The Up-regulation of Human Caspase-8 by Interferon-γ in Breast Tumor Cells Requires the Induction and Action of the Transcription Factor Interferon Regulatory Factor-1*

Carmen Ruiz-Ruiz‡§, Carmen Ruiz de Almodóvar‡¶, Antonio Rodriguez**‡‡, Gustavo Ortiz-Ferrón‡§§, Juan Miguel Redondo***¶¶, and Abelardo López-Rivas††††

From the Instituto de Parasitología y Biomedicina, CSIC, Calle Ventanilla 11, 18001 Granada, (Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Madrid E-28049, and **Centro Nacional de Investigaciones Cardiovasculares, Ronda de Poniente 5, Tres Cantos, Madrid 28760, Spain

Received for publication, December 1, 2003, and in revised form, February 13, 2004
Published, JBC Papers in Press, March 1, 2004, DOI 10.1074/jbc.M313023200

The Journal of Biological Chemistry
Vol. 279, No. 19, Issue of May 7, pp. 19712–19720, 2004
Printed in U.S.A.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

1 The abbreviations used are: TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; EMSA, electrophoretic mobility shift assay; GAS, γ-interferon activation sequence; ISRE, interferon stimulatory response element; IRF-1, interferon regulatory factor-1; STAT, signal transducers and activators of transcription; DISC, death-inducing signaling complex; IFN, interferon; ISGF, interferon-stimulated gene factor.

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L), a member of the tumor necrosis factor superfamily, is a potent inducer of apoptosis upon binding to its death receptors TRAIL-R1 and TRAIL-R2 (also known as DR4 and DR5, respectively) (1). Cytoplasmic death domains in both receptors serve to recruit intracellular adapter molecules that in turn engage procaspase-8, thereby forming the death-inducing signaling complex (DISC). Caspase-8 is activated in the DISC, allowing the initiation of a cascade that leads to apoptotic cell death (2). Death receptor-mediated apoptosis can occur by at least two different pathways (3). In one, caspase-8-mediated cleavage of Bid generates a truncated form that translocates to mitochondria and promotes the release of cytochrome c and Smac/DIABLO via a Bax/Bak-dependent mechanism, thus allowing the activation of caspase-9 and effector caspases (4–6). In contrast, in the mitochondria-independent pathway, activated caspase-8 directly processes and activates the effector caspase-3 (3, 6). In both pathways, caspase-8 is an indispensable mediator for death receptor-induced apoptosis.

Unlike TNF-α or CD95L, TRAIL selectively induces apoptosis in tumor cells without being toxic to most normal cells in culture (1, 7). Moreover, preclinical studies in mice and nonhuman primates have shown no systemic toxicity upon injection of recombinant versions of TRAIL at doses that effectively suppress solid tumors such as colon and mammary carcinomas (7). These data suggest that TRAIL could be a suitable target in cancer therapy.

However, not all types of tumor cell are sensitive to TRAIL-induced apoptosis. Different mechanisms of resistance exist because apoptotic control can be exerted at many stages, such as the levels of death receptors at the cell surface or the expression of intracellular pro- and antiapoptotic proteins. Recently, several reports have shown that caspase-8 expression and activation may play a critical role in the sensitivity of tumor cells to TRAIL-mediated apoptosis. The expression of the cellular Flice-like inhibitory protein, which blocks the activation of caspase-8 at the DISC, has been suggested to be a major cause of TRAIL resistance (8, 9). Furthermore, loss of caspase-8 expression has been described in a range of different tumor cells (9–16). In most of these tumors, treatments that up-regulate the levels of caspase-8 expression induce sensitization to TRAIL-mediated apoptosis (9–11, 13, 16–18).
Interferons (IFNs) are a family of natural glycoproteins that play a key role in antiviral, antiproliferative, and immunomodulatory responses. IFN-γ, or type II IFN, is produced mainly by cells of the immune system in response to activation (19) and has an important antitumoral role. The antitumoral response induced by IFN-γ is the result of different coordinated events that involve both immunologic and nonimmunologic processes. IFN-γ activates cells of the innate immune system such as NK cells, NKT cells, and macrophages to proliferate, produce cytokines, and acquire lytic activity against tumor cells (20–22). In addition, IFN-γ promotes the development of a Th1 antitumor response that involves cytotoxic CD8+ T cells (23). Furthermore, IFN-γ can exert direct antiproliferative and proapoptotic effects on a wide variety of tumor cells by the induction of cell cycle inhibitors and different apoptosis-related proteins such as death receptors and their ligands, caspases, and several members of the Bel-2 family (24–26). The double-stranded RNA-activated protein kinase, PKR, is an IFN-γ-inducible protein that has also been shown to function as an inducer of apoptosis and a tumor suppressor protein (27).

