IRF-5 Is a Mediator of the Death Receptor-induced Apoptotic Signaling Pathway

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The efficient and regulated response to cellular stress is coordinated by a genetic regulatory network in which a given transcription factor controls the expression of diverse target genes depending on the cell type and/or nature of the stimuli. The tumor suppressor p53 is thought to preferentially regulate the balance between cell survival and death. The interferon regulatory factor 5 (IRF-5), known to be involved in the innate immune response to pathogens, is also a critical regulator of DNA damage-induced apoptosis. Here, we provide direct evidence that IRF-5 promotes apoptosis upon signaling through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptors (DR). We report that IRF-5 sensitizes tumor cells to TRAIL-induced apoptosis and cell death that is further enhanced by type I interferons. Cells deficient of IRF-5 gave a significantly diminished response to these agents. IRF-5 is involved in DR signaling upstream of caspase 8, in part because of an IRF-5-dependent increase in caspase 8 activation. We provide evidence that TRAIL induces a signaling cascade that leads to the phosphorylation and nuclear localization of IRF-5, resulting in transactivation of key DR signaling components. The results presented here identify IRF-5 as a new mediator of DR signaling and provides molecular insight into the mechanism of TRAIL-induced IRF-5 signaling.

Unlike conventional cancer therapeutics, death receptor (DR) ligands trigger tumor cell apoptosis independent of p53. The DR Apo2 ligand or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) is a type II membrane-bound molecule of the TNF family. Members of this family share diverse biological effects: induction of apoptosis and/or promotion of cell survival. Interest in TRAIL has grown tremendously after evidence emerged that it induces apoptosis in malignant while sparing normal cells. TRAIL stimulation is characterized by interaction with death domain-containing receptors following adaptor recruitment and caspase activation.

TRAIL triggers apoptosis by binding to receptors DR4 and/or DR5, which engage the cell extrinsic pathway (1). The receptors then recruit and activate apoptosis-initiating proteases caspase 8 and 10 through the death domain-containing adaptor molecule Fas-associated death domain (FADD), leading to formation of the death-inducing signaling complex (DISC). Caspase 8 and 10 activate downstream caspases 3, 6, and 7, which execute the apoptotic demise of the cell. In some cells, TRAIL-induced activation of caspase 3 is further augmented through engagement of the intrinsic pathway. In this case, caspase 8/10 cleaves and activates the pro-apoptotic Bcl-2 family member Bid, which then interacts with two other family members, Bax and Bak. tBid translocates to the mitochondria leading to the release of cytochrome c and Smac/Diablo to the cytosol. Cytochrome c, together with Apaf-1, activates the initiator protease caspase 9, which contributes to activation of caspases 3, 6, and 7.

Regulation of the TRAIL-induced apoptotic signaling pathway is not well understood. Numerous genes and signaling pathways are thought to be involved in its regulation (2–6). The DR4 promoter has several AP-1-binding sites, and DR5 has two Sp1 sites that can be activated by chemotherapeutic agents (7). The presence of distinct binding sites in these promoter regions suggests that expression may be differentially regulated.

The family of interferon regulatory factors (IRFs) has been implicated as critical transcription factors that sense a variety of environmental stresses (8). IRFs mediate their biological activity by binding to promoters of target genes. They have been extensively studied for their role in host immune response to pathogens; less is known of their role in cell growth regulation. IRF-1 and -5 possess tumor suppressor activity (9, 10); IRF-3 and -5 mediate virus-induced apoptosis (8, 11). Type I and II IFNs are capable of sensitizing tumor cells to TRAIL-induced apoptosis (6, 12). Although the molecular mechanism(s) of action is not fully understood, data indicate that IRF-1 and -3 may play important roles by regulating TRAIL expression (13, 14). More recent data suggest a role for IRF-1 in the nuclear colocalization of caspase 8 and FADD (15). Much less is known...
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of IRF-5 apoptotic signaling pathways. Data from our laboratory and others indicate that IRF-5 is critical for the induction of apoptosis in response to DNA damage (9, 10, 16, 17). Early studies suggested a role for IRF-5 in p53-mediated apoptosis (10); later data revealed that IRF-5 acts on a pathway that is distinct from p53 (9, 16, 17). Data from microarray analyses indicate that components of the DR signaling pathway are regulated by IRF-5 (9, 16, 18).

In the present study, we investigated the contribution of IRF-5 to TRAIL-mediated DR signaling. We demonstrate that IRF-5 overexpression sensitizes tumor cells to TRAIL-induced apoptosis; signaling was diminished in cells lacking IRF-5. The involvement of IRF-5 in DR signaling occurs upstream of caspase 8 activation. We also provide evidence that TRAIL induces a signaling cascade that leads to the phosphorylation and activation of IRF-5, resulting in transactivation of DR signaling machinery. The results indicate that IRF-5 is a previously unidentified mediator of DR signaling and provides a mechanistic insight into IRF-5-mediated apoptosis by TRAIL.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Treatments—HCT116 isogenic cell lines and gfp-IRF5 stable expressing cells were described (9, 19). IRF-5−/− MEFs (20) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The caspase 8 promoter reporter (pC8−1588) was from Dr. Abela Lopez-Rivas; the TRAIL promoter (Apo1056) was from Dr. Alex Almasan; the DR5 promoter (pDR5/SacI) was from Dr. Toshiyuki Sakai; the FLIP promoter was from Dr. Wafik El-Diery; and the HA-tagged murine IRF-5 was from Dr. Taniguchi. Human IFNβ was purchased from PBL Biomedical Laboratories (Piscataway, NJ); human TRAIL was from Axxora, LLC (San Diego, CA); and mouse TRAIL was from Biomol International, LP (Plymouth Meeting, PA). The cells were treated with 1000 units/ml of IFNβ at 37°C for 24 h, 10 ng/ml human recombinant TRAIL unless otherwise indicated for 3 h. The cells were incubated with 2 μM MG132 (Calbiochem) for 16 h.

