

# Stat1-dependent Induction of Tumor Necrosis Factor-related Apoptosis-inducing Ligand and the Cell-Surface Death Signaling Pathway by Interferon $\beta$ in Human Cancer Cells<sup>1</sup>

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## ABSTRACT

Type I IFNs are known to inhibit tumor cell growth and stimulate the immune system. However, little is known of the mechanism of type I IFN-induced apoptosis in human cancer cells. In this study, we have IFN- $\beta$  treatment of a human colorectal cell line (KM12L4) and a resistant clone of this cell line, L4RIFN. We demonstrate the induction of apoptosis in the parent cell line. This process was associated with the induction of the Jak-Stat signaling pathway, induction of the proapoptotic mediator tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and activation of procaspase-3, -8, -9, and -10. Additionally, we evaluated the role of Stat1 in mediating IFN- $\beta$  induction of these proapoptotic signals in a fibrosarcoma cell line (2ftgh) and a Stat1-deficient clone (U3A). Our results demonstrate that IFN- $\beta$  induction of apoptosis and the induction of proapoptotic mediator TRAIL is Stat1 dependent. Evaluation of a stable transfectant of the KM12L4 cell line expressing c-FLIP supports the role of TRAIL and the cell-surface death signaling pathways in IFN- $\beta$  induction of apoptosis. Studies evaluating the TRAIL promoter indicate induction of TRAIL promoter activity by IFN- $\beta$ . These results may represent a novel pathway by which IFN- $\beta$  may induce therapeutic effects.

## INTRODUCTION

Colorectal cancer affects 134,000 patients yearly and is the second leading cause of cancer death in the United States, accounting for over 54,000 deaths annually (1). Many patients present with metastatic disease and an even greater number subsequently develop metastases. For patients with metastases from colorectal cancer, prognosis and survival vary with the extent of disease and performance status. Systemic chemotherapy offers potential palliation, with minimal to no chance of cure for these patients. Novel strategies are required to address this significant clinical problem (2).

Type I IFNs, IFN- $\alpha$  and IFN- $\beta$ , are known to inhibit tumor cell growth and stimulate the immune system (3). Type I IFNs exert their response via a common receptor in which interaction with this receptor results in phosphorylation of receptor-associated tyrosine kinases (Jak1 and Tyk2), which in turn phosphorylate and activate a family of proteins described as signal transducers and activators of transcription (STAT; Ref. 3). Many investigators have demonstrated significant growth inhibition by IFN- $\beta$  of various cancers, including colorectal cancer (4–8). Whereas little is known of the mechanism of type I IFN-induced apoptosis in human cancer cells, recent reports have

demonstrated the induction of TRAIL<sup>4</sup> and/or the death receptor apoptotic pathway in human T cells, monocytes, macrophages, and virally infected cells (9–11).

These observations led us to postulate that the death receptor pathway and TRAIL may play a pivotal role in IFN- $\beta$ -induced apoptosis in human cancers. We have previously reported the induction of TRAIL after adenovirus-mediated IFN- $\beta$  gene therapy (12). In the present study, we have evaluated recombinant IFN- $\beta$  treatment of human colorectal cancer cells and subsequent induction of apoptosis. This process is associated with the induction of the Jak-Stat signal pathway, activation of procaspase-3, -8, -9, and -10 and the induction of TRAIL. To evaluate the role of Stat1 in mediating the IFN- $\beta$  induction of apoptosis, we evaluated a fibrosarcoma cell line 2ftgh and a Stat1-deficient clone of this cell line, U3A (13). IFN- $\beta$ -induction of TRAIL was observed in the sensitive 2ftgh cell line but was absent in the IFN-resistant Stat1-deficient U3A clone. Evaluation of a stable transfectant of the KM12L4 cell line overexpressing c-FLIP demonstrated an attenuation of the apoptotic response to IFN- $\beta$ , supporting the role of TRAIL and the cell-surface signaling pathways in IFN- $\beta$  induction of apoptosis. TRAIL promoter studies indicate induction of promoter activity after IFN- $\beta$  treatment. These data indicate IFN- $\beta$ -induced apoptosis, and induction of the proapoptotic mediator TRAIL are Stat1 dependent and may represent a novel pathway for IFN- $\beta$  therapeutic effects.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** Human colorectal adenocarcinoma cell lines SW620 and COLO205 were obtained from our laboratory or were purchased from the American Tissue Culture Collection. The KM12L4 cell line was kindly provided by Dr. I. Fidler (M. D. Anderson Cancer Center, Houston, TX). Dr. George Stark (The Lerner Research Institute, The Cleveland Foundation, Cleveland, OH) provided the human fibrosarcoma cell line 2ftgh and the Stat1 knockout derivative of 2ftgh, U3A. These cell lines were maintained in MEM or RPMI 1640 containing 10% FBS and antibiotics. The IFN- $\beta$ -resistant KM12L4 cells (L4RIFN) were maintained in 10% FBS supplemented with 2000 units/ml recombinant IFN- $\beta$  (Biogen, Inc., Cambridge, MA). Cells were harvested by removing the growth medium, washing with NaCl solution, and briefly digesting the cells with 0.25% trypsin. Complete serum was added to the digested cells, which were then pelleted at 1000 rpm in a Beckman centrifuge at 4°C and resuspended in full medium to an appropriate concentration for *in vitro* cell counts, PhiPhiLux G<sub>1</sub>D<sub>2</sub> kit (Onco-immunin, Inc., Gaithersburg, MD), and *in situ* fluorescent TUNEL staining (Roche Diagnostics, Indianapolis, IN).