Several reports have shown that IFN-γ can induce caspase-8 up-regulation in a variety of human tumor cell lines derived from breast cancer, colon carcinoma, myeloid leukemia, neuroblastoma, medulloblastoma, or Ewing sarcoma (17, 28–30). Moreover, we have recently described that modulation of caspase-8 expression might be the main mechanism for IFN-γ-induced sensitization of breast tumor cells to apoptosis mediated by CD95 and TRAIL receptors (6, 28). The transcription factors STAT-1 and IRF-1 have been suggested to be involved in the induction of caspase-8 by IFN-γ, but the molecular mechanism underlying this regulation has not been elucidated (17, 18). We have now studied the regulation of human caspase-8 gene expression in IFN-γ-treated MCF-7 breast tumor cells in detail. We demonstrate that IFN-γ up-regulates caspase-8 mRNA levels without altering mRNA stability, in a protein synthesis-dependent manner. We have also studied the human caspase-8 gene promoter to determine the mechanism by which IFN-γ regulates its transcriptional activity. We identify and characterize an IFN-stimulated response element (ISRE) in the promoter region that binds the IFN-γ-inducible transcription factor IRF-1 and is essential for IFN-γ-induced caspase-8 gene promoter activation in breast tumor cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—RPMI 1640 medium and fetal bovine serum were obtained from Invitrogen. All restriction enzymes were from Roche Applied Science. T4 DNA ligase was purchased from New England Biolabs (Beverly, MA). DNA polymerase. The oligonucleotide primers were 5'-GATGGAGGG-3' and 5'-TGCCCATCTA-3' which span the 5'-end of the first exon of the longest isoform of caspase-8 cDNA. This oligonucleotide was end-labeled with [γ-32P]ATP with polynucleotide kinase (Roche Applied Science) and then purified on a G25 spin Column (Roche Applied Science). Fifty microliters of total RNA from MCF-7 cells were precipitated and resuspended into 15 μl of 2× hybridization buffer. The RNA solution was then mixed with 10 μg of poly (A)+ and 15 μl of formaldehyde, heated at 68 °C for 10 min, and incubated overnight at 37 °C. After this incubation, samples were treated with S1 nuclease (Invitrogen) for 30 min at 37 °C according to the manufacturer's instructions. The S1-digested products were resolved on a denaturing 8% polyacrylamide gel. Four microliters of the nondigested and diluted (1:800) labeled oligonucleotide probe (i.e., the probe) were also run at the same time.

**Primer Extension Assay**—The antisense oligonucleotides PE1 (5'-CACCGCAGCCACCATCCTCCTTT-3') and PE2 (5'-CACCTTCT-TCTGAACTGCTGTTGGCCG'-3'), which correspond to sequences located within the 5'-end of the noncoding exon 1 of the human caspase-8 gene, were end-labeled with [γ-32P]ATP to a specific activity of 4× 1010 cpm μl−1. 1.5× 106 pmol of labeled oligonucleotide were mixed with 20 μl of total RNA from control or IFN-γ-treated MCF-7 cells, 2 μl dNTPs, 10 μm dithiothreitol, and First Strand Buffer. RNA and total RNA from mouse liver cells were used in place of MCF-7 RNA as negative controls. The samples were heated at 68 °C for 5 min and then allowed to cool to 42 °C, at which point the annealed primers were then extended with Molenoy murine leukemia virus reverse transcriptase (Invitrogen) for 1 h at 42 °C. The extended products were resolved on denaturing 8% polyacrylamide gels. Sequencing reactions, performed on a promoter-containing plasmid with the same oligonucleotide (Sequenase Quick-Denature sequencing kit; Upstate Biotechnology, Lake Placid, NY), were run at the same time in order to accurately determine the 5'-end termini.

