\gamma$  (10 U/ml), and/or LPS (1 ng/ml) for an additional 24 h. Luciferase and  $\beta$ -galactosidase activity were measured as described previously (22), and  $\beta$ -galactosidase activity was used for normalization of the luciferase activity within each treatment.

**Western Blot Analysis.** Whole cell extracts were prepared from peritoneal macrophages after treatment with different stimuli using a lysis buffer containing 20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (Complete<sup>TM</sup>; Boehringer). Crude extracts were passed through a 23-gauge needle 10 times to disrupt cells completely, and were centrifuged to remove debris. Supernatants were subjected to 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with the indicated antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and reagents for enhanced chemiluminescence detection were obtained from Amersham Pharmacia Biotech.

**Bead Assay for Detection of DNA-binding Proteins.** For DNA capture using magnetic beads, nuclear extracts from IFN- $\gamma$ -treated RAW 264.7 cells were obtained using the method of Dignam et al. (24), with modifications. Cells were washed with PBS and incubated for 15 min in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and protease inhibitors) at 4°C. Cells were pelleted and resuspended in fresh buffer A and homogenized using a Dounce homogenizer for 10 strokes. The nuclear pellet was finally resuspended in buffer C (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors) and incubated for 15 min at 4°C, with rotation. Nuclear debris was pelleted, and supernatants were snap-frozen at -70°C. Biotinylated DNA fragments containing different regions of the Cox-2 promoter were prepared by PCR reactions using the different luciferase reporter constructs and GL2 and biotinylated GL1 primers. Approximately 30 pmol of each fragment was immobilized onto magnetic beads (Dynabeads M280 streptavidin; Dynal) according to the manufacturer's procedure. In all cases, 95% of the biotinylated DNAs were attached to the beads. For capturing DNA binding proteins, 500  $\mu$ g of nu-

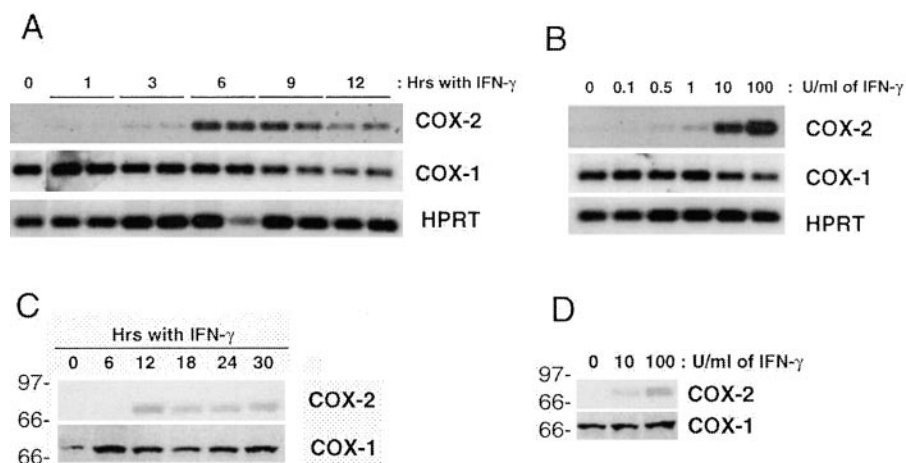
clear proteins was incubated with 200  $\mu$ g of DNA-conjugated beads for 2 h at 4°C. Beads were washed three times using buffer C, and were resuspended in SDS-loading buffer and subjected to SDS-PAGE and Western blot analysis.

**Electrophoretic Mobility Shift Assay.** Primers for Cox-2 ISRE I (5'-TTCCCGCATTAAAATAAAACAGAAAACAAGAACT-ATTTAAA-3' and complementary strand) and Cox-2 ISRE II (5'-TGTGGATATTCTCCCCTTTCATTTTTGTTTTGTTT-TGTTCT 3' and complementary strand) were annealed and <sup>32</sup>P-endlabeled with T4 polynucleotide kinase (Promega). Nuclear extracts (5  $\mu$ g) were incubated with 0.2 ng of the indicated DNA probe and 2  $\mu$ g of poly(dI-dC) (Amersham Pharmacia Biotech) in a binding buffer containing 20 mM Hepes, pH 7.9, 4 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol at room temperature for 15 min. For competition experiments, 50-fold excess of primers containing GBP-ISRE, IFN- $\gamma$ -stimulated protein 15 (ISG15)-ISRE (23), or nuclear factor (NF)- $\kappa$ B binding site (21) were included in the binding reaction. For supershift assays, a total of 2 ng of antibodies against specific members of the IRF family of transcription factors was also included in the reaction. DNA-protein complexes were resolved by electrophoresis in a 5% polyacrylamide gel as described previously (25).

**PGE<sub>2</sub> Detection.** For quantification of PGE<sub>2</sub> release from macrophages,  $5 \times 10^5$  cells per well were seeded on a 24-well plate. The next day, cells were incubated with 500  $\mu$ l of medium only or medium supplemented with IFN- $\gamma$  (100 U/ml), and the presence of PGE<sub>2</sub> in the supernatants was measured by ELISA 24 h later (Cayman Chemical; no. 514010).

## Results

**IFN- $\gamma$  Activates Transcription of the Cox-2 Gene.** IFN- $\gamma$  is a major activator of macrophages, a cell type in which Cox-2 has been reported to be highly upregulated upon exposure to several proinflammatory signals. Initially, we characterized the effect of IFN- $\gamma$  on Cox-2 gene expression. Peritoneal exudate macrophages were isolated from C57BL/6 mice and treated in vitro with IFN- $\gamma$  for different periods of time. RNA isolated from each treatment was converted to cDNA and amplified by PCR for Cox-2, Cox-1, and HPRT as control. Cox-2 transcripts were detected within 1 h after incubation with IFN- $\gamma$ , peaked at 6 h,

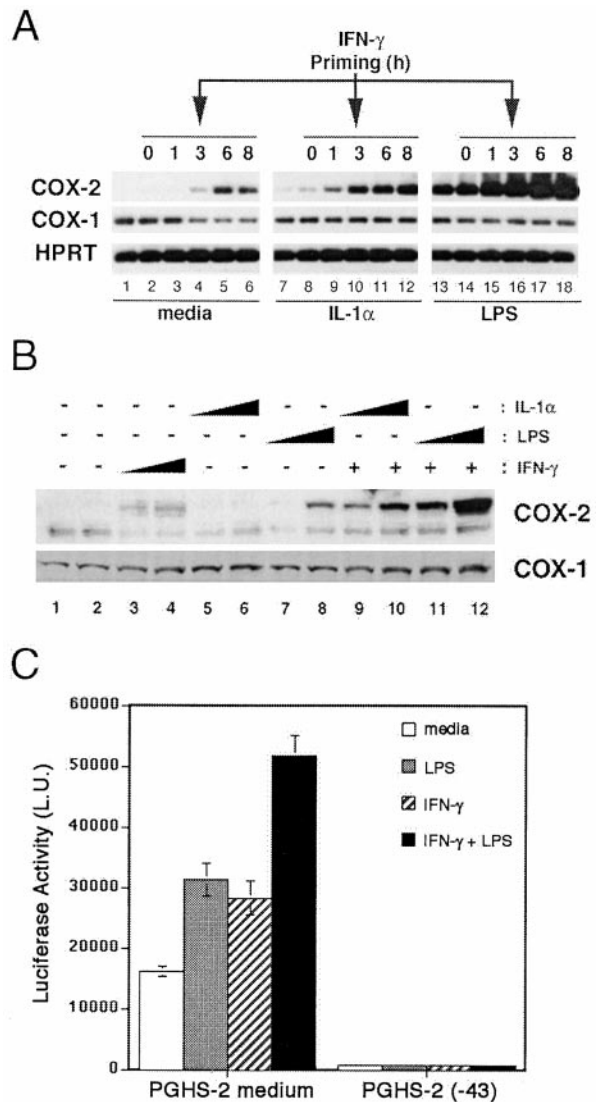


**Figure 1.** IFN- $\gamma$  activation of Cox-2 gene expression. (A) Peritoneal macrophages from C57BL/6 mice were treated for the indicated amounts of time with 10 U/ml of murine IFN- $\gamma$ . Total RNA was isolated from duplicate wells and subjected to RT-PCR reactions with specific primers for Cox-1, Cox-2, and HPRT. (B) Dose response analysis of Cox-2 gene expression for IFN- $\gamma$ . (C) Western blot analysis of total protein extracts from peritoneal macrophages incubated for different periods of time with 100 U/ml of IFN- $\gamma$  were performed using anti-Cox-1 and anti-Cox-2 antibodies. (D) Dose response analysis of Cox-1 and Cox-2 protein expression after 12 h of treatment with medium only (0) or IFN- $\gamma$ .