FACS Analysis—Analysis of apoptosis and cell death was carried out as described (9, 16). The cells were treated with the indicated concentrations of TRAIL for 12–24 h unless otherwise stated. Cell viability was determined by propidium iodide (PI; BD Pharmingen, Lexington, KY) staining of trypsin-harvested cells and FACS (Becton Dickinson, Palo Alto, CA). The average percentage of positive cells (means ± S.E.) was calculated from three independent experiments. Apoptosis was quantified by single annexin V fluorescein isothiocyanate-PI or annexin V-phycocerythrin (PE) staining (BD Pharmingen).

Caspase 8 Activity—The cells were left untransfected or transfected with IRF-5 siRNA for 24 h and treated with 20 ng/ml TRAIL for 4 h. Caspase 8 activity was determined in cell lysates using the Caspase 8 Colorimetric activity assay kit (Chemicon International, Temecula, CA) according to the manufacturer’s specifications.

Immunoprecipitations (IP) and Immunoblot (IB) Analysis—Cell lysates for IP and IB analysis were prepared as described (16) with the addition of half phosphatase inhibitor mixture (Pierce). For IP, 300 μg of extracts were incubated with either (1:50) Thr(P) (Cell Signaling, Beverly, MA), Ser(P) (Zymed Laboratories Inc., Inc.), Tyr(P) (Cell Signaling, Beverly, MA), IRF-5 antibodies (Abcam, Cambridge, MA), or IgG controls as previously described (16). The proteins were resolved by SDS-PAGE and immunoblotted. For direct IB, 60-μm dishes were transfected with 2 μg of expression vector encoding Myc-IRF5 or empty Myc pcDNA3.1 (Invitrogen) using FuGENE 6 (Roche Applied Science). After 48 h, the cells were harvested, and whole cell lysates were resolved. Antibodies against IRF-5, cleaved poly(ADP-ribose) polymerase, cleaved caspase 3, caspase 6, caspase 7, caspase 8, FADD, phospho-FADD (Cell Signaling, Beverly, MA), I-FLICE/FLIP, cytochrome c, tBid (R&D Systems, Inc., Minneapolis, MN), c-Myc, DR5, and HA (Roche Applied Science) were used. Immunoreactive protein complexes were visualized with ECL reagents (Amersham Biosciences). The membranes were stripped and reprobed using Restore Western blot stripping buffer (Pierce).

RNA Interference—IRF-5 gene silencing experiments were performed as described (16) with Lipofectamine 2000 or Oligofectamine transfection reagent (Invitrogen). 21 h post-transfection, the cells were treated with TRAIL for an additional 3 h and then harvested. The effect of IRF-5 siRNA, shRNA, empty vector shRNA, or nontargeting control siRNAs (Dharmacon, Inc., Lafayette, CO) was analyzed by IB or microarray. IRF-5 siRNA was previously described (16); IRF-5 shRNA was from Ambion (catalog number 16708) and cloned to pRNAiIn-H1.2 from GenScript Corp.

Subcellular Fractionation—The cells were homogenized in isotonic mitochondrial buffer (210 mM sucrose, 70 mM mannitol, 10 mM Hepes, pH 7.4, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitors and centrifuged at 1,000 × g for 10 min to discard nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × g for 15 min to pellet mitochondria-enriched heavy membrane fraction, and the supernatant was centrifuged further at 100,000 × g for 30 min to obtain cytosolic (S-100) fraction. The protein extracts were analyzed by IB.

Microarray Analysis—p53−/− cells were treated with 20 ng/ml TRAIL and 1000 units/ml IFNβ for 3 h after transfection of IRF-5 or control nontargeting siRNAs. Total RNA (5 μg) was isolated, and probes were generated as described (9). Comparison of gene expression was made in treated, transfected cells using the GEArray Q Series human apoptosis gene array (SuperArray Inc., Bethesda, MD) that contains 112 genes involved in apoptosis (16). The level of gene expression was determined by statistical analysis using the GEArray Expression Analysis Suite (SuperArray Inc., Bethesda, MD). Three independent experiments were performed. To assess differences in gene expression, we selected genes that had a greater than 2.5-fold change and a p value of 0.001. The p values were calculated by using the two-sample t test on log-transformed data.

Subcellular Localization and Extraction—gfp-IRF5-expressing cells were grown on coverslips and treated with TRAIL for the indicated time period. The cells were fixed, mounted, and stained with 4′,6′-diamino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA), and the images were obtained on a
Zeiss Axiovision 200 microscope equipped with ApoTome. The normalized nuclear fluorescence intensities (nuclear gfp/4'-6'-diamino-2-phenylindole) were plotted with time. Cytosolic and nuclear extracts were purified and analyzed as described (21).

**Dual Luciferase Assay**—The cells were transiently transfected in 6-well plates using FuGENE 6. Dual luciferase assays were carried out and normalized to internal pRL and protein as described (22). The experiments were repeated at least three times in duplicate.