**Plasmid Constructs**—Different caspase-8 promoter fragments were generated by PCR amplification with KlenTaq LA polymerase mix (Clontech) with genomic DNA from Jurkat cells as template. The following sense primers were used for the indicated fragments: −1588, 5'-CATTGCACACAACCTAGTGGC-3' and −1568, 5'-GCAAGAATCTACTATGGCG-3'; −197, 5'-CGAACAATCAGTGTCG-3'; −132, 5'-GTTGATGAAAAG-GTTG-3'; −82, 5'-GTTGTTTAGGACAGAAGG-3'. A single antisense primer, 5'-GCTCCACCCACCTCCATC-3' (+230), was used to obtain all of the promoter fragments. Numbering is relative to the first nucleotide of exon 1. Constructs pC8-1588Luc and pC8-82Luc were generated by cloning the corresponding PCR fragments into the BamHI/HindIII sites of the promoterless pXP2 luciferase reporter plasmid, whereas pC8-688Luc, pC8-197Luc, and pC8-162Luc plasmids were created by subcloning the respective PCR promoter fragments into the HindIII site of pXp2-Luc vector.

**Mutant versions of the luciferase reporter plasmids** pC8−197 (pC8-197mut) and pC8-82 (pC8-82mut) were prepared by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by DNA polymerase. The oligonucleotide primers were 5'-GCTCTGAGT-TTTGTGATGTTTCCACCTGTTGCTGACG-3' and its complementary antisense oligonucleotide. Mutated positions are underlined. The final products of the reactions were sequenced to confirm the introduction of the expected mutations. The pCDNA-IFN-1 plasmid was generously provided by Dr. T. Maniatis (University of Massachusetts Medical School, Worcester, Massachusetts).

**Cell Transfections and Luciferase Assays**—Cells were plated at a density of 2× 105 cells/35-mm tissue culture dish the day before transfection with FuGENE reagent (Roche Applied Science) according
to the manufacturer’s instructions. Cells were co-transfected with 0.5 μg of the luciferase reporter plasmid described above as appropriate and 0.25 μg of β-galactosidase expression plasmid as an internal control for transfection efficiency. Twenty hours after transfection, cells were harvested, and luciferase activity was measured in an F8T2 luminescence spectrometer (Berthold Detection Systems) according to the instructions of the Luciferase system kit (Promega). Where indicated, cells were treated with IFN-γ for an additional period before measuring luciferase activity. For co-transfection experiments, 0.25 μg of luciferase reporter plasmid, 0.5 μg of the IFN-1 expression plasmid or the empty vector, and 0.25 μg of β-galactosidase vector were mixed with the FuGENE reagent. All transfection experiments were carried out in duplicate. Transfection efficiency was always normalized by measuring β-galactosidase activity and expressed as relative luciferase units.

Electrophoretic Mobility Shift Assays (EMSAs)—For nuclear protein extractions, MCF-7 cells were lysed in 400 μl of hypotonic buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na3MoO4, protease inhibitors, 0.75 mM spermine, and 0.15 mM spermidine) containing 0.6% Nonidet P-40. Nuclei were then centrifuged and washed once with a detergent-free hypotonic buffer and incubated in 50 μl of high salt buffer (20 mM HEPES, pH 7.6, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na3MoO4, and protease inhibitors) for 30 min on a rocking platform at 4 °C. Nuclei were centrifuged at 13,000 rpm in a microcentrifuge for 10 min, and supernatants containing the nuclear extracts were immediately stored at −80 °C. The protein concentration was determined by the Bradford procedure.

Electrophoretic mobility shift assays were performed as previously described (32). Nuclear proteins (4 μg) were incubated with 2.0 μg of poly(dI-dC) DNA carrier and 4 μl of 5× DNA binding buffer (10% polyvinylpyrrolidone, 12.5% (v/v) glycerol, 50 mM Tris (pH 8), 2.5 mM EDTA, and 2.5 mM dithiothreitol) in a final volume of 20 μl for 10 min on ice. Next, 2 μl (5 × 106 cpm/μl) of 32P-labeled double-stranded oligonucleotide was added to the reaction mixture, which was then incubated at room temperature for 40 min. For competition assays, the nuclear extracts were incubated with a 100-fold molar excess of unlabeled double-stranded oligonucleotide for 10 min at 4 °C before the addition of the 32P-labeled probe. Where indicated, 2 μg of rabbit polyclonal antisera against IRF-1 (sc-4979X) or STAT-1α p91 (sc-345X) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the nuclear extracts, which were then maintained for 30 min at 4 °C prior to adding the 32P-labeled probe. The sequences of the oligonucleotides used in these studies were as follows: 5′-gatcGTTTTTTTGTTTCTGGTACCTCTTGTG-3′ (ISRE sequence located within the promoter of the human caspase-8 gene) and 5′-agtctACAACACGCTGATTTCCCCGAAA-TGGCGCGC-3′ (GAS sequence from −138 to −107 of the IRF-1 promoter) (33).