and slowly decreased after 9 h (Fig. 1 A). In contrast to Cox-2, IFN- $\gamma$  treatment resulted in a very modest decrease in the steady-state levels of Cox-1 mRNA after 9 h, consistent with previous observations (26). Equivalence of RNA loading was verified by the consistency of HPRT mRNA housekeeping signals. Moreover, previous studies from our laboratory, also carried out with peritoneal exudate macrophages, indicate that over this period of time, IFN- $\gamma$  fails to induce either macrophage apoptosis or necrosis (27). Treatment of macrophages with different doses of IFN- $\gamma$  for 6 h showed a peak in mRNA with 10–100 U/ml (Fig. 1 B), although detectable levels of Cox-2 transcript were observed after treatment with as little as 0.5 or 1 U/ml IFN- $\gamma$ . To analyze the effect of IFN- $\gamma$  on Cox-2 protein production, we performed Western blot analyses using Cox-2-specific antibodies (Fig. 1, C and D). Cox-2 protein peaked after 12 h of IFN- $\gamma$  treatment, reaching a plateau that persisted for >30 h. This induction was detectable with concentrations as low as 10 U/ml of IFN- $\gamma$ . Taken together, these data indicate that steady-state Cox-2 mRNA expression and protein synthesis are upregulated by IFN- $\gamma$  in mouse peritoneal macrophages.

**IFN- $\gamma$  Synergizes with LPS and IL-1 $\alpha$  to Induce Cox-2 mRNA and Protein Expression.** LPS (28–32) and IL-1 $\alpha$  (33, 34) are two powerful inducers of Cox-2 expression in several cell types. We sought to analyze if pretreatment of macrophages with IFN- $\gamma$  would affect activation of Cox-2 by these two inducers. When we analyzed steady-state levels of Cox-2 mRNA after 2 h of treatment with IL-1 $\alpha$  or LPS in the absence of IFN- $\gamma$ , clearly detectable amounts of Cox-2 transcript were observed in LPS-treated macrophages, yet only a barely detectable signal was observed in response to IL-1 $\alpha$  (Fig. 2 A; compare lanes 1 and 7 for IL-1 $\alpha$ , and lanes 1 and 13 for LPS). Concurrent treatment of cells with IFN- $\gamma$  and IL-1 $\alpha$  (lane 8) or LPS (lane 14) slightly augmented the signal seen in the absence of IFN- $\gamma$ . Preincubation of macrophages with IFN- $\gamma$  for longer time periods produced a significant enhancement of both IL-1 $\alpha$ - (lanes 9–12) and LPS-dependent (lanes 15–18) Cox-2 mRNA induction. This coactivation peaks after 6–8 h of pretreatment with IFN- $\gamma$ , enhancing mRNA accumulation  $\sim$ 10-fold over levels detected after 2 h of treatment with either inducer alone (compare lanes 7 and 12 for IL-1 $\alpha$  and lanes 13 and 18 for LPS).

Analysis of Cox-2 protein revealed similar results (Fig. 2 B). Although IFN- $\gamma$  alone activated the expression of Cox-2 (lanes 3 and 4), it strikingly increased the levels of Cox-2 induced by IL-1 $\alpha$  (compare lanes 5 and 6 with lanes 9 and 10) or LPS (compare lanes 7 and 8 with lanes 11 and 12) alone. To investigate whether IFN- $\gamma$  and LPS or IL-1 $\alpha$  coactivate Cox-2 expression transcriptionally, transient transfections using a luciferase reporter containing  $\sim$ 2.1 kb of the murine Cox-2 promoter (PGHS-2 medium) were performed in RAW 264.7 macrophages (Fig. 2 C). IFN- $\gamma$  alone increased the activity of this reporter almost twofold, which is comparable to the activation obtained with a low dose of LPS (1 ng/ml). However, when transfected cells were preincubated with IFN- $\gamma$  for 6 h, activation by LPS



**Figure 2.** IFN- $\gamma$  synergizes with IL-1 $\alpha$  and LPS for Cox-2 expression. (A) Peritoneal macrophages were either untreated (lanes 1, 7, and 13) or pretreated with 10 U/ml of IFN- $\gamma$  (lanes 2–6, 8–12, and 14–13) for the indicated periods of time (top) before the addition of media (lanes 1–6), IL-1 $\alpha$  (10 ng/ml, lanes 7–12), or LPS (0.1 ng/ml, lanes 13–18) for 2 h. Time 0 of IFN- $\gamma$  priming represents the point at which IL-1 $\alpha$  or LPS was added to the cultures for 2 h. (B) Western blot analysis of priming experiment for Cox-1 and Cox-2 proteins. Cells were primed with 10 U/ml of IFN- $\gamma$  for 12 h (1) and subsequently incubated with two concentrations of IL-1 $\alpha$  (triangle; 1 and 10 ng/ml) or LPS (triangle; 0.1 and 0.5 ng/ml) for another 12 h. Results shown are representative of three independent experiments. (C) Transient transfection assay using the Cox-2 luciferase reporter (PGHS-2 medium) or a minimal Cox-2 promoter (PGHS-2 [–43]) in RAW 264.7 cells. After transfection, cells were left untreated (media) or were pretreated with IFN- $\gamma$  (10 U/ml) for 6 h. The media was replaced with IFN- $\gamma$  alone, LPS alone, or IFN- $\gamma$  plus LPS (1 ng/ml), and incubation continued for an additional 18 h. Results are the means of six independent experiments  $\pm$  SD.