**In Vivo ChIP Assay**—The ChIP assay was performed according to the manufacturer’s instructions (Upstate, Lake Placid, NY) (23). The samples were immunoprecipitated with 2 μg of NF-κB p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or IRF-5 antibodies for 16 h at 4 °C. RT-PCR was performed using the following promoter primer sets (Invitrogen): DR5 ISRE, 5’-ACACACCATCTACATCT-3’ and 5’-TGAGGTTCAGCTCGCCATT-3’; DR5 NF-κB, 5’-ACCCTTGTGCTCTGTTG-3’ and 5’-TTCAGCGGACTTACG-3’; TRAIL ISRE1, 5’-ATAAGAGGCAAGAGGCAGGAA-3’ and 5’-TGGCACTGGATCTTAAACC-3’; TRAIL ISRE2, 5’-CAGATAAGGCTGCTGGATT-3’ and 5’-CCCAACAACATCTATGGA-3’; and Caspase 8 ISRE, 5’-CAGACTGCGAGGAATGGAGGA-3’ and 5’-CCAGCTCAAGCACGATG-3’. 2 μl of input DNA or 4 μl of ChiP DNA was used for PCR. Optimal PCR cycling conditions for each promoter was as follows: one cycle at 94 °C (4 min); 35 cycles at 94 °C (1 min), 58.5 °C (1 min), and 72 °C (1 min); and one cycle at 72 °C (5 min). The amplified promoter regions are shown in supplemental Fig. S1.

**RNA Analysis**—RNA extraction and RT-PCR analysis were performed as described (9, 16). Quantitative real time RT-PCR analysis was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) or IRF-5 antibodies for 16 h at 4 °C. RT-PCR was performed as described (9, 16). Quantitative real time RT-PCR analysis was performed using the following promoter primer sets (Invitrogen): ISRE, 5’-ACACCACCGTTCACCAATCT-3’ and 5’-CACCTTCTACAATGAGCTGCGTGTG-3’; and ISRE1, 5’-ACACCACCGTTCACCAATCT-3’ and 5’-ACCCTTGTGCTCTGTTG-3’.

**RESULTS**

**IRF-5 Sensitizes Tumor Cells to TRAIL**—To investigate whether IRF-5 plays a role in DR signaling, we compared the sensitivity of HCT116 isogenic cells expressing ectopic gfp-IRF5 to TRAIL. p53−/− and p53−/− cells were equally sensitive; overexpression gave a significant increase in the percent of cells undergoing apoptosis (Fig. 1a). Similar findings were made in BJAB, Jurkat, and A549 tumor cell lines generated to overexpress IRF-5 (supplemental Fig. S2). To determine the mechanism of IRF-5-induced sensitivity to TRAIL, we first examined activation of caspase 8. TRAIL-treated cells resulted in significant caspase 8 cleavage (Fig. 1b, first lane). Overexpression of Myc-tagged IRF-5 gave a >2-fold increase in caspase 8 activation, as determined by densitometric analysis and normalization with actin levels (Fig. 1b, second lane).

The effect of silencing IRF-5 expression on TRAIL-induced caspase 8 activation was next investigated. Efficient silencing of endogenous IRF-5 was established using either 25 nM targeted siRNAs (siRNA1) or 100 ng of IRF-5 shRNA (Fig. 1c). At these concentrations, IRF-5 siRNA/shRNA was specific for IRF-5 alone; type I IFN levels were unchanged, as well as other IRFs or interferon-stimulated genes, as previously reported (16). It is important to note that at higher concentrations of IRF-5 siRNA (100 nM) or shRNA (500 ng), IRF-5 expression was not repressed (Fig. 1c), and interferon-stimulated genes (20 and 54) were up-regulated, indicating induction of the IFN signaling pathway (data not shown). The results in Fig. 1c show >95% knockdown of IRF-5 proteins by siRNA or shRNA. Similar to untransfected cells, treatment of nontargeting siRNA or empty vector shRNA control cells with TRAIL augmented IRF-5 expression and caspase 8 cleavage. Transfection of either IRF-5 siRNA or shRNA decreased endogenous IRF-5 levels as well as inhibited TRAIL-induced caspase 8 cleavage. Because IRF-5 siRNA or shRNA gave similar results, we next examined the effect of IRF-5 siRNA on caspase 8 enzymatic activity. Similar to immunoblot data, silencing of endogenous IRF-5 gave ~70% decrease in TRAIL-induced caspase 8 activity (Fig. 1d).

**Silencing of IRF-5 Desensitizes Tumor Cells to TRAIL**—To further assess the effect of IRF-5 siRNA on TRAIL-mediated signaling, we measured apoptosis by annexin V-PE staining. p53−/− or stable expressing gfp-IRF5/p53−/− cells were left untransfected or transfected with siRNAs for 24 h and treated with TRAIL. The percent of PE-positive cells was determined by FACS (Fig. 2a). In accordance with data shown in Fig. 1a, gfp-IRF5/p53−/− cells were significantly more sensitive to TRAIL than parental. Knockdown of IRF-5 resulted in a dramatic decrease in TRAIL-induced apoptosis.