RESULTS

IFN-γ Enhances TRAIL-induced Caspase-8 Activation and Caspase-8 Expression in Breast Tumor Cells—We recently reported that IFN-γ sensitizes breast tumor cells to TRAIL-mediated apoptosis by enhancing the activation of mitochondria-dependent and -independent apoptotic pathways (6). Here, we have further examined this sensitization process by determining the time course of TRAIL-induced caspase-8 activation in MCF-7 cells preincubated with IFN-γ. The results presented in Fig. 1 show that processing of procaspase-8 was first observed at 4 h after the addition of TRAIL to MCF-7 cells. In contrast, upon pretreatment with IFN-γ, procaspase-8 cleavage could be clearly detected as early as 2 h after TRAIL receptor activation. This suggests that facilitation by IFN-γ of apical procaspase-8 activation by TRAIL may play a pivotal role in the sensitization observed.

The significance of IFN-γ-activated procaspase-8 processing in the mechanism of IFN-γ-induced sensitization to TRAIL-mediated apoptosis in breast tumor cells was also suggested by our previous results demonstrating that IFN-γ up-regulates caspase-8 mRNA as well as protein expression (28). To further define the observed modulation of caspase-8 mRNA by IFN-γ, we tested whether IFN-γ affected the stability of this mRNA. MCF-7 cells were harvested at different times over a 12-h period after the addition of the RNA polymerase II inhibitor actinomycin D, and the level of caspase-8 mRNA was analyzed. As shown in Fig. 2A, actinomycin D induced a significant decrease in basal caspase-8 mRNA levels after 6 h of exposure, whereas a more substantial reduction was detected at 12 h. Moreover, in cells preincubated with IFN-γ for 12 h before the addition of actinomycin D, a similar time course of reduction in caspase-8 mRNA was observed (Fig. 2B). Quantitative analysis of mRNA levels in Fig. 2, A and B, confirmed that pretreatment with IFN-γ did not increase caspase-8 mRNA stability (data not shown). These data suggest that the up-regulation of caspase-8 mRNA levels by IFN-γ in breast tumor cells is likely to be the result of the induction of caspase-8 gene transcription.

Identification and Analysis of the 5′ Upstream Region of the Human Caspase-8 Gene—The human caspase-8 gene maps to chromosome 2q33-3q34 and contains 13 putative exons, with the ATG start codon located in exon 5 (34, 35). Two very recent reports have described the promoter region of human caspase-8 (36, 37). However, at the time this work was initiated, the human caspase-8 promoter region had not been characterized. To identify the genomic sequence upstream of the human caspase-8 gene, we conducted a GenBank database search of the caspase-8 DNA sequence within band q33-q34 of chromosome 2. This identified a 1785-bp region located 5′ upstream of the non-coding exon 1 of human caspase-8 and 3′ downstream of the exon 3.
11 of caspase-10 (accession number NT_005403) (Fig. 3A). Computer-based analysis of this region with the TRANSFAC program (38) highlighted several potential binding sites for known transcription factors, such as NF-κB, NF-AT, Sp1, GATA, and AP-1, as well as consensus sequences for binding of transcription factors regulated by interferon-γ (Fig. 3, A and B; data not shown). Although no consensus TATA or CAAT boxes were found, this analysis suggested that the identified genomic region probably matched the human caspase-8 promoter. To map the transcriptional start site of the caspase-8 gene, we carried out S1 nuclease protection analysis and primer extension assays (see “Experimental Procedures.”) Three sites located 15 nucleotides upstream of the first nucleotide of exon 1 were thus identified (Fig. 3B). These results are in agreement with the heterogeneous transcription start sites described for other TATA-less promoters controlled by the Sp1 transcription factor (39–41). Consistent with this, a different start site in the caspase-8 gene has recently been described by other authors (37).