**Generation of Resistant KM12L4 (L4RIFN).** KM12L4 cells were treated and grown in medium initially supplemented with 300 units/ml recombinant IFN- $\beta$  in 75 mm<sup>3</sup> flasks. Cells were harvested and passed at least once every 7 days. When surviving cells grew confluent at a certain IFN- $\beta$  concentration,

Received 3/18/03; revised 5/30/03; accepted 6/2/03.

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<sup>1</sup> Supported in part by a grant from the American Cancer Society (to F. R. S.) and by NIH Grant 1-R01-CA-090919-01A2 (to F. R. S.).

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<sup>4</sup> The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase nick end labeling; RPA, ribonuclease protection assay; FBS, fetal bovine serum; ISRE, IFN stimulation response element; ISG, IFN-stimulated gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

resistant cells were treated and grown in medium with increasingly higher IFN- $\beta$  concentrations (500, 750, 1000, 1250, and 2000 units/ml) for at least 2–3 weeks. At 2000 units/ml concentration of IFN- $\beta$ , the resistant KM12L4 cells were harvested and expanded.

**Generation and Verification of c-FLIP-expressing Clones of the KM12L4 Cell Line.** For the creation of the stable clone of the KM12L4 cell line expressing c-FLIP (KM12FLIP) along with the control plasmid clone (KM12pcDNA), the minimum concentration of G418 required to kill the KM12L4 cells was determined at 800  $\mu\text{g/ml}$  by substitution of culture medium with medium containing various concentrations of G418 (0, 50, 100, 200, 400, 600, 800  $\mu\text{g/ml}$ ). The plasmids of pcDNA3.1-c-FLIP (short form; kindly provided by Dr. Wafik S. El-Deiry) and pcDNA3.1 vector were linearized, and 5  $\mu\text{g}$  of plasmid DNA was transfected into KM12L4 cells plated in 60-mm culture dishes using Superfect Reagent (Qiagen). Forty-eight h after transfection, the cells were split and grown in G418 at 800  $\mu\text{g/ml}$ . The cells were fed every 3–4 days until G418-resistant colonies were formed. Twenty single colonies were picked and expanded through several passages. To verify the colonies, PCR technique was used. The genomic DNA were isolated with the reagents of G NOME (BIO 101), and 1  $\mu\text{g}$  of the genomic DNA was used in PCR with the specific primers. The primer pairs were used for c-FLIP: 5'-CAGATGAGAAGGAGATGCTGC-3' and 5'-CTCAACCACAAGGTC-CAAGAA-3'. The primer pairs of neomycin were used to confirm the pcDNA3.1 vector: 5'-ATGATTGAACAAGATGGATTGCACG-3' and 5'-TCAGAAGAACTCGTCAAGAAGCGGA-3'. The plasmid DNA of c-FLIP were used as positive control. The cell lines containing c-FLIP plasmids confirmed by PCR were picked and expanded to use for *in vitro* cell count and to provide cell extract for Western blotting, as described in the section below.