We next investigated the effect of siRNAs on cell viability by PI staining after treatment with TRAIL or a combination of TRAIL plus IFNβ. We previously reported that IFNβ up-regulates IRF-5 expression and enhances sensitivity of p53−/− and p53−/− tumor cells to DNA damage-induced apoptosis and cell death (16). The percentage of cells undergoing cell death was quantitated by FACS (Fig. 2b). siRNA alone had no effect on cell death. Treatment with TRAIL gave >60% of cells stained positive for PI, whereas treatment with IFNβ augmented the sensitivity (>90%). Treatment of IFNβ alone gave ~15% cell death (data not shown). Knockdown of IRF-5 substantially decreased cell sensitivity to TRAIL and the combination. These data indicate that IRF-5 is a critical transducer of TRAIL-induced apoptosis and cell death and contributes to the observed combinatorial effects of IFNβ and TRAIL.

**IRF-5 Mediates Extrinsic and Intrinsic Apoptotic DR Signaling**—To gain further insight into the molecular mechanism underlying IRF-5-mediated sensitization to TRAIL, we first examined IRF-5 transcriptional regulation by TRAIL. The results in Fig. 3a show a dose-response increase in IRF-5 transcripts that was independent of p53. Next, we examined expression of DR signaling components by immunoblot. The data
indicate that the extrinsic apoptotic signaling pathway is fully engaged in p53<sup>-/-</sup> cells treated with TRAIL (Fig. 3b, first and fourth lanes). IRF-5 protein levels were also up-regulated by TRAIL. Upon silencing IRF-5, TRAIL-induced cleavage of caspase 3, 7, 8, and 9 and poly(ADP-ribose) polymerase were significantly reduced, suggesting that IRF-5 is acting upstream
of these events (Fig. 3b, first and second lanes). No significant change in caspase 6 cleavage was observed. Analysis of further upstream components, DR5, FADD, and the cellular FLICE-like inhibitory protein (cFLIP), revealed that IRF-5 may be acting on multiple levels leading to the observed sensitization. Knockdown of IRF-5 led to a decrease in FADD and phospho-FADD levels, along with a substantial decrease in DR5 expression. FLIP levels were up-regulated.

The effect of IRF-5 siRNA on the translocation of intrinsic mitochondrial cofactors was examined. TRAIL treatment led to the cleavage of Bid to tBid and translocation of tBid to the mitochondria with concomitant release of cytochrome c to the cytosol (Fig. 3c). Knockdown of endogenous IRF-5 decreased tBid translocation and release of cytochrome c. A corresponding decrease in Bak, but not Bax, levels was observed in whole cell lysates (Fig. 3b). tBid translocation and cytochrome c release are triggered in cooperation with Bax or Bak.

**IRF-5 Mediates Death Receptor Signaling**

In the past 3 years, new light has been shed on the relationship between apoptosis and IFN signaling pathways. Although some studies have posited that cross-talk is p53-dependent (27), many investigators have concluded that other pathways link the type I IFNs to apoptosis. The mediator(s) responsible for cross-talk and the mechanism(s) for integration of these two pathways remains to be determined. Here, we examined IRF-5-dependent gene expression by focused microarray in p53−/− cells treated with the combination TRAIL plus IFNβ. Gene expression was profiled using the Oligo GEArray® human apoptosis microarray (SABiosciences, Frederick, MD) that contains 112 genes involved in apoptosis, including TNF ligands and their receptors, Bcl-2 family members, caspases, death domain genes, death effector family members, and genes involved in the p53 and DNA damage response pathway. A detailed list of the genes analyzed can be found on line. The cells were transfected with nontargeting or IRF-5 siRNAs. Data from three independent experiments was used for subtraction analyses and background normalizations to identify IRF-5 target genes (see “Experimental Procedures”). The pattern of expression was remarkably similar between the three independent analyses with a p value of 0.001. Table 1 summarizes the list of genes in which expression was dramatically reduced by a loss of endogenous IRF-5 signaling. Genes showing a ≥2.5-fold alteration in expression levels included death domain family members (MYD88 and RIPK1), p53 and ATM pathway (GADD45 and CHEK2), CARD family (RIPK2, NOL3, and BCL10), TNF receptor family (TNFRSF14, TNFRSF1A, TNFRSF7, TNFRSF8, and TNFRSF9), the TNF ligand TNFSF8, TRAF family (TRAF2, TRAF3, TRAF6, and TRIP), and caspases (Caspases 3, 7, and 8). Fewer genes...
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IRF-5 target genes induced by Apo2L/TRAIL and IFNβ

The Oligo GEArray® human apoptosis microarray (Frederick, MD) profiling 112 genes involved in apoptosis was used. The results are from three independent experiments. The genes listed show a >2.5- or 4.5-fold modulation in the presence or absence of IRF-5 siRNA with p < 0.001.

| ≥2.5-fold | ≥4.5-fold |
|-----------|-----------|
| MYD88     | TNFRF10B  |
| GADD45    | CARD4(NOD1)|
| CHEK2     | RIPK1     |
| RIPK2     | TANK      |
| BCL10     | TNF       |
| NOL3(Nop30)| TNFRF10B(DR5)|
| TNFRF14   | FAS       |
| TNFRF51A  | TNFRF1B   |
| TNFRF57(CD27)| Caspase 13|
| TNFRF58(CD30)| TRAIL    |
| TNFRF59   |           |
| TNFRF8(CD30L)|         |
| TRAF2     |           |
| TRAF3     |           |
| TRAF6     |           |
| TRIP      |           |
| Caspase 3 |           |
| Caspase 7 |           |
| Caspase 8 |           |

The data presented here clearly demonstrate that endogenous IRF-5 is phosphorylated in response to TRAIL.