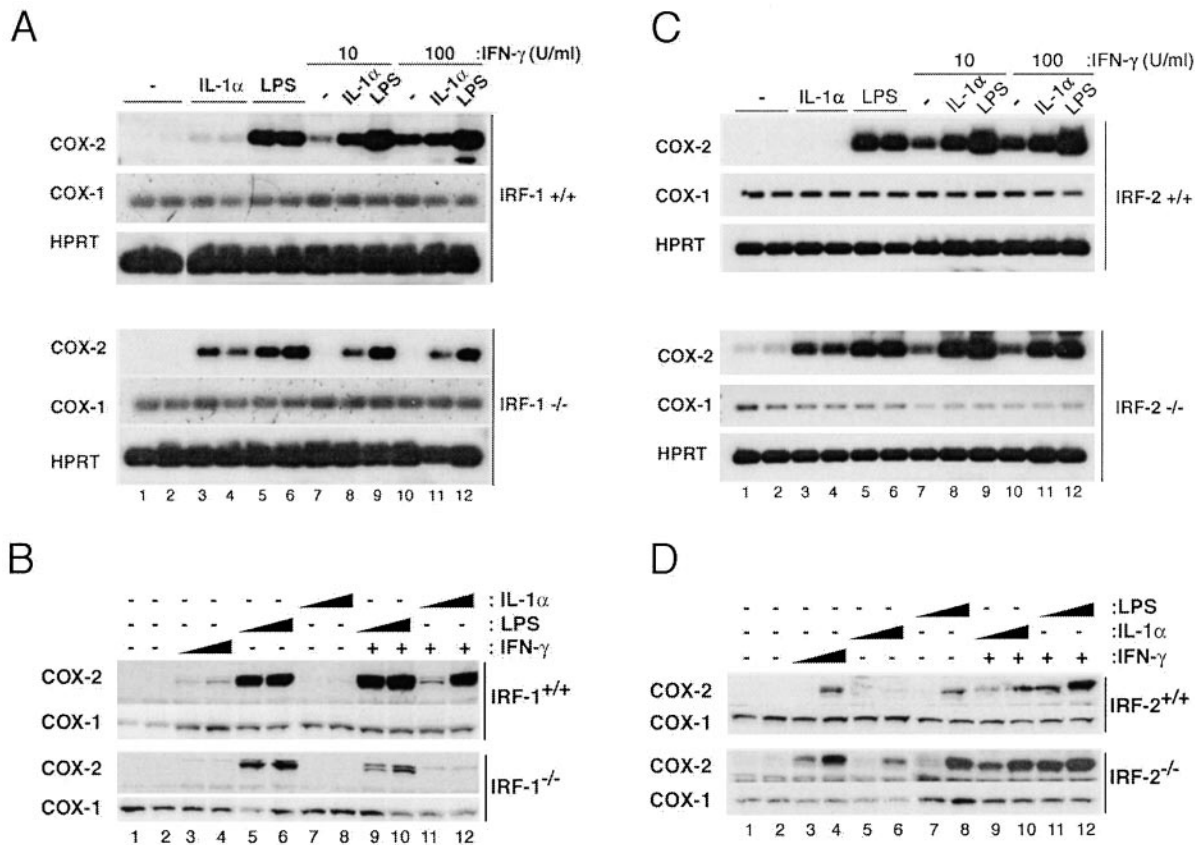
was substantially augmented. Similar treatments did not affect luciferase activity in cells transfected with a minimal Cox-2 promoter (Fig. 2C; PGHS-2 [–43]). Although similar experiments were conducted using IL-1 $\alpha$  as the in-

ducer, the effect on Cox-2 RNA and protein levels seen in peritoneal macrophages could not be mimicked in luciferase reporter assay, which is likely to reflect the predominant posttranscriptional regulation of Cox-2 expression by IL-1 $\alpha$  (34). However, IL-1 $\alpha$  treatment did not affect the IFN- $\gamma$ -dependent activation of the Cox-2 luciferase promoter construct (data not shown). Together, these data indicate that IFN- $\gamma$  enhances Cox-2 expression induced by both IL-1 $\alpha$  and LPS in mouse peritoneal macrophages, rendering it more sensitive to induction by these activators.

*IRFs Are Involved in the Regulation of Cox-2 Gene.* Members of the IRF family of transcription factors have been demonstrated to play a major role in the regulation of several inflammatory genes including iNOS (12, 35, 36) and IL-12 p40 and IL-12 p35 (13, 37). IRF-1 is induced early by IFN- $\gamma$  (38) through a STAT-1-dependent pathway (39) and acts as a transcriptional activator, whereas IRF-2 is expressed constitutively and is commonly described as an antagonist of IRF-1-dependent activation (7, 8, 40). We took advantage of mice with targeted disruptions in IRF-1 or IRF-2 genes for the analysis of the ex-

pression of Cox-2 after treatment with IFN- $\gamma$  alone or in combination with LPS or IL-1 $\alpha$ .

IFN- $\gamma$  was unable to activate Cox-2 mRNA expression in IRF-1 $^{-/-}$  macrophages (Fig. 3 A; IRF-1 $^{-/-}$ , lanes 7 and 10) when compared with the control IRF-1 $^{+/+}$  macrophages. Moreover, synergy for steady-state Cox-2 mRNA expression between IL-1 $\alpha$  or LPS and either concentration of IFN- $\gamma$  was abrogated in IRF-1 $^{-/-}$  macrophages (compare lanes 3 and 4 with lanes 8 and 11 for IL-1 $\alpha$ , and lanes 5 and 6 with lanes 9 and 12 for LPS). Cox-2 protein expression was also analyzed in IRF-1 $^{-/-}$  macrophages (Fig. 3 B). We found that after IFN- $\gamma$  treatment, these cells were unable to produce any Cox-2 (Fig. 3 B; lanes 3 and 4). Moreover, IFN- $\gamma$  priming did not result in IL-1 $\alpha$ - or LPS-dependent synergy in Cox-2 protein expression (Fig. 3 B; compare lanes 5–8 with 9–12 from IRF-1 $^{-/-}$  and IRF-1 $^{+/+}$ ), consistent with the steady-state mRNA data. One additional point to be made is that Cox-2 mRNA was upregulated in IRF-1 $^{-/-}$  macrophages treated with IL-1 $\alpha$  only (lanes 3 and 4), although this was not observed at the level of protein.



**Figure 3.** Regulation of the expression of Cox-2 in IRF-1- and IRF-2-deficient macrophages. (A) RT-PCR analysis of the expression of Cox-1, Cox-2, and HPRT in IRF-1-deficient macrophages (IRF-1 $^{-/-}$ ) compared with background-matched controls (IRF-1 $^{+/+}$ ). Cells were treated with IL-1 $\alpha$  (lanes 3 and 4; 10 ng/ml), LPS (lanes 5 and 6; 1 ng/ml), and IFN- $\gamma$  (lanes 7 and 10; 10 and 100 U/ml, respectively) alone for 2 h or after pretreatment with IFN- $\gamma$  for 6 h (lanes 7–12). (B) Western blot analysis of Cox-1 and Cox-2 protein expression in IRF-1-deficient macrophages after stimulation with IFN- $\gamma$  (triangle; 10 and 100 U/ml), LPS (0.1 and 0.5 ng/ml), and IL-1 $\alpha$  (1 and 10 ng/ml) alone or after priming for 12 h with 10 U/ml of IFN- $\gamma$  (1). Results presented are representative of two independent experiments. (C) and (D) Similar to A and B, but using IRF-2-deficient macrophages (IRF-2 $^{-/-}$ ) or their background-matched controls (IRF-2 $^{+/+}$ ). Results shown are representative of four independent experiments.

In the case of IRF-2<sup>-/-</sup> macrophages (Fig. 3 C), higher levels of expression of steady-state Cox-2 mRNA were observed in the absence of inducers compared with control macrophages (compare lanes 1 and 2 in IRF-2<sup>-/-</sup> versus IRF-2<sup>+/+</sup>), as well as in the IL-1 $\alpha$ -dependent activation (lanes 3 and 4). This increase in the basal level was also evident at the level of Cox-2 protein expression (Fig. 3 D), in which IRF-2-deficient macrophages are more responsive to these inducers (compare IRF-2<sup>-/-</sup> versus IRF-2<sup>+/+</sup> for all treatments).