**In Vitro Cell Count.** The KM12L4 and L4RIFN cells were harvested as described above, plated at a density of  $2 \times 10^5$  cells/well/2 ml of complete medium, and cultured at 37°C and 5% CO<sub>2</sub> overnight. Twenty-four h later, the cells were treated with fresh medium with or without recombinant IFN- $\beta$  (2000 units/ml) or recombinant TRAIL (Biomol Research Laboratories, Plymouth Meeting, PA) and were cultured for 48 h. The cell numbers were determined by hemocytometer using trypan blue exclusion method in triplicate wells.

**Cell Death Detection ELISA.** KM12L4 and L4RIFN were plated into a 96-well plate,  $4 \times 10^4$  cells/100  $\mu\text{l}$  of complete medium/well in triplicate. The plate was cultured overnight in a 37°C incubator with 5% CO<sub>2</sub>. Medium was removed by suction and was replaced with fresh medium with or without recombinant IFN- $\beta$  (2000 units/ml); cells were cultured for an additional 48 h. Cell apoptosis was measured with a commercially available ELISA kit (Roche) that detects DNA-histone fragments according to the provided protocol. The 96-well plates were centrifuged at  $200 \times g$  for 10 min. The medium was removed by suction carefully to avoid disrupting the cell pellet. After lysis of the cells, the microplate was again centrifuged. The supernatant was then incubated with anti-DNA and anti-histone antibodies in a streptavidin-coated 96-well plate for 2 h. A chromogenic substrate was added to each well and was incubated until color development. The absorbance was then measured at a wavelength of 405 nm on a spectrophotometer, and induction of apoptosis was calculated by dividing the  $A_{405 \text{ nm}}$  from the treated cells by the  $A_{405 \text{ nm}}$  from saline control cells.

**In Situ TUNEL Staining.** KM12L4 and L4RIFN were plated in chamber slides at a density of  $5 \times 10^5$  cells/2 ml of complete medium/well in triplicate. The plate was cultured overnight in a 37°C incubator with 5% CO<sub>2</sub>. Medium was removed by suction and replaced with fresh medium with or without recombinant IFN- $\beta$  (2000 units/ml); cells were cultured for an additional 48 h. The chamber slides were then fixed in 4% paraformaldehyde in PBS without calcium for 1 h at room temperature. The slides were also permeabilized with a buffer containing 0.1% Triton X-100 (Sigma, St. Louis, MO) and 0.1% sodium citrate for 2 min on ice. The slides were then incubated in 20 mg/ml of Proteinase K (Sigma) in 10 mM Tris-HCl buffer (pH 7.0) for 15 min at room temperature. Fifty  $\mu\text{l}$  of the TUNEL (Roche) were placed on the slides, and the slides were covered with strips of parafilm and were incubated at 37°C for 2 h. Samples were then analyzed with an Olympus IX70 fluorescence microscope with an excitation wavelength of 488 nm.

**Caspase-3 Activity by Flow Cytometry.** Caspase-3 activity, as a marker of apoptosis, was measured using the PhiPhiLux G<sub>1</sub>D<sub>2</sub> kit (Oncoimmunin, Inc., Gaithersburg, MD) by flow cytometry according to manufacturer's recommendations. Briefly, 48 h after treatment, cells were incubated with 50  $\mu\text{l}$  of

PhiPhiLux substrate supplemented with 10% FBS in CO<sub>2</sub> incubator at 37°C for 60 min after the cells were harvested, and then were washed with flow cytometry dilution buffer provided in the kit and analyzed by flow cytometry within 60 min.