To determine whether the 5’ upstream region of caspase-8 gene contains the minimal elements for basal promoter activity, different sized fragments were generated and cloned into the promoterless pXP2 luciferase reporter vector (Fig. 3A). MCF-7 cells were transiently transfected with these reporter plasmids, and 12 h later they were treated with or without 10 ng/ml IFN-γ for an additional 8 h. Control cultures were transfected with empty vector (pXP2). Luciferase activity was determined and normalized to the β-galactosidase activity. The error bars represent S.D. from three independent experiments.
next larger (pC8–162), suggesting that the region between nucleotides −82 and −162 plays an important role in regulating basal caspase-8 promoter activity. These results are in agreement with recent data indicating that an Sp1 binding site located within this region is important for the basal activity of this promoter (37). Despite its relatively low activity, the pC8-82 construct appears to contain the minimal promoter elements of the human caspase-8 gene, since its activity was still 3-fold higher than that of the control pXP2 promoterless luciferase vector.

Regulation of Human Caspase-8 Promoter Activity in Breast Tumor Cells by IFN-γ—Computer-based analysis revealed four GAS and two ISRE sites within the 1785-bp caspase-8 upstream sequence (Fig. 3A). Although other authors have reported the up-regulation of caspase-8 by IFN-γ in various cell types, data on the involvement of GAS and ISRE elements, and therefore the role of STAT-1 and IRF-1 transcription factors, are contradictory (17, 18). We therefore decided to analyze the importance of the identified GAS and ISRE motifs within the caspase-8 promoter in the induction of caspase-8 expression by IFN-γ in breast tumor cells. MCF-7 cells were transiently transfected with the caspase-8 promoter-containing constructs described above (Fig. 3A), and luciferase activity was measured after IFN-γ treatment. Results shown in Fig. 3C show that the activities of all five constructs analyzed were stimulated by IFN-γ. Interestingly, the pC8-82 construct, which contained the shortest promoter frag-
ment, exhibited an induction of activity by IFN-γ similar to that of the other constructs, suggesting that a key element responsive to IFN-γ must be located within the 82-bp 5' proximal region of the human caspase-8 promoter.

Characterization of an ISRE Element Essential for IFN-γ-induced Caspase-8 Promoter Activity—An ISRE motif was the only putative IFN-γ-responsive element in the 82-bp region of the human caspase-8 promoter identified by computer analysis (Fig. 3B). Site-directed mutagenesis of conserved residues in this ISRE motif was performed on the pC8-197 and pC8-82 constructs (Fig. 3B). The two mutated constructs, named pC8-197mt and pC8-82mt, completely lacked IFN-γ inducibility upon transfection in MCF-7 cells (Fig. 4A). Moreover, mutation of the ISRE site decreased but did not abolish the basal activity of the caspase-8 promoter, suggesting that this site is also involved in the constitutive transcriptional activity of the caspase-8 gene. A role of this kind for an ISRE element has been previously described in relation to the promoter region of other genes (41–43). To exclude the possibility that the reduction in the basal activity of the mutated constructs could affect the inducibility by any stimulus, and therefore by IFN-γ, MCF-7 cells were transfected with pC8-197 or pC8-197mt plasmids, and luciferase activity was assayed after treatment with a calcium ionophore and a phorbol ester. These stimuli are
known activators of the transcription factors NF-κB and AP-1 (44, 45), which have putative binding sites within the pC8-197 promoter fragment (Fig. 3B). As shown in Fig. 4A (upper panel), despite the loss in basal activity, the stimulation of luciferase activity by calcium ionophore and phorbol ester was similar in cells transfected with wild-type or mutant constructs, indicating that abrogation of IFN-γ-inducible transcriptional activity in the ISRE mutated constructs was not due to a general impediment of transcriptional stimulation.

To further analyze the involvement of this ISRE motif, we used a probe containing this element in EMSA experiments with nuclear extracts from MCF-7 cells treated with IFN-γ for different times. As shown in Fig. 4B, a major DNA-protein complex and two minor complexes were clearly induced upon treatment with IFN-γ for 4 h. We also analyzed the time course of IFN-γ-induced transcriptional activity by transfecting MCF-7 cells with the pC8-82 plasmid and measuring luciferase activity after incubation with IFN-γ. Increased luciferase activity was first observed 4 h after the addition of IFN-γ in comparison with untreated cells (Fig. 4C). This agrees with the time course of caspase-8 mRNA induction determined by Northern blot analysis (Fig. 4D), with elevated mRNA levels first detected after 4-h exposure to IFN-γ.