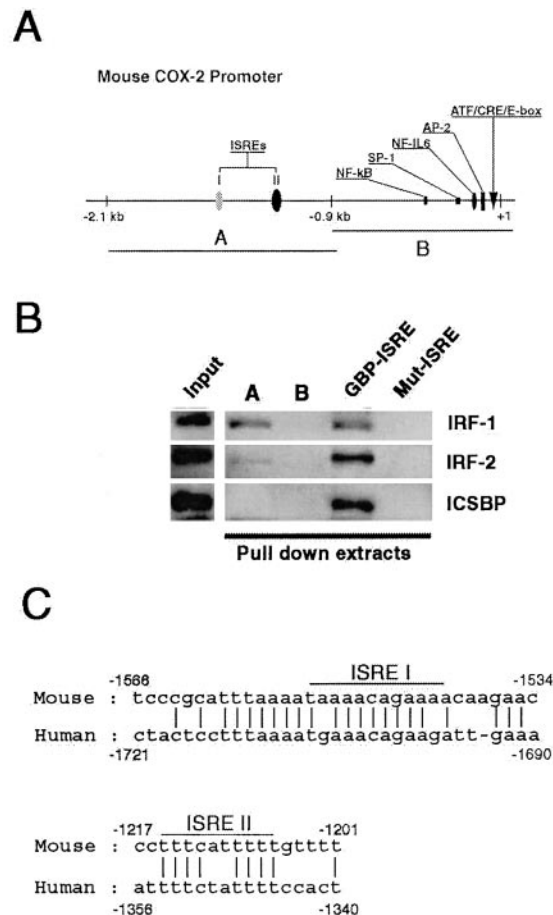
To analyze how the absence of IRF-1 or IRF-2 affects final production of PGs, we measured PGE<sub>2</sub>, one of several possible prostanoid products that is dependent on Cox-2 activity, in supernatants of IRF-1<sup>-/-</sup>, IRF-2<sup>-/-</sup>, and C57BL/6<sup>+/+</sup> macrophages after stimulation with IFN- $\gamma$  (Table I). No significant difference in PGE<sub>2</sub> production by these macrophages was observed, either basally or in the first 12 h after treatment with IFN- $\gamma$  (data not shown). However, after 24 h of IFN- $\gamma$  treatment, IRF-2<sup>-/-</sup> macrophages showed the highest release of PGE<sub>2</sub>, followed by C57BL/6<sup>+/+</sup>, whereas IRF-1<sup>-/-</sup> macrophages showed no PGE<sub>2</sub> production, as expected. In fact, IRF-1<sup>-/-</sup> macrophages failed to exhibit increased PGE<sub>2</sub> release in response to IFN- $\gamma$  together with LPS (data not shown). Taken together, these data indicate that IRF-1 and IRF-2 are directly involved in the regulation of Cox-2 gene expression and, ultimately, PGE<sub>2</sub> production. Thus, IRF-1 acts as an inducible transcriptional activator, as IRF-1<sup>-/-</sup> macrophages are deficient in (a) IFN- $\gamma$ -dependent activation of the Cox-2 gene, (b) IFN- $\gamma$ -dependent coactivation of IL-1 $\alpha$ - or LPS-dependent Cox-2 transcription, and (c) Cox-2 protein expression and release of PGE<sub>2</sub>. In contrast, IRF-2 is involved in the repression of Cox-2 gene transcription. This statement is supported by (a) the observed increase in the basal and inducible transcription of Cox-2 gene, (b) the increased levels of Cox-2 protein expression, and (c) the elevated levels of PGE<sub>2</sub> after 24 h of activation of IRF-2<sup>-/-</sup> macrophages by IFN- $\gamma$ .

**Table I.** PGE<sub>2</sub> Production in C57BL/6<sup>+/+</sup>, IRF-1<sup>-/-</sup>, and IRF-2<sup>-/-</sup> Macrophages

Macrophage type	PGE <sub>2</sub> released	
	Medium	IFN- $\gamma$
		(ng/ml)
C57BL/6 <sup>+/+</sup>	5.6 $\pm$ 0.5	7.4 $\pm$ 0.5
IRF-1 <sup>-/-</sup>	4.5 $\pm$ 0.6	5.6 $\pm$ 0.8
IRF-2 <sup>-/-</sup>	5.0 $\pm$ 1.1	9.5 $\pm$ 1.0

Macrophages were cultured for 24 h at a density of 5  $\times$  10<sup>5</sup> cells in 24-well plates in a final volume of 0.5 ml with medium only or medium containing 100 U/ml of IFN- $\gamma$ . Results represent the mean  $\pm$  SD of duplicate wells and were derived from a single experiment representative of three independent experiments.

**IRF-1 and IRF-2 Bind to the Cox-2 Promoter.** Two overlapping fragments (labeled "A" and "B" on Fig. 4 A), similar in size and spanning  $\sim$ 2.1 kb of the mouse Cox-2 promoter (sequence data available from EMBL/GenBank/DBJ under accession no. S82456; reference 20), were synthesized and independently conjugated to magnetic beads. The beads were then incubated with nuclear extracts from the RAW 264.7 macrophage cell line pretreated with IFN- $\gamma$  for 3 h. The proteins that bound to the immobilized DNA fragments were analyzed by Western blot analysis using antibodies against IRF-1, IRF-2, or ICSBP (Fig. 4 B), three members of the IRF family of transcription factors that are expressed at high levels in these cells after IFN- $\gamma$