**Western Blot Analysis.** KM12L4 cells treated with IFN- $\beta$  for 48 h were lysed in radioimmunoprecipitation assay buffer [10 mM Tris (pH 7.4), 1% NP40, 0.1% SDS, 0.1% deoxycholic acid, 150 mM NaCl, 1 mM EDTA, and 10  $\mu\text{g/ml}$  aprotinin]. Equal amounts of total protein (50  $\mu\text{g/lane}$ ) were separated by SDS PAGE and then were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS (50 mM Tris, 150 mM NaCl) and then were incubated with specific antibodies. After incubation, the membranes were washed four times for 10 min each in TBS/0.1% Tween 20 and then were incubated with horseradish peroxidase-conjugated affinity-purified goat antimouse or goat antirabbit IgG (BMB) for 2 h. Membranes were then washed 5 times, and the antigen-antibody complexes bound to the membranes were detected using the enhanced chemiluminescence (ECL) reagent system (Amersham Pharmacia, Piscataway, NJ). Mouse antihuman Stat1, Stat2, and ISGF3 $\gamma$  (p48) antibodies (Transduction Laboratories, Franklin Lakes, NJ) were used to label the protein blots. In addition, antibodies against caspase-3, caspase-7, caspase-8, caspase-9, and caspase-10 (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA) were also used. TRAIL protein expression was detected with a polyclonal anti-TRAIL rabbit antibody (ProSci, Poway, CA). c-FLIP protein expression was detected with a monoclonal antibody (Dave-2; Alexis, San Diego, CA) Equal loading of protein was determined by labeling with a goat polyclonal human  $\beta$ -actin antibody (Santa Cruz Biotechnology).

**Caspase-Inhibition Assays Using Recombinant Human IFN- $\beta$ .** A general caspase inhibitor (Z-VAD-FMK) was obtained from Calbiochem (La Jolla, CA). The inhibitor was dissolved in DMSO to make stock solutions of 50 mM and was stored at -20°C until use. Cells were seeded into 96-well plates in 100  $\mu\text{l}$  of full growth media at  $4 \times 10^4$  cells/well. At 24 h, media was removed by suction and replaced with 100  $\mu\text{l}$  of full-growth medium containing 1000 units/ml recombinant (r)IFN- $\beta$  with caspase inhibitor at molar concentrations of 100  $\mu\text{M}$ , 50  $\mu\text{M}$ , 10  $\mu\text{M}$ , or 1  $\mu\text{M}$  (appropriate concentrations of DMSO alone are added to control groups). Twenty-four h later, medium was removed and again replaced with full-growth medium containing 1000 units/ml rIFN- $\beta$  with identical various concentrations of individual caspase inhibitors. After an additional twenty-four h, the cell-death detection ELISA was performed in triplicate for each inhibitor concentration according to the protocol above.

Cells were seeded in two-chamber slides in 2 ml of full growth media at  $5 \times 10^5$  cells/chamber. At 24 h, media was removed by suction and replaced with full-growth medium containing 1000 units/ml rIFN- $\beta$  with individual caspase inhibitors at molar concentrations of 100  $\mu\text{M}$ . Twenty-four h later, medium was removed and again replaced with full-growth medium containing 1000 units/ml rIFN- $\beta$  with identical various concentrations of individual caspase inhibitors. After an additional twenty-four h, the *in situ* TUNEL staining protocol outlined above was used to stain duplicate samples.

**RPA.** RiboQuant multi-Probe RNase Protection Assay (PharMingen, Franklin Lakes, NJ) was used for detecting and quantifying RNA transcripts for apoptosis-related molecules. Human apoptosis-related multiprobe template set hAPO-3c, containing the mRNA of genes involved the apoptosis receptor signal transduction, was used. <sup>32</sup>P-labeled antisense RNA probes were synthesized by *in vitro* transcription and were extracted twice by phenol-chloroform. The purified multiriboprobes were hybridized with 12  $\mu\text{g}$  of vacuum-dried total RNA isolated from KM12L4, L4RIFN, SW620, COLO205, 2fgh, and U3A cells with or without IFN- $\beta$  treatment (2000 units/ml). After hybridization for 16 h at 56°C, the mixtures were subjected to RNase digestion and Proteinase K treatment followed by phenol-chloroform extraction. The resulting protected probe and undigested template probes were resolved in 5% urea-denaturing polyacrylamide gel. The gels were dried under vacuum for 2 h at 80°C and were visualized by autoradiography. With the undigested probes as markers, a standard curve was plotted on a semi-log scale of migration distance *versus* log nucleotide length. This curve was used to establish the identity of RNase-protected bands in the samples. To evaluate the levels of expression of individual mRNA in different target RNA (PBS and IFN- $\beta$ ), the genes of interest were analyzed and quantified by UN-SCAN-IT digitizing software (Silk Scientific, Inc., Orem, UT).