The Proximal ISRE Element of the Human Caspase-8 Promoter Specifically Binds IRF-1 upon Treatment with IFN-γ—ISRE elements can be bound by different members of the IRF family of transcription factors, such as IRF-1 and the ISGF-3 complex. Whereas IFN-γ signals through IRF-1 by inducing its expression, the components of ISGF-3, STAT-1α, and IRF-9/p48 are usually expressed constitutively and are activated by IFN-γ treatment (46). In order to identify the transcription factors that bind to the proximal ISRE motif from the caspase-8 promoter, we performed a set of EMSA experiments. First of all, competition experiments using an excess of either the ISRE probe or an oligonucleotide containing the GAS element of the IRF-1 promoter (a high affinity STAT-1α-binding site) (33) indicated that only the ISRE oligonucleotide was able to compete for binding to the IFN-γ-induced complexes (Fig. 5A, left panel). Moreover, when nuclear extracts were incubated with an anti-STAT-1α antibody prior to EMSA analysis, we could not observe any effect on the ISRE binding complexes induced by IFN-γ (Fig. 5A, middle panel). As a control, additional EMSA experiments were performed with the GAS motif of the IRF-1 promoter to confirm that a GAS-specific complex could in fact be supershifted by the anti-STAT-1α antibody (Fig. 5A, right panels). These results suggest that STAT-1α, and hence the ISGF-3 complex, does not bind the ISRE element of the caspase-8 promoter. We also found that the level of caspase-8 induction by IFN-γ in the IRF-9/p48-deficient cell line U2A is similar to that observed in the control 2TGH parental cell line (data not shown), further confirming that the ISGF-3 transcription factor is not involved in IFN-γ-mediated regulation of caspase-8 expression.

Since IFN-γ signaling via IRF-1 depends on the de novo synthesis of the transcription factor, we determined the level of caspase-8 mRNA in IFN-γ-treated MCF-7 cells that were preincubated with the inhibitor of protein synthesis cycloheximide. As shown in Fig. 5B, IFN-γ was not able to induce the expression of caspase-8 mRNA in cycloheximide-treated cells above the level achieved with cycloheximide alone, probably indicating that new protein synthesis was required for the induction by IFN-γ. Moreover, when EMSA analysis was performed with nuclear extracts from cycloheximide-treated, IFN-γ-induced MCF-7 cells, none of the ISRE-binding complexes were observed (Fig. 5C, left panel), thus revealing that new protein synthesis was also required for complex formation. In view of these data, we next examined the potential binding of IRF-1 to the caspase-8 promoter ISRE by incubating nuclear extracts with an antibody against IRF-1 prior to EMSA assays. As observed in Fig. 5C, right panel, the major IFN-γ-induced DNA-protein complex and one of the minor complexes completely disappeared in the presence of anti-IRF-1 antibody, whereas the lower minor band was unaffected. We next confirmed that treatment with IFN-γ induced the expression of IRF-1 in MCF-7 cells. IRF-1 mRNA and protein were markedly induced upon incubation of MCF-7 cells with IFN-γ for 2 h and remained elevated up to 8 h, the latest incubation time analyzed (Fig. 5D) (results not shown). IRF-1 induction preceded the formation of the ISRE-nuclear protein complex (Fig. 4B) and the expression of caspase-8 mRNA (Fig. 4D), consistent with a role of newly synthesized IRF-1 in the regulation of caspase-8 expression by IFN-γ.

These results thus suggest that the IFN-γ-inducible transcription factor IRF-1 must be the transactivator that binds the ISRE motif of the caspase-8 promoter in response to IFN-γ in breast tumor cells. To further substantiate the role of IRF-1, we conducted co-transfection experiments with an IRF-1-expressing plasmid and the promoter constructs pC8-82 or pC8-82-mt. As shown in Fig. 6, IRF-1 clearly stimulated the transcriptional activity of the pC8-82 plasmid above the base-line activity of empty vector-transfected cells (Fig. 6). In contrast, no induction could be detected in co-transfections with the construct containing the mutant ISRE motif (Fig. 6). Interestingly, the transcriptional activity of the pC8-82 promoter fragment induced by IRF-1 was of a similar magnitude to that achieved with IFN-γ (Fig. 4A), a further indication of the importance of this factor in mediating the effects of IFN-γ.