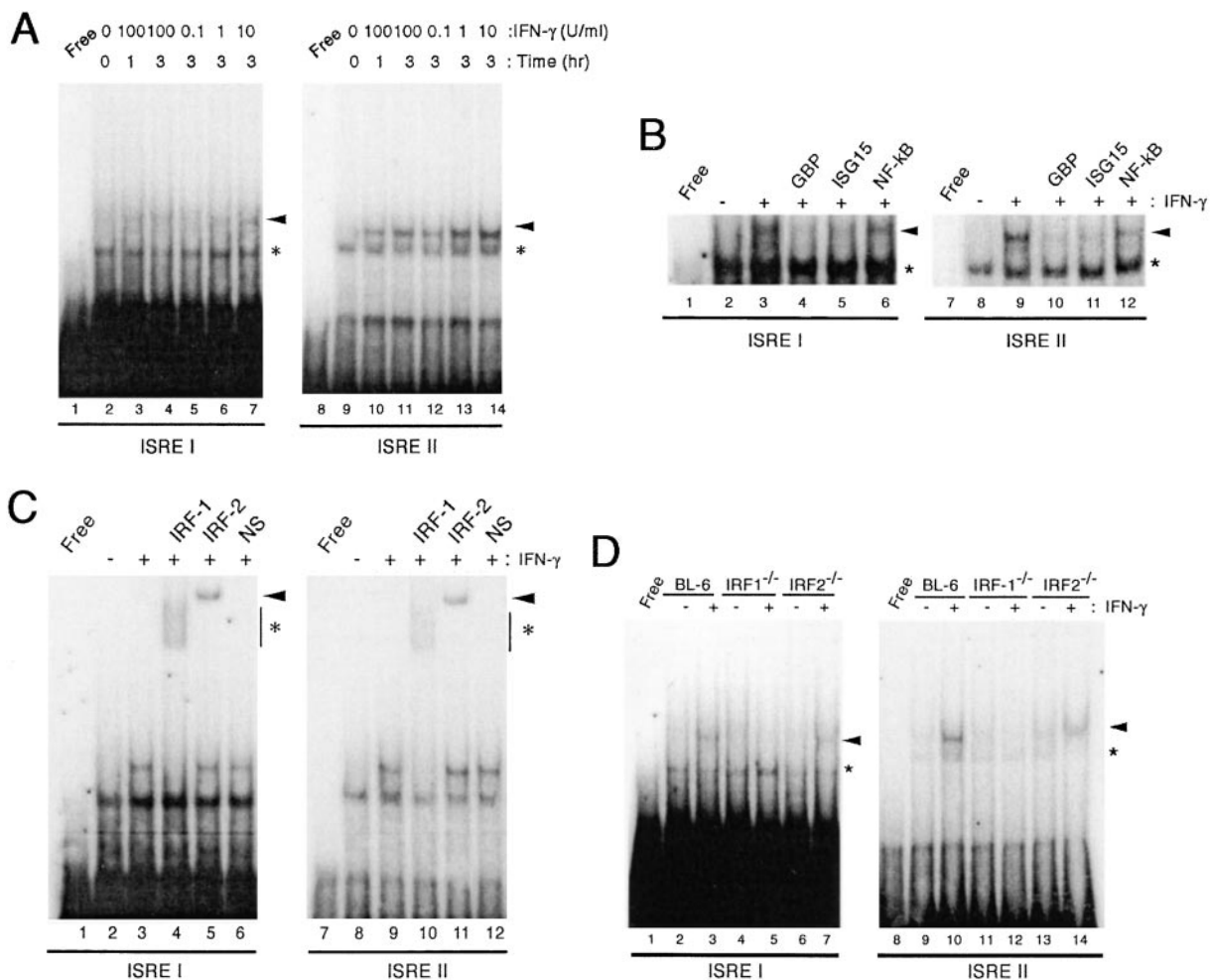


**Figure 4.** Two ISREs are located in the promoter region of the Cox-2 gene. (A) Schematic representation of the  $\sim$ 2.1-kb promoter region of the murine Cox-2 gene with the location of characterized binding sites for several transcription factors, as well as two ISREs described in this paper. A and B represent two overlapping DNA fragments used to locate potential ISREs in the promoter. (B) Magnetic beads containing fragment A or B, as well as beads containing the GBP-ISRE sequence (positive control) or a mutant ISRE (Mut-ISRE; negative control), were incubated with nuclear extracts of IFN- $\gamma$ -treated RAW 264.7 cells. Eluates were analyzed by Western blot for the presence of IRF-1, IRF-2, or ICSBP. The input lane represents 20% of the nuclear extract used for each lane (50  $\mu$ g). (C) Depiction of the sequence for the two ISREs found in the mouse Cox-2 promoter (top) and their conservation with those found in the human counterpart (bottom). In each case, numbers represent their nucleotide position from the +1 transcription start site.

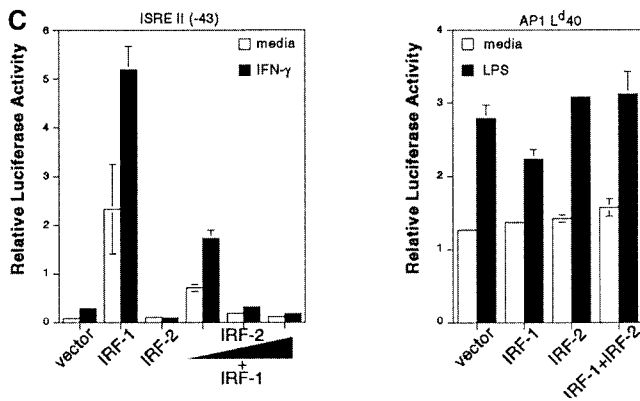
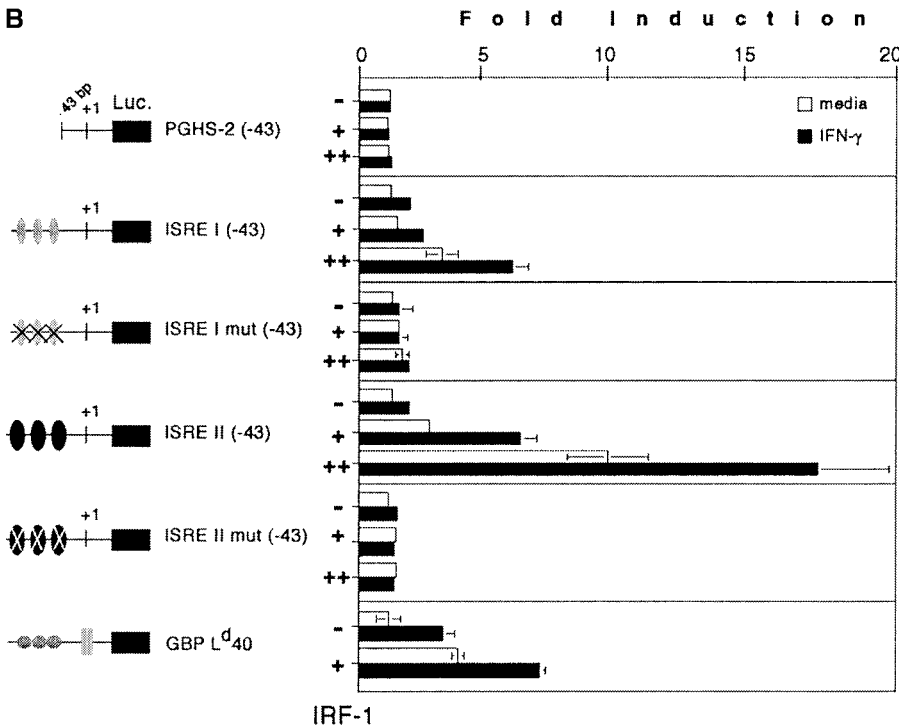
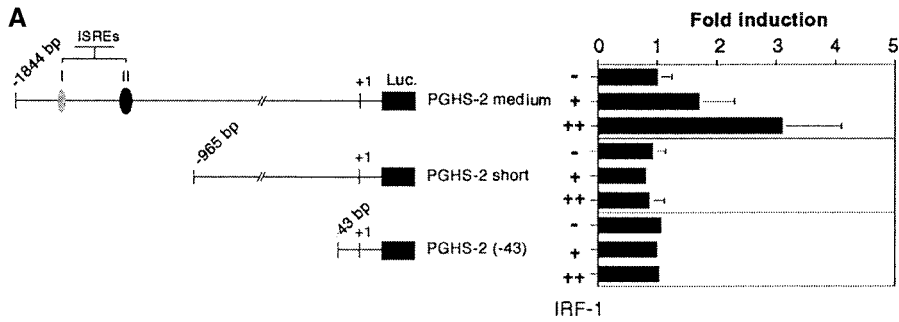
treatment (input lane represents 20% of total nuclear protein used for each assay). To control for the specificity of this assay, beads containing DNA with three copies of the ISRE found in the GBP promoter were used as a positive control, whereas a mutated version of this ISRE was used for controlling nonspecific binding (GBP-ISRE and Mut-ISRE, respectively). Materials precipitated with beads containing fragment A, which spans the region between  $\sim -2.1$  and  $\sim -0.9$  kb of the promoter, showed detectable levels of IRF-1 and IRF-2, but not ICSBP. The amount of IRF-1 precipitated by this fragment was similar to the amount precipitated by three in-tandem copies of the GBP-ISRE beads, indicating similar binding capacities for IRF-1. However, at least 10-fold less IRF-2 was precipitated by fragment A, indicating a lesser affinity of this site for IRF-2. No binding of these proteins was detected in beads containing fragment B, which encompasses the TATA box, initia-

tor sequence, and almost all sites involved in the activation of the Cox-2 gene described to date (diagram in Fig. 4 A). These data clearly demonstrate that the upstream portion ( $-2.1$  to  $-0.9$  kb) of the Cox-2 promoter is responsible for the recruitment of IRF-1 and IRF-2.

**Two ISRE Sites Found in Cox-2 Promoter.** A computer search for potential ISREs in the Cox-2 promoter region using MatInspector 2.2 (41) resulted in two sequences that partially match canonical ISREs (referred to as ISRE I for the more upstream site and ISRE II for the downstream site; Fig. 4 C). They are both located in the region of the promoter that we previously defined as fragment A in our bead assay (Fig. 4 A), indicating their potential role in the binding of IRF family members to this fragment. Furthermore, these two sequences were highly conserved in the human Cox-2 promoter (Fig. 4 C), especially in those nucleotides that define the potential ISREs.