Because phosphorylation of a protein can often alter its cellular localization (16, 17, 20–23, 28), we determined IRF-5 localization after TRAIL treatment. In p53+/− cells, gfp-IRF5 translocated to the nucleus at 1 h post-treatment (Fig. 4c). At 2 h, the majority of gfp-IRF5 was expressed in the nucleus (data not shown). gfp-IRF5 was not detected in the nucleus of p53−/− cells till 2 h post-treatment (Fig. 4d). At 3 h, the majority of cells had IRF-5 in the nucleus (data not shown). Quantitation of nuclear fluorescence is shown in Fig. 4e. Similar results were found by immunoblot analysis of nuclear extracts (Fig. 4f). The kinetics of gfp-IRF5 translocation in either cell type correlated well with phosphorylation data, revealing that IRF-5 is activated by TRAIL.

IRF-5 regulates DR signaling machinery—Data from immunoblot (Fig. 3) indicated that IRF-5 is acting at a step early in the initiation of DR signaling. A reduction in protein levels of critical DISC components DR5, caspase 8, and FADD was shown in conjunction with a reduction of IRF-5 (Fig. 3b), suggesting that IRF-5 may contribute to their regulation. In addition, TNFRF10B(DR5), caspase 8, and TRAIL were identified by microarray as potential IRF-5 target genes. The DR5 (GenBank™ accession number AB054004) and caspase 8 (GenBank™ accession number MYD88) gene promoters have recently been characterized; FADD has not. The caspase 8 promoter contains functional IRF-E/ISRE-binding sites (29); DR5 contains p53 and NF-κB sites (5). A computer-based analysis of the DR5 promoter with TRANSFAC® identified two potential IRF-E/ISRE sites residing close together. We also examined the TRAIL promoter (GenBank™ accession number AF178756) because it contains functional ISRE/IRF-E sites (13, 14). To examine the role of IRF-5 in DR5, caspase 8 and TRAIL gene transcription, we conducted promoter reporter assays. The data in Fig. 5a show that IRF-5 can transactivate each promoter, albeit to different extents. Treatment with TRAIL further enhanced IRF-5 transactivation of DR5 and caspase 8 promoters (data not shown).

To confirm whether IRF-5 could regulate endogenous expression, we examined TRAIL, DR5, and caspase 8 transcript levels by quantitative real time RT-PCR (Fig. 5b). The cells were transiently transfected with empty vector (mock) or IRF-5 expression plasmid and treated with TRAIL. As shown in Fig. 5b, TRAIL, DR5 and caspase 8 mRNA induction was significantly enhanced by overexpression of IRF-5 and treatment with TRAIL. In p53+/− cells, overexpression alone rarely gave an increase in mRNA levels; yet combined with TRAIL, the levels were further up-regulated compared with mock transfected cells. In comparison, similar to the promoter reporter studies shown in Fig. 5a, overexpression of IRF-5 in p53−/− cells gave a significant increase in transcript levels; further up-regulation was observed after treatment with TRAIL. Similar results were obtained by semi-quantitative RT-PCR (supplemental Fig. S3).

To determine whether IRF-5 is a direct regulator of DR5, TRAIL, or caspase 8 gene transcription, we performed ChIP assays. The ability of endogenous IRF-5 to bind to putative sites in each promoter was examined after treatment with TRAIL.
Chromatin was immunoprecipitated with the indicated antibodies and analyzed by PCR using primers that amplify each putative site (see “Experimental Procedures” and supplemental Fig. S1). As a positive control, NF-κB p65 antibodies were used to immunoprecipitate the DR5 promoter (5). IRF-5 antibodies were specific for the putative DR5 ISRE/IRF-E binding site but not the NF-κB site, indicating specificity for the antibodies and IRF-5 binding (Fig. 5c). IRF-5 bound to only one of the putative ISRE sites in the TRAIL promoter, ISRE1 and not ISRE2. We also detected binding to the ISRE site in the caspase 8 promoter. No amplification was observed from any primer set in nonimmune IgG controls. Together, these results support an important role for IRF-5 in the transcriptional regulation of DR5, TRAIL, and caspase 8 by its recruitment to ISRE/IRF-E sites in each promoter.

**IRF-5-deficient Cells Are Insensitive to TRAIL.**—To unambiguously determine whether IRF-5 is an essential mediator of TRAIL-induced DR signaling, we examined MEFs from IRF-5−/− mice (20). wt MEFs showed a time- and dose-dependent increase in the number of cells undergoing apoptosis by TRAIL (Fig. 6a). IRF-5−/− MEFs did not confer sensitivity to TRAIL. Multiple passages of primary IRF-5−/− MEFs were examined (from passages 2 to 8) for their sensitivity to TRAIL; all were resistant compared with wt. Complementation of the null MEFs with murine HA-IRF-5 resensitized cells to TRAIL (Fig. 6b); similar to levels observed in wt MEFs (Fig. 6a). Although MEFs express functional murine death receptors (mDR5), they are not very sensitive to TRAIL-induced apoptosis. It has been suggested that mDR5 may be susceptible to proteasome degradation, as was observed in the ability of E1A to stabilize DR5 through inhibition of the proteasome (30). To address this, we examined apoptosis in MEFs treated with the proteasome inhibitor MG132. Enhanced sensiti-
As expected, after 3 h of TRAIL treatment, DR5, FADD, and p53 remained sensitive, indicating that a deficiency in IRF-5 and not DR5 expression or stability was responsible for the observed effects (Fig. 6c).