**Northern Blotting Analysis and Preparation of Probes.** Thirty  $\mu\text{g}$  of total RNA isolated from KM12L4, L4RIFN, SW620, and COLO205 cells as well as 2ftgh and the U3A cell lines were size-fractionated in 1% agarose/formaldehyde gels and transferred to Hybond-N<sup>+</sup> nylon membranes (Amersham Life Science) by standard procedures. The membranes were cross-linked and air-dried for 2 h and then were prehybridized in hybridization buffer (50% formamide, 2% SDS, 100  $\mu\text{g}/\text{ml}$  freshly denatured salmon sperm DNA, 6 $\times$  SSC, and 5 $\times$  Denhardt's solution) for 2 h at 42°C, followed by hybridization for 18 h with random labeled [ $\alpha$ -<sup>32</sup>P]-cDNA probes at 2  $\times$  10<sup>6</sup> cpm/ml. The 600-bp cDNA fragment of hTRAIL and 983-bp cDNA fragment of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were constructed in our laboratory and were used as probes. After hybridization, the blots were progressively washed with buffers containing 2 $\times$  SSC, 0.1% SDS (2  $\times$  10 min at room temperature), 1 $\times$  SSC, 0.1% SDS (2  $\times$  30 min at 55°C), and 0.1 $\times$  SSC, 0.1% SDS (2  $\times$  20 min at 55°C). Bands were visualized by autoradiography. To establish equal loading, the blots labeled with the specific probes were stripped and relabeled with the GAPDH probe.

**Promoter Activity Assay.** KM12L4 or Km12L4RIFN cells were plated in 24-well plates 1 day before transfection. One  $\mu\text{g}$  of the hTRAIL promoter construct containing the  $\sim$ 1.5-kb segment 5' upstream of the start codon (1523 pGL2m) was cotransfected with 0.01  $\mu\text{g}$  of pRL-SV40 plasmid using Superfect Reagent (Qiagen). Cells were then treated with 1000 units/ml of IFN- $\beta$  or PBS. Twenty-four h after treatment, cells were extracted with 100  $\mu\text{l}$  of passive lysis buffer (PROMEGA) and firefly and renilla luciferase activity was measured using the Dual-Luciferase reporter Assay System.

## RESULTS

**Recombinant IFN- $\beta$  Treatment of a Colorectal Cancer Cell Line Is Associated with Induction of Apoptosis, Activation of the ISGF3 Complex Components, and Induction of TRAIL.** The colorectal cancer cell line KM12L4 was treated with recombinant IFN- $\beta$  (0–2000 units/ml). Treatment resulted in significant cell death and was associated with the induction of apoptosis as measured by *in situ* TUNEL staining, and flow cytometric analysis of caspase-3 activity (Fig. 1). An IFN- $\beta$  resistant clone of the KM12L4 cell line was developed by serially exposing the cells to increasing concentrations of IFN- $\beta$ . The  $IC_{50}$  of this IFN- $\beta$  resistant clone (L4RIFN) is 7609 units/ml as compared with 690 units/ml of the parent cell line. As demonstrated in Fig. 1, this clone is resistant to IFN- $\beta$ -induced apoptosis as measured by *in situ* TUNEL and by flow cytometric analysis of caspase-3 activity.

To evaluate the role of the Jak-Stat signaling pathway in IFN- $\beta$ -induced apoptosis, Western blot analysis was performed on the KM12L4 and L4RIFN cells after IFN- $\beta$  treatment. The results of the analyses of the members of the Jak-Stat signal transduction pathway are presented in Fig. 2A. Increased protein expression of the ISGF3 transcription complex members Stat1, Stat2, and ISGF3 $\gamma$  were observed after IFN- $\beta$  treatment in the parent cell line, but minimal changes were observed in the resistant cell line, indicating disruption of the IFN receptor/Jak-Stat signaling pathway as a potential mechanism of resistance in the L4RIFN cell line.