**Figure 5.** IRF-1 and IRF-2 bind to both Cox-2 ISREs. (A) EMSAs performed using probes containing the ISRE I and ISRE II from the murine Cox-2 promoter and nuclear extracts from C57BL/6 macrophages incubated with IFN- $\gamma$  for different periods of time or doses, as indicated. "Free" lanes represent the mobility of the probe without any extract added. Black arrowheads point to the IFN- $\gamma$ -inducible complex. Asterisks indicate the constitutive complex. (B) DNA binding specificity experiments on ISRE I and ISRE II using for competition 50-fold excess of cold ISREs (GBP-ISRE; lanes 4 and 10, and ISG15-ISRE; lanes 5 and 11) or a nonspecific competitor (NF- $\kappa$ B binding site; lanes 8 and 12). (C) Supershift experiments on ISRE I and II using IRF-1- or IRF-2-specific antibodies as indicated. NS, nonspecific antiserum. (D) EMSA using control C57BL/6<sup>+/+</sup> or IRF-1<sup>-/-</sup> and IRF-2<sup>-/-</sup> macrophages.



**Figure 6.** Transcriptional regulation of the Cox-2 promoter. (A) RAW 267.4 cells were transiently transfected with the indicated PGHS-2 luciferase reporters (left, 200 ng/each) along with control empty vector to complete 200 ng (-) or increasing amounts of IRF-1 expression plasmid (+ = 100 ng and ++ = 200 ng). Results are expressed as fold induction relative to the activity of each reporter in the absence of IRF-1 plasmid, and represent the means from three independent experiments  $\pm$  SD. Luciferase (Luc.) activity measured for PGHS-2 medium in the absence of the IRF-1 construct and after normalization by  $\beta$ -galactosidase activity was twice that obtained for PGHS-2 short, whereas PGHS-2 (-43) activity was <1% of PGHS-2 medium. (B) RAW 267.4 cells were transfected with the indicated reporter plasmid (left) in the absence (-, control vector) or the presence of increasing amounts of IRF-1 (+ = 100 ng and ++ = 200 ng), and were completed to 200 ng with control vector. After transfection, cells were treated with media or IFN- $\gamma$  (10 U/ml) as indicated. Results are expressed as in A. Luc., luciferase; ISRE II Mut, mutant ISRE II. (C) Cells were cotransfected with the ISRE II (-43) reporter along with either control vectors (500 ng), IRF-1 (125 ng), IRF-2 (375 ng), or IRF-1 expression plasmids in the presence of increasing amounts of IRF-2 (125, 250, and 375 ng). Results represent the means from three independent experiments  $\pm$  SD.



**Both ISREs Found in the Cox-2 Promoter Form IFN- $\gamma$ -inducible Complexes.** Oligonucleotides containing both ISRE sequences found in the Cox-2 promoter were end labeled and used as probes for electrophoretic mobility shift assay (EMSA). Incubation of these probes with nuclear extracts from untreated peritoneal macrophages resulted in a distinct band (asterisk in Fig. 5 A; lanes 2 and 9) not present in lanes containing free probes (lanes 1 and 8). Incubation of cells with IFN- $\gamma$  for 1 or 3 h produced in both cases the induction of a second band with slower mobility (black arrow in Fig. 5 A; lanes 3 and 4 for ISRE I, and lanes 10 and 11 for ISRE II). This band was more intense in ISRE II than ISRE I, indicating a stronger binding activity of ISRE II. Different doses of IFN- $\gamma$  were tested in the formation of the inducible upper band (lanes 5–7 and 12–14). We determined that as little as 1 U/ml IFN- $\gamma$  for ISRE I or 0.1 U/ml for ISRE II was sufficient to stimulate the formation of the upper complex. These data clearly indicate that both ISREs found in the Cox-2 promoter are able to form constitutive and IFN- $\gamma$ -inducible complexes with nuclear proteins from murine macrophages. In both ISREs, the IFN- $\gamma$ -inducible band was clearly competed with both GBP and ISG15 cold ISRE probes, but not by a probe containing NF- $\kappa$ B binding site (Fig. 5 B).

**IRF-1 and IRF-2 Form Complexes with Both Cox-2 ISREs.** Our previous data with IRF-1<sup>-/-</sup> and IRF-2<sup>-/-</sup> macrophages (Fig. 3) indicated that IRF-1 and IRF-2 were involved in the regulation of the expression of Cox-2 by binding to its promoter (Fig. 4). Preincubation of EMSA reactions with anti-IRF-1 antibody produced a clear supershift of both ISRE I- and ISRE II-inducible bands (Fig. 5 C; lanes 4 and 10, asterisks), indicating the presence of IRF-1 in these complexes. Antibodies against IRF-2 also produced a supershift (lanes 5 and 11, arrows), although displacement of the inducible band was less complete than observed for anti-IRF-1 antibody.

To dissect further the composition of each complex, nuclear extracts from C57BL/6<sup>+/+</sup>, IRF-1<sup>-/-</sup>, and IRF-2<sup>-/-</sup> macrophages, untreated or treated with IFN- $\gamma$ , were used for EMSA (Fig. 5 D). IRF-1<sup>-/-</sup> extracts were unable to form the inducible upper complex seen in C57BL/6<sup>+/+</sup> extracts in the presence of IFN- $\gamma$  (compare lanes 3 and 5 for ISRE I and lanes 10 and 12 for ISRE II, arrows), demonstrating that IRF-1 is part of this complex and is absolutely necessary for its formation. IRF-2, on the other hand, is not required for the formation of the inducible bands, as these bands are present with IRF-2<sup>-/-</sup> extracts and are enhanced in the presence of IFN- $\gamma$  (lanes 7 and 14, arrows). However, a clear diminution of the constitutive band formed by IRF-2<sup>-/-</sup> extracts and ISRE II in the presence of IFN- $\gamma$  indicates that IRF-2 is part of this faster-migrating complex (lane 14, asterisk). Taken together, these data indicate that IRF-1 and IRF-2 bind through two different ISREs located in the promoter of the Cox-2 gene. IRF-2 is part of the constitutive complex, whereas an IRF-1-associated complex is induced by IFN- $\gamma$ .

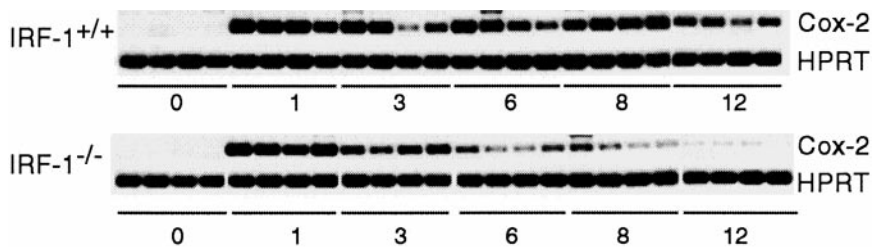
**Transcriptional Regulation of Cox-2 through ISREs.** In the transfection experiment shown in Fig. 2 C, we found

that IFN- $\gamma$  induced and coactivated LPS-dependent gene transcription from a reporter driven by 2.1 kb of the Cox-2 promoter (PGHS-2 medium). To analyze the potential transactivation characteristics of the ISREs located within the Cox-2 promoter, RAW 264.7 cells were cotransfected with a series of luciferase reporters (shown in Fig. 6 A, left), along with increasing amounts of either an IRF-1 expression vector or a control plasmid. IRF-1 activated the PGHS-2 medium reporter construct (Fig. 6 A, top), which contains both ISRE I and ISRE II, in a dose-dependent manner, and reached a greater than threefold transactivation with the highest concentration of IRF-1 used (++; Fig. 6 A, right). Removal of both ISREs, as is the case for the PGHS-2 short construct or the Cox-2 basic promoter (PGHS-2 [-43]), completely abrogated IRF-1-dependent transactivation, indicating the importance of these ISREs in the whole promoter context. Although the PGHS-2 short construct showed similar levels of activation compared with PGHS-2 medium when cells were treated with IFN- $\gamma$  alone, no coactivation with LPS and IFN- $\gamma$  was seen in PGHS-2 short (data not shown), again indicating the importance of the ISRE sequences.