**DISCUSSION**

The IRF family is well known for its contribution to the regulation of immunity and oncogenesis (12). Although there are some overlapping functions, each family member appears to have distinct cellular activities that are dependent on cell type-specific expression, cellular stimuli, and interacting partners. Human IRF-5 was initially characterized for its role in virus-induced type I IFN signaling (21). Subsequent studies showed that it plays a critical role in the overall pathogen-induced immune response as a key mediator of Toll-like receptor signaling (20, 22). IRF-5 is critical for the induction of interleukin-6, interleukin-12, TNFα, and type I IFN depending on cell type (17, 20, 21, 31). Data from multiple laboratories indicate that another important role for IRF-5 is in cell growth regulation and apoptosis (9, 10, 16, 17). IRF-5 is regulated by type I and II IFNs and is therefore a candidate mediator of IFN-induced apoptosis (16, 31).

Even though IRF-5 is a direct target of p53 (10), its cell cycle regulatory and pro-apoptotic effects are p53-independent (9, 16). These results were recently confirmed in IRF-5−/− mice, supporting our earlier finding that IRF-5 acts on a pathway that is distinct from p53 (9, 16, 17). A molecular mechanism for IRF-5 action is its ability to regulate pro- and anti-apoptotic genes. Caspase 8 and DR5, both critical for initiating DR signaling, were repeatedly identified by microarray analysis as potential IRF-5 target genes, suggesting a new role for IRF-5 in DR signaling (9, 16, 18).

An interesting link between DR and IFN signaling has recently emerged in the literature suggesting cross-talk between these two pathways (1, 6, 12). TRAIL appears to play a role in the early phases of IFN-dependent host defense against viral infection through its regulation by IFNs (1). FADD is an essential mediator of type I IFN induction and other genes important for host defense (32). IRF-1 and -3 have been implicated as potential mediators of IFN-induced TRAIL expression through binding to ISRE in the promoter (13, 14). Although it has been observed for some time that type I and II IFNs can sensitize tumor cells to TRAIL, the detailed mechanism(s) and the mediators responsible remain to be characterized.

In this report, we investigated the role of IRF-5 in DR signaling. Cancer cell apoptosis in response to TRAIL ligand requires IRF-5. This was confirmed in other immortalized cell types such as BJAB, Jurkat, and A549 (supplemental Fig. S2). We find that IRF-5 acts at a step upstream of caspase 8 activation (Figs. 1 and 3). Abolition of IRF-5 by siRNA/shRNA inhibited TRAIL-induced caspase 8 cleavage (Fig. 1, c and d), resulting in the subsequent inhibition of apoptosis and cell death (Fig. 2). A similar decrease in caspase 8 cleavage was observed in MEFs from IRF-5−/− mice compared with wt cells (supplemental Fig. S4). A closer examination of upstream DISC components, DR5 and FADD, indicated that IRF-5 may be involved in their regulation, as shown by the distinct ablation of protein by IRF-5 deficiency. Another possibility is that IRF-5 serves as a scaffold- or interacting partner upstream of caspase 8, possibly playing a role in formation of the DISC complex. To this end, we examined DISC formation in untreated and treated cells by immunoprecipitation of whole cell lysates with anti-His or anti-FLAG antibodies recognizing the tagged recombinant TRAIL. As expected, after 3 h of TRAIL treatment, DR5, FADD, and
cleaved caspase 8 were bound in a complex. To our surprise, we also pulled down IRF-5 (data not shown). This interaction was specific to IRF-5 because IRF-1, -3, or -7 was not detected. Unfortunately, when we tried to confirm this by purifying the DISC complex, as described (33), we no longer observed interaction with IRF-5. This may be a result of IRF-5 interacting instead with the secondary complex involved in TRAIL-mediated kinase activation (11). The secondary complex retains the primary DISC components FADD and caspase 8 and recruits RIP1, TRAF2, and NEMO/IKK (11). The exact contribution of IRF-5 to TRAIL-induced DISC formation requires further attention. Current data suggest another potential mechanism involving cFLIP, which blocks DR-induced apoptosis by inhibiting caspase 8 activation at the DISC. A lack of IRF-5 expression actually up-regulated cFLIP levels, suggesting that IRF-5 may regulate cFLIP in some manner or regulate an inhibitor of cFLIP. One determinant of IRF-5 expression actually up-regulated cFLIP levels, suggesting that IRF-5 may regulate cFLIP in some manner or regulate an inhibitor of cFLIP. One determinant of IRF-5 up-regulation is the direct repression of FLIP by c-Myc (34); however, IRF-5 siRNA had little effect on c-Myc protein levels (Fig. 3b). Even though the FLIP promoter contains an ISRE1, we did not detect binding to or transactivation by IRF-5 (data not shown). We did, however, observe transactivation of the TRAIL, DR5, and caspase 8 promoters by IRF-5; overexpression up-regulated endogenous transcript levels (Fig. 5, a and b, and supplemental Fig. S3). These data independently confirm results from our microarray analysis pointing to TRAIL, DR5, and caspase 8 as new IRF-5 target genes. By ChIP assay we confirmed recruitment of IRF-5 to the promoters after treatment (Fig. 5c). Binding of IRF-5 to ISRE1 and not ISRE2 of the TRAIL promoter was distinct from IRF-3 binding, because both sites were utilized by IRF-3 after virus infection (14). These data designate IRF-5 as a new transcriptional regulator of TRAIL, DR5, and caspase 8. Further studies are required to determine the exact region in each promoter that IRF-5 binds to, along with potential interacting partners. As illustrated in supplemental Fig. S1, in addition to an ISRE/IRF-E site in each amplified promoter region, the DR5 promoter also contains multiple Ets-2 binding sites; TRAIL ISRE2 contains an Sp1 site; and caspase 8 contains an NF-κB site, all of which may contribute to IRF-5 binding to these promoters.