**DISCUSSION**

For the present study, we set out to examine the mechanism of IFN-γ-induced up-regulation of caspase-8 expression. To achieve this, we initially cloned the human caspase-8 promoter and characterized both the transcriptional start site and the minimal sequence requirement for basal transcription. Contrary to what has been recently reported (37), we found several transcription initiation sites located in a region of 15 bp 5’ upstream of the first nucleotide of exon 1. This is not surprising, since heterogeneous transcriptional initiation is known to occur in other TATA-less promoters, of housekeeping and IFN-regulated genes (39–41, 47). Moreover, transcription from two
Regulation of Human Caspase-8 Expression by IRF-1

of these sites was up-regulated after IFN-γ treatment (data not shown), suggesting that these sites are probably important in the regulation of caspase-8 gene expression by the cytokine. Our results also indicate that the promoter region between −162 and −82 bp is important for maintaining the basal transcriptional activity. Sequence analysis of this region indicates the presence of a Sp1 binding site that may play an important role in sustaining the basal activity of the human caspase-8 promoter, as recently indicated (37).

Loss of caspase-8 expression has been demonstrated in malignant neuroblastomas, medulloblastomas, Ewing tumor, rhabdomyosarcomas, retinoblastomas, primitive neuroectodermal brain tumors, and small cell lung carcinomas (9, 11–16). In many instances, gene hypermethylation has been proposed as the mechanism of silencing caspase-8 gene expression, because DNA methylase inhibitors up-regulate caspase-8 expression (10, 16). However, methylation of the region upstream of exon 1 is not associated with caspase-8 silencing (36), suggesting that other mechanisms of gene inactivation are also present in these tumor cells (12). In this respect, inactivating caspase-8 gene mutations have also been described in some types of tumor cells such as colorectal carcinoma and squamous carcinoma cells (48, 49).

Modulation of caspase-8 expression by IFN-γ has been described in several human tumor models (6, 11, 17, 18, 28–30). Furthermore, IFN-γ-regulated caspase-8 expression is also a key step in activation-induced death of T lymphocytes (50), suggesting that this cytokine may function not only in cell-mediated immunity and antitumor responses but also as a regulator of T-cell survival.

Regarding the molecular mechanism of human caspase-8 gene regulation by IFN-γ, a GAS element located 204 nucleotides upstream from the transcription initiation site has been reported as important for the up-regulation observed in neuroblastoma cells (18). In that study, up-regulation of caspase-8 mRNA by IFN-γ was first observed at 12 h, although it appears not to require protein synthesis (18). However, it is well known that activation of gene expression by the STAT-1/GAS system occurs very early after the activation of the IFN-γ receptor and involves the phosphorylation of the STAT-1α protein by JAK kinases and its translocation as a dimer to the nucleus, where it binds to GAS sites in the promoter of responsive genes (51). We have shown in the current study that deletion of the caspase-8 promoter region harboring the GAS element does not abrogate inducibility by IFN-γ. We have also demonstrated that an ISRE located 18 bp from the initiation of exon 1 is essential for the induction of promoter activity by IFN-γ. Moreover, our results indicate that IFN-γ-induced elevation of caspase-8 mRNA expression in breast tumor cells requires protein synthesis and is first observed at 4 h after the addition of IFN-γ. These data are in accordance with the EMSA results, which show that formation of the ISRE complex requires protein synthesis and is also first seen 4 h after IFN-γ treatment. Importantly, in this work we have demonstrated that the formation of the ISRE complex is competed by an antibody to the IRF-1 transcription factor. Additionally, in transient transfection experiments with a luciferase reporter plasmid containing the minimal promoter required for IFN-γ responsiveness, we found that co-transfection of an IRF-1-expressing vector transcriptionally activates the luciferase vector to a similar extent as IFN-γ, further demonstrating the role of the IRF-1/ISRE system in the regulation of human caspase-8 expression in breast tumor cells. These results also provide a molecular explanation to the recently reported induction of human caspase-8 mRNA by IRF-1 in neuroblastoma (17). Interestingly, sequence analysis of the murine caspase-8 promoter reveals the presence of an ISRE-like element in the region proximal to the beginning of exon 1, as observed in the human promoter, thus supporting the important role of this element in the transcriptional regulation of this gene. IFN-γ also up-regulates the expression of other members of the caspase family in tumor cells (25). It is therefore interesting that an ISRE found in the promoter region of human caspase-1 gene is responsible for the reported up-regulation of the expression of this caspase by IFN-γ in tumor cells (42). Although the promoter elements responsible for activating the expression of other caspase genes have not been identified, a common regulation of this family of apoptotic cysteine proteases by IFN-γ is an attractive possibility.