To analyze further the transcriptional potential and specificity of each Cox-2 ISRE, reporter plasmids containing three copies of each of these sites or mutated versions of the same sequences were cloned in front of the basic Cox-2 promoter and tested (Fig. 6 B, left). Transcription could be activated through both ISREs by IFN- $\gamma$  or by increasing amounts of IRF-1, with levels comparable to those seen for GBP-ISRE (Fig. 6 B, bottom), whereas the basic promoter activity was not affected (Fig. 6 B, top). ISRE II showed greater IFN- $\gamma$ - or IRF-1-dependent activation than ISRE I, in agreement with the binding activity observed for these ISREs in EMSA (compare ISRE I [-43] versus ISRE II [-43] in Fig. 6 B). Mutation of two nucleotides located in the core TTTC of both ISRE sequences, which destroyed the ISRE motif, completely abrogated IFN- $\gamma$ - or IRF-1-dependent activation.

We showed previously that IRF-2<sup>-/-</sup> macrophages possess elevated Cox-2 mRNA and protein levels (Fig. 3, C and D), suggesting a suppressive role of IRF-2 in this promoter. Cotransfection of IRF-2 along with ISRE II (-43) reporter abrogated IFN- $\gamma$ -dependent activation of the luciferase activity (Fig. 6 C, right; IRF-2). Moreover, it completely inhibited IRF-1-dependent activation when the highest dose of IRF-2 was used, without affecting the LPS-dependent activation of an unrelated AP1 reporter (Fig. 6 C, left). Taken together, these data demonstrate that both ISREs located upstream in the Cox-2 promoter are essential for IRF-1-dependent activation. ISRE I and II are individually able to activate transcription from a basic Cox-2 promoter in the presence of IRF-1, and mutations in these ISREs designed to prevent IRF-1 binding were effective at abrogating transactivation. Finally, we showed that IRF-2 works as a negative regulator of Cox-2 promoter by inhibiting IFN- $\gamma$ - and IRF-1-dependent activation by binding these ISREs.

**IRF-1<sup>-/-</sup> Mice Are Unable to Sustain the LPS-induced Expression of the Cox-2 Gene in the Liver.** We showed in



**Figure 7.** IRF-1<sup>-/-</sup> mice exhibit diminished hepatic Cox-2 mRNA expression in response to LPS in vivo. Control (IRF-1<sup>+/+</sup>) and IRF-1<sup>-/-</sup> mice were injected with 25  $\mu$ g LPS intraperitoneally, and livers were collected at the indicated times after injection. Total RNA was prepared from individual mice (four per time point) and was analyzed for Cox-2- and HPRT-specific transcripts by RT-PCR.

vitro that macrophages respond to LPS and IFN- $\gamma$  synergistically to activate the expression of Cox-2. In vivo, intraperitoneal injection of mice with a sublethal dose of LPS (25  $\mu$ g/mouse) sharply increased expression of Cox-2 mRNA in liver within 1 h, with no detectable difference between IRF-1<sup>-/-</sup> and IRF-1<sup>+/+</sup> responses (Fig. 7). Strikingly, by 3 h after injection, IRF-1<sup>-/-</sup> mice exhibited a progressive decline in the steady-state levels of Cox-2 mRNA, approaching almost undetectable levels at 12 h after injection, whereas levels of Cox-2 mRNA remained fairly constant in the IRF-1<sup>+/+</sup> mice. This data clearly indicates that the transcription factor IRF-1 plays a critical role in vivo in the maintenance of Cox-2 expression over time.

## Discussion

IFN- $\gamma$  is a well-characterized macrophage differentiation signal. Macrophages pretreated with IFN- $\gamma$  achieve a higher level of activation, e.g., IFN- $\gamma$ -primed macrophages become more sensitive to other stimuli, such as bacterial products and cytokines, as evidenced by the synergistic induction of many inflammatory gene products, e.g., iNOS (12, 35, 36) and IL-12 p40 (13, 37, 42, 43). In this paper, we show that activation with IFN- $\gamma$ , as well as its priming effect, can be extended to Cox-2, another gene with a central role in inflammatory responses. Although IFN- $\gamma$  regulation of Cox-2 has been described previously (26, 44, 45), little is known of the molecular mechanisms that underlie activation of this gene by IFN- $\gamma$ . For example, in bronchial epithelial cells (44) and in normal human epidermal keratinocytes (26), Cox-2 induction is controlled through autocrine loops via the epidermal growth factor receptor and its ligands, such as TGF- $\alpha$ , resulting in a delayed activation. However, in macrophages, Cox-2 mRNA induction was detected as early as 1 h after IFN- $\gamma$  treatment, and the protein expression was detected after 6 h (Fig. 1), giving us the first indication of a distinct inductive mechanism.

Strikingly, IRF-1<sup>-/-</sup> macrophages were unable to activate IFN- $\gamma$ -dependent transcription or synergize with LPS or IL-1 to elicit Cox-2 (Fig. 3). The lack of Cox-2 expression was also reflected in the low levels of PGE<sub>2</sub> produced by these cells 24 h after treatment. Conversely, in the case of IRF-2<sup>-/-</sup> macrophages, we showed an increase in the basal level of Cox-2 transcription and Cox-2 protein, as well as in the release of IFN- $\gamma$ -stimulated PGE<sub>2</sub>. We presented data that showed dysregulated PGE<sub>2</sub> release in IRF-1<sup>-/-</sup> and IRF-2<sup>-/-</sup> macrophages as one of the possible outcomes of Cox-2 dysregulation. However, Cox-2 ex-

pression and PGE<sub>2</sub> release are not always coordinated, and other PGs or thromboxanes may be dysregulated as well. Transfection studies have shown that IRF-1 can function as an activator, inducing Cox-2 promoter constructs that contain ISREs (6), whereas IRF-2 antagonizes the function of IRF-1 by working as a repressor (7, 8). Both IRF-1 and IRF-2 are normally expressed at very low levels in the cell (7, 46). An increase in the intracellular concentration of IRF-1 significantly alters the IRF-1/IRF-2 ratio because of the fact that IRF-2 levels remain fairly stable throughout the cell cycle. It has been shown that the modification of this ratio can produce a dramatic change in the transcription pattern of cells (46). Interestingly, increased levels of Cox-2 expression have been associated with oncogenic and inflammatory processes. For example, Cox-2 protein is undetectable in normal intestine (47), but its levels are elevated in up to 85% of colorectal adenocarcinomas (48–50), coincident with an increase in metastatic potential (51). It is tempting to speculate that an altered IRF-1/IRF-2 ratio may exist in those cells, which could anomalously activate gene expression.

We found that Cox-2 gene regulation by IRF-1 and IRF-2 is achieved through two unique ISREs located upstream of the Cox-2 promoter. Although the consensus sequence for IRF-1/IRF-2 binding motif, (G/C)(A)AAA(N)<sub>2-3</sub>AAA(G/C)(T/C) (6, 9), did not precisely match the sequences of the ISREs described here, ISRE II partially matched sequences previously cloned for their affinity for IRF-1 and IRF-2 binding (52), but with a single base change in the core sequence (GTA instead of GTG). ISRE I and ISRE II were able to bind IRF-1 and IRF-2 in the context of the whole promoter (Fig. 4), recruiting similar amounts of IRF-1 compared with three copies of the GBP-ISRE (53). Moreover, removal of these ISRE sequences from the promoter context completely abolished IRF-1-dependent Cox-2 gene transcription (Fig. 6 A). We found that PGHS-2 short, which does not contain the novel ISREs described, is nonetheless able to direct transcription induced by IFN- $\gamma$  alone, but unable to coactivate transcription by IFN- $\gamma$  and LPS (data not shown). This finding supports previous studies showing that IFN- $\gamma$  also triggers secondary signals that enhance transcription through downstream binding sites (non-ISRE; references 26, 36, 44, 54, 55). When analyzed separately, ISRE II was more efficient at binding IRF-1 and IRF-2 complexes than ISRE I, and although both ISREs were able to enhance transcription of the basic Cox-2 promoter in an IFN- $\gamma$  and IRF-1 dose-dependent manner, their different binding ac-

tivities clearly correlated with distinct transactivation potentials. Interestingly, homologous sites were also found in the human *Cox-2* promoter; they are located at a similar distance from the transcription start site and follow a similar orientation with respect to the entire promoter (Fig. 4 C).