Although low levels of endogenous or transfected IRF-5 can be detected in the nucleus of unstimulated cells (21, 23), multiple stimuli including virus (17, 21, 23), CpG oligonucleotides (20), R848 (22), and DNA-damaging agents (16) enhance nuclear localization. Data from in vitro kinase and in vivo phosphorylation assays indicate a requirement for IRF-5 phosphorylation that precedes nuclear localization. Here, we show that treatment of cells with TRAIL induces IRF-5 phosphorylation and nuclear translocation (Fig. 4). The fact that we could pull down phosphorylated IRF-5 after TRAIL treatment with phospho-specific antibodies or IRF-5 antibodies increases the likelihood that IRF-5 itself is phosphorylated and not a TRAIL-induced phosphorylated protein that is associating with IRF-5. However, this is an interesting question that we are currently
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...mechanism of TRAIL-induced IRF-5 signaling through its (i) up-regulation of IRF-5 transcript and protein levels, (ii) induction of apoptotic activities or DR signaling, p53, or components of this pathway may play a role in IRF-5 activation (16). p53 is known to regulate DR5 expression (35). We are currently in the process of dissecting further the molecular mechanism of IRF-5 activation by TRAIL.

While this manuscript was in preparation, a paper by Couziniet al. (36) was published indicating that IRF-5 serves as a factor that sensitizes cells to Fas-induced apoptosis. Although no detailed mechanism was given, using IRF-5−/− mice, they showed that IRF-5 is involved in a stage of Fas DR signaling that precedes the activation of caspase 8. In addition, the lack of IRF-5 contributed to impaired cFLIP down-regulation. These data are similar to findings presented here using IRF-5 siRNAs in human cells and cells from IRF-5−/− mice (Figs. 2 and 3 and supplemental Fig. S4). Combined, results reveal for the first time an important role for IRF-5 in DR signaling by multiple inducers. Here, we provide insight into the molecular mechanism of TRAIL-induced IRF-5 signaling through its (i) up-regulation of IRF-5 transcript and protein levels, (ii) induction of IRF-5 phosphorylation, (iii) promotion of IRF-5 nuclear localization, and (iv) binding of IRF-5 to IRF-E/ISRE sites in the promoters of target genes. IRF-5 acts as a novel mediator of DR signaling by providing signal amplification through up-regulation by TRAIL and its subsequent up-regulation of TRAIL, DR5, and caspase 8. Examination of TRAIL-mediated apoptosis in MEFs from wt or IRF-5−/− mice further supports an important role for IRF-5 in DR signaling. Although MEFs are not extremely sensitive to human or mouse TRAIL, significant differences in sensitivity were observed at high concentrations and extended time periods. Similar results were found in FADD- or caspase 8-deficient MEFs after TRAIL treatment (37–39). The role of IRF-5 in TRAIL-mediated DR signaling was further confirmed by complementation of IRF-5-deficient MEFs with exogenous IRF-5 or treatment with the proteasome inhibitor MG132, which helps to stabilize DR5 (Fig. 6).