The importance of the up-regulation of caspase-8 expression observed in breast tumor cells treated with IFN-γ to the sensitization of these cells to TRAIL-induced apoptosis can be inferred from the mechanism of procaspase-8 processing and activation at the DISC. After recruitment to the DISC, procaspase-8 is autoprocessed to generate the active form that can cleave other substrates, including executioner caspases. According to the induced proximity model for caspase-8 activation, a locally high concentration of the caspase-8 zymogen would promote its autoprocessing and the release of the active caspase (52). It is therefore possible that the increased expression of caspase-8 found in IFN-γ-treated breast tumor cells might facilitate formation of the DISC upon death receptor activation, with subsequent activation of an apoptotic cascade. Moreover, in cells such as neuroblastoma, where caspase-8 expression is inhibited by hypermethylation of the gene, IFN-γ renders these cells sensitive to TRAIL, FasL, and TNF-α (17), although no direct effect of IFN-γ on the methylation status of regulatory sequences of the caspase-8 gene was found. Induction of caspase-8 might facilitate not only death receptor-mediated apoptosis but also certain drug-inducible apoptotic pathways in various tumor cells (17, 53–55). Therefore, modulation of caspase-8 by IFN-γ might be envisaged as a more widespread mechanism for the sensitization of different tumor cells to apoptosis. Altogether, the available data also suggest that caspase-8 may act as a tumor suppressor gene and that inhibition of the death receptor pathway to apoptosis may play an important role in the pathogenesis of many types of tumors.

Acknowledgments—We acknowledge Gema Roldo for excellent technical assistance. We are very grateful to Dr. S. Bartlett for critical reading of the manuscript and editorial assistance.

REFERENCES

1. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and Goodwin, R. G. (1995) Immunology 3, 673–682
2. Kischkel, F. C., Lawrence, D. A., Chnutherapai, A., Chow, P., Kim, K. J., and Ashkenazi, A. (2000) Immunity 12, 611–620
3. Scaffidi, C., Fulda, S., Srivivasan, A., Pissene, C., Li, F., Tomasselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687
4. Yamada, H., Tada-Oikawa, S., Uchida, A., and Kawashani, S. (1999) Biochem. Biophys. Res. Commun. 265, 130–133
5. Deng, Y., Lin, Y., and Wu, X. (2002) Genes Dev. 16, 33–45
6. Ruiz-Ruiz, C., and Lopez-Rivas, A. (2002) Biochem. J. 365, 825–832
7. Walczak, H., Miller, R. E., Arai, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. (1999) Nat. Med. 5, 157–163
8. Siegmund, D., Hadwiger, P., Piferenmaier, K., Vornlocher, H. P., and Wajant, H. (2002) Mol. Med. 8, 725–732
9. Mistad, O., Mistad, G., Fouladi, K., Anderson, K. C., and Treon, S. P. (2002) Blood 99, 2162–2171
10. Teita, T., Wei, T., Valentine, M. B., Vanin, E. F., Grenet, J., Valentine, V. A., Behm, F. G., Look, A. T., Lahti, J. M., and Kidd, V. J. (2000) Nat. Med. 6, 529–535
11. Hopkins-Donaldson, S., Bodmer, J. L., Bourlou, K. B., Brograna, C. B., Tschopp, J., and Gross, N. (2000) Cancer Res. 60, 4315–4319
12. Harada, K., Toyooka, S., Shivapurkar, N., Maitra, A., Reddy, J. L., Matta, H., C. Ruiz-Ruiz and A. López-Rivas, unpublished data.