IFN- $\gamma$  was also found to be a powerful coactivator of IL-1 $\alpha$ - and LPS-mediated induction of *Cox-2* in mouse peritoneal macrophages (Fig. 2). Pretreatment of macrophages with IFN- $\gamma$  clearly enhanced the activation induced by IL-1 $\alpha$  and LPS by >10-fold over that seen with either of these inducers alone. Whereas LPS induction of *Cox-2* is controlled in large measure at the level of transcription (28, 32, 56), IL-1 $\alpha$  regulates *Cox-2* expression largely through stabilization of *Cox-2* mRNA (34), consistent with our inability to detect IL-1 $\alpha$ -mediated response in transient transfection assays using the *Cox-2* promoter. The activation of *Cox-2* by a combination of IFN- $\gamma$  and LPS was described previously using the macrophage cell line RAW 264.7 (45), although this effect was not seen in human peripheral blood monocytes (31). The synergy of IFN- $\gamma$  with IL-1 $\alpha$  for activation of *Cox-2* protein expression and PGE<sub>2</sub> release has also been controversial (57, 58). Recently, Barrios-Rodiles and Chadee (58) described an inhibitory effect of IFN- $\gamma$  priming on IL-1 $\beta$ -dependent activation of *Cox-2*. These differences may possibly be attributable to the different states of differentiation/activation of the cells used in the different studies (monocytic or promyelocytic cells treated with PMA compared with thioglycollate-elicited macrophages or the macrophage cell line RAW 264.7). In addition to the two novel ISRE sites described herein, other sites have been identified in the promoter region of the *Cox-2* gene, e.g., stimuli that activate G proteins and protein kinases A and C can stimulate the synthesis of *Cox-2* by a process involving the cyclic AMP response element (CRE) and E-box (Fig. 4 A; references 55, 59–61). Other inducers of *Cox-2*, like IL-1, TNF, GM-CSF, and LPS, elicit their activity through the usage, to varying extents, of NF- $\kappa$ B, NF-IL-6, and the CRE binding sites also located within the *Cox-2* promoter, and are conserved among different species (32, 62–64). One possible mechanism of IFN- $\gamma$  coactivation is the cooperation between IRF-1, bound to both ISREs located upstream in the promoter, and the transcription factors that bind to these downstream sites. Because IRF members have been involved in the recruitment of histone acetylases to the promoter environment (23), one additional but not exclusive possibility is that IRF-1, after binding to the ISREs, increases the concentration of these chromatin-modifying activities in the promoter, making it more accessible for the binding of other factors (65).

Moreover, IRF-1 is inducible by several cytokines (e.g., IL-1, TNF, IL-6 [52, 66, 67], IL-12 [68, 69]), and by LPS (70), which have also been demonstrated to increase *Cox-2* expression. This suggested the possibility that IRF-1 also contributes to the regulation of *Cox-2* gene expression by these activators. Our findings using IRF-1- and IRF-2-deficient macrophages confirm this relationship, as IL-1- and LPS-dependent transcription and protein synthesis of

*Cox-2* are clearly modified with respect to the response of macrophages from normal control mice (Fig. 3; compare stimulation with IL-1 $\alpha$  and LPS individually).

With the data presented here, we have identified clear parallels in the expression of iNOS and *Cox-2* genes. Both of these genes play essential roles in innate immune responses and inflammatory processes, being regulated at transcriptional (36, 54, 71), posttranscriptional, and posttranslational levels (72, 73). iNOS gene expression is transcriptionally induced by IFN- $\gamma$  and LPS in peritoneal macrophages (74). Two ISREs in the promoter region of the iNOS gene are involved in its IFN- $\gamma$ -dependent transcriptional activation (12, 36). They are arranged in opposite orientations, binding IRF-1 and activating transcription in combination with p50/cRel and NF- $\kappa$ B sites in the promoter (36, 54). IRF-1<sup>-/-</sup> macrophages are deficient in the expression of iNOS and the production of NO after IFN- $\gamma$  treatment (12, 13). Although iNOS regulation also shows a dependency on ICSBP for its induction (75), we did not detect binding of endogenous ICSBP to the *Cox-2* promoter under conditions employed. However, we cannot rule out the possibility of a weak binding and a functional role for this factor in the regulation of *Cox-2* gene expression. Nevertheless, we found that IRF-2 is part of the constitutive complex that binds to the *Cox-2* ISREs. In the absence of IRF-2, increases in basal and stimulated *Cox-2* gene and protein expression were observed, with an associated increase in the PGE<sub>2</sub> release upon IFN- $\gamma$  stimulation. Overexpression of IRF-2 completely abolished IRF-1- as well as IFN- $\gamma$ -dependent activation of the *Cox-2* promoter, indicating a silencing role for IRF-2. To date, the negative regulation of *Cox-2* gene expression by IRF-2, and its potential for attenuating inflammatory responses, has not been explored.

Focal cerebral ischemia has been associated with the upregulation of both iNOS and *Cox-2* genes, which have been implicated in the brain injury associated with stroke (18, 76–78). In a mouse model of cerebral ischemia, it has been shown that during the ischemic process, IRF-1 mRNA was upregulated in normal mice, whereas in IRF-1<sup>-/-</sup> mice, there was attenuation of brain injury after ischemia (79). Although the authors point out that a decrease in NO production may be one cause of this attenuation, the data presented herein suggest that a decrease in PG release secondary to attenuated *Cox-2* production may contribute to the decrease in the ischemic injury in IRF-1<sup>-/-</sup> mice. Moreover, we found that IRF-1<sup>-/-</sup> mice are unable to sustain steady-state mRNA production of *Cox-2* in the liver after intraperitoneal injection of LPS (Fig. 7). We have confirmed that these mice are severely deficient in the production of serum IFN- $\gamma$  after LPS treatment when compared with IRF-1<sup>+/+</sup> controls (80; data not shown). The peak of IFN- $\gamma$  production is at 8–12 h after LPS injection (data not shown), which correlates strongly with the sustained gene activation in IRF-1<sup>+/+</sup> mice. Along the same lines, it has been reported that IRF-1<sup>-/-</sup> mice are less susceptible to LPS-induced mortality (80; data not shown), which again suggests a role for *Cox-2*

and PG in the inflammatory response associated with septic shock.

We have presented herein a detailed biological and mechanistic analysis of the direct activation of Cox-2 gene by IFN- $\gamma$  in freshly isolated macrophages, a major cellular target for IFN- $\gamma$  and an important source of inflammatory PGs. We have demonstrated for the first time that two members of the IRF family of transcription factors, IRF-1 and IRF-2, regulate Cox-2 gene expression by their opposite actions through two ISRE sites found in the promoter. Moreover, we found that IRF-1 is necessary for the maintenance of hepatic Cox-2 gene expression by LPS in vivo. Given the recent increase in interest in Cox-2 regulation in the search for new antiinflammatory drugs, our data provide critical new information that may result in novel therapeutic intervention strategies.

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