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REFERENCES
1. Kelley, S. K., and Ashkenazi, A. (2004) Curr. Opin. Pharmacol. 4, 333–339
2. Horak, P., Pils, D., Haller, G., Pribill, I., Roessler, M., Tomek, S., Horvat, R., Zeillinger, R., Zielinski, R., and Krainer, M. (2005) Mol. Cancer Res. 3, 335–343
3. Izeradjene, K., Douglas, L., Delaney, A., and Houghton, J. A. (2005) Oncogene 24, 2050–2058
4. Ren, Y. G., Wagner, K. W., Knee, D. A., Aza-Blanc, P., Nasoff, M., and Deveraux, Q. L. (2004) Mol. Biol. Cell 15, 5064–5074
5. Shetty, S., Graham, B. A., Brown, J. G., Hu, X., Vegh-Yarema, N., Harding, G., Paul, J. T., and Gibson, S. B. (2005) Mol. Cell. Biol. 25, 5404–5416
6. Shigeno, M., Nakao, K., Ichikawa, T., Suzuki, K., Kawakami, A., Abiru, S., Mitazoe, S., Nakagawa, Y., Ishikawa, H., Hamasaki, K., Nakata, K., Ishii, N., and Eguchi, K. (2003) Oncogene 22, 1653–1662
7. Yoshida, T., Maeda, A., Tani, N., and Sakai, T. (2001) FEBS Lett. 507, 381–385
8. Sato, M., Taniguchi, T., and Tanaka, N. (2001) Cytokine Growth Factor Rev. 12, 133–142
9. Barnes, B. J., Kellum, M. J., Pinder, K. E., Frisancho, J. A., and Pitha, P. M. (2003) Cancer Res. 63, 6424–6431
10. Mori, T., Anazawa, Y., Iizumi, M., Fukuda, S., Nakamura, Y., and Arakawa H. (2002) Oncogene 21, 2914–2918
11. Varfolomeev, E., Macek, H., Sharp, D., Lawrence, D., Renz, M., Vucic, D., and Ashkenazi, A. (2005) J. Biol. Chem. 280, 40599–40608
12. Sato, K., Hida, S., Takayanagi, H., Yokochi, T., Kayagaki, N., Takeda, K., Yagita, H., Okumura, K., Tanaka, N., Taniguchi, T., and Ogasawara, K. (2001) Eur. J. Immunol. 31, 3138–3146
13. Clarke, N., Jimenez-Lara, A. M.,Voltz, E., and Gronemeyer, H. (2004) EMBO J. 23, 3051–3060
14. Kirshner, J. R., Karpova, A. Y., Kops, M., and Howley, P. M. (2005) J. Virol. 79, 9320–9324
15. Stang, M. T., Armstrong, M. J., Watson, G. A., Sung, K. Y., Liu, Y., Ren, B., and Yim, J. H. (2007) Oncogene 24, 6420–6430
16. Hu, G., Manci, M. E., and Barnes, B. J. (2005) Cancer Res. 65, 7403–7412
17. Yanai, H., Chen, H. M., Inuzuka, T., Kondo, S., Mak, T. W., Takaoka, A., Honda, K., and Taniguchi, T. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 3402–3407
18. Barnes, B. J., Richards, J., Manci, M., Hanash, S., Beretta, L., and Pitha, P. M. (2004) J. Biol. Chem. 279, 45194–45207
19. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Science 282, 1497–1501
20. Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T. W., and Taniguchi, T. (2005) Nature 434, 243–249
21. Barnes, B. J., Moore, P. A., and Pitha, P. M. (2001) J. Biol. Chem. 276, 23382–23390
22. Schoenemeyer, A., Barnes, B. J., Manci, M. E., Latz, E., Goutagny, N., Pitha, P. M., Fitzgerald, K. A., and Golenbock, D. T. (2005) J. Biol. Chem. 280, 17005–17012
23. Barnes, B. J., Kellum, M. J., Field, A. E., and Pitha, P. M. (2002) Mol. Cell. Biol. 22, 5721–5740
24. van Valen, F., Fulda, S., Schafer, K., Truchenebrot, B., Hotzfeld, M., Poremba, C., Debatin, K. M., and Winkelmann, W. (2003) Int. J. Cancer 107, 929–940
25. Yang, P., Pears, J. I., Tano, R., Zhang, N., Tyrell, J., and Jaffe, G. I. (2007) Invest. Ophthalmo. Vis. Sci. 48, 3341–3349
26. Korz, C., Pscherer, A., Benner, A., Mertens, D., Schaffner, C., Leupolt, E., Dohner, H., Stilgenbauer, S., and Lichter, P. (2002) Blood 99, 4554–4561
27. Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shibue, T., Honda, K., and Taniguchi, T. (2003) Nature 424, 516–523
28. Liu, R., Yang, L., Arguello, M., Penafuerte, C., and Hiscott, J. (2005) J. Biol. Chem. 280, 3088–3095
29. Casciano, I., De Ambrosio, A., Croce, M., Pagnan, G., Di Vincenzo, A., Allemani, G., Bannelli, B., Ponzoni, M., Romani, M., and Ferrari, S. (2004) Cell Death Differ. 11, 131–134
30. Finnberg, N., Gruber, J. I., Fei, P., Rudolph, D., Bric, A., Kim, S. H., Burns, T. F., Ajuba, H., Page, R., Wu, G. S., Chen, Y., McKenna, W. G., Bernhard, E., Lowe, S., Mak, T., and El-Diery, W. S. (2005) Mol. Cell. Biol. 25, 2000–2013
31. Manci, M. E., Hu, G., Sangster-Guity, N., Olshalsky, S. L., Hoops, K., Fitzgerald-Bocarsly, P., Pitha, P. M., Pinder, K., and Barnes, B. J. (2005) J. Biol. Chem. 280, 21078–21090
32. Balachandran, S., Thomas, E., and Barber, G. N. (2004) Nature 432,
33. Suliman, A., Lam, A., Datta, R., and Srivastava, R. K. (2001) Oncogene 20, 2122–2133
34. Ricci, M. S., Jin, Z., Dews, M., Yu, D., Thomas-Tikhonenko, A., Dicker, D. T., and El-Diery, W. S. (2004) Mol. Cell. Biol. 24, 8541–8555
35. Takimoto, R., and El-Diery, W. S. (2000) Oncogene 19, 1735–1743
36. Couzinet, A., Tamura, K., Chen, H., Nishimura, K., Wang, Z., Morishita, Y., Takeda, K., Yagita, H., Yanai, H., Taniguchi, T., and Tamura, T. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 2556–2561
37. Kuang, A. A., Diehl, G. E., Zhang, J., and Winoto, A. (2000) J. Biol. Chem. 275, 25065–25068
38. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, F., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Groncharov, T., Holtmann, H., Lonai, P., and Wallach, D. (1998) Immunity 9, 267–276
39. Yeh, W. C., Pompa, J. L., McCurrach, M. E., Shu, H. B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Diery, W. S., Lowe, S. W., Goeddel, D. V., and Mak, T. W. (1998) Science 279, 1954–1958