The induction of TRAIL in normal hematopoietic cells by type I IFNs led us to evaluate cell-surface receptors and ligands involved in the cell death pathway in this apoptotic process. RPA after IFN- $\beta$  treatment of the KM12L4 cells was used to evaluate the potential contribution of the process of the death receptor pathway-related molecules. As demonstrated in Fig. 2B, a significant increase in TRAIL transcript was observed after IFN- $\beta$  treatment. The induction of TRAIL message by IFN- $\beta$  was confirmed by Northern blot analysis (Fig. 2C). This significant induction of TRAIL by IFN- $\beta$  was absent in L4RIFN. Western blot analysis demonstrated a similar induction of TRAIL protein levels in the KM12L4 parent cell line after IFN- $\beta$  treatment (Fig. 2D). This IFN- $\beta$  induction of TRAIL protein levels was again absent in the resistant clone.

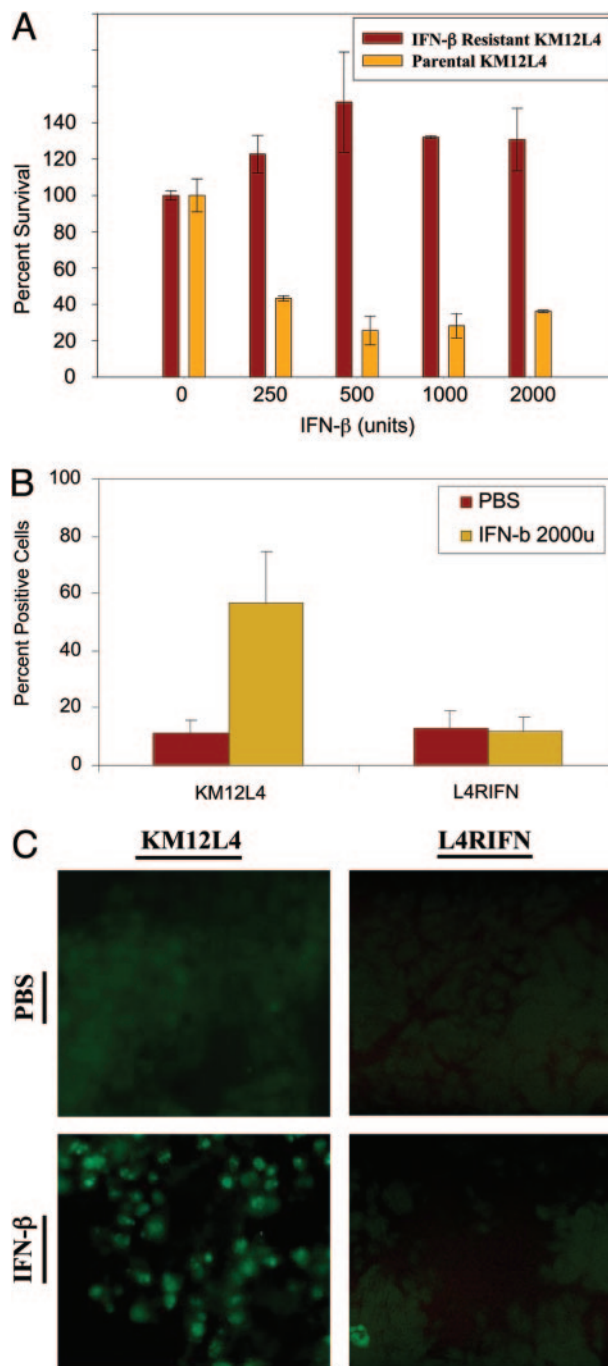


Fig. 1. IFN- $\beta$  treatment of KM12L4 cell line is associated with induction of apoptosis. A, columns, cell survival percentage compared with the PBS control groups; error bars, SD. B, apoptosis was evaluated by flow cytometric analysis of caspase-3 activity. Data presented as percentage positive cells; error bars, SD. C, apoptosis was demonstrated by *in situ* TUNEL staining and analysis with fluorescent microscopy.

**IFN- $\beta$ -induced Apoptosis Is Associated with Induction and Activation of Caspase-3, -8, and -10, and Is Inhibited by a Non-specific Caspase and by Caspase-8 Inhibitors.** With the identification of the induction of TRAIL after IFN- $\beta$  treatment, we evaluated the activation of procaspase-3, -8, -9 and -10. Procaspase-8 and -10 are associated with the activation of the death receptor pathway, whereas procaspase-9 is associated with the mitochondrial apoptotic pathway, and procaspase-3 is a downstream effector caspase and can be activated in both pathways. IFN- $\beta$  treatment was associated with cleavage and activation of all four caspases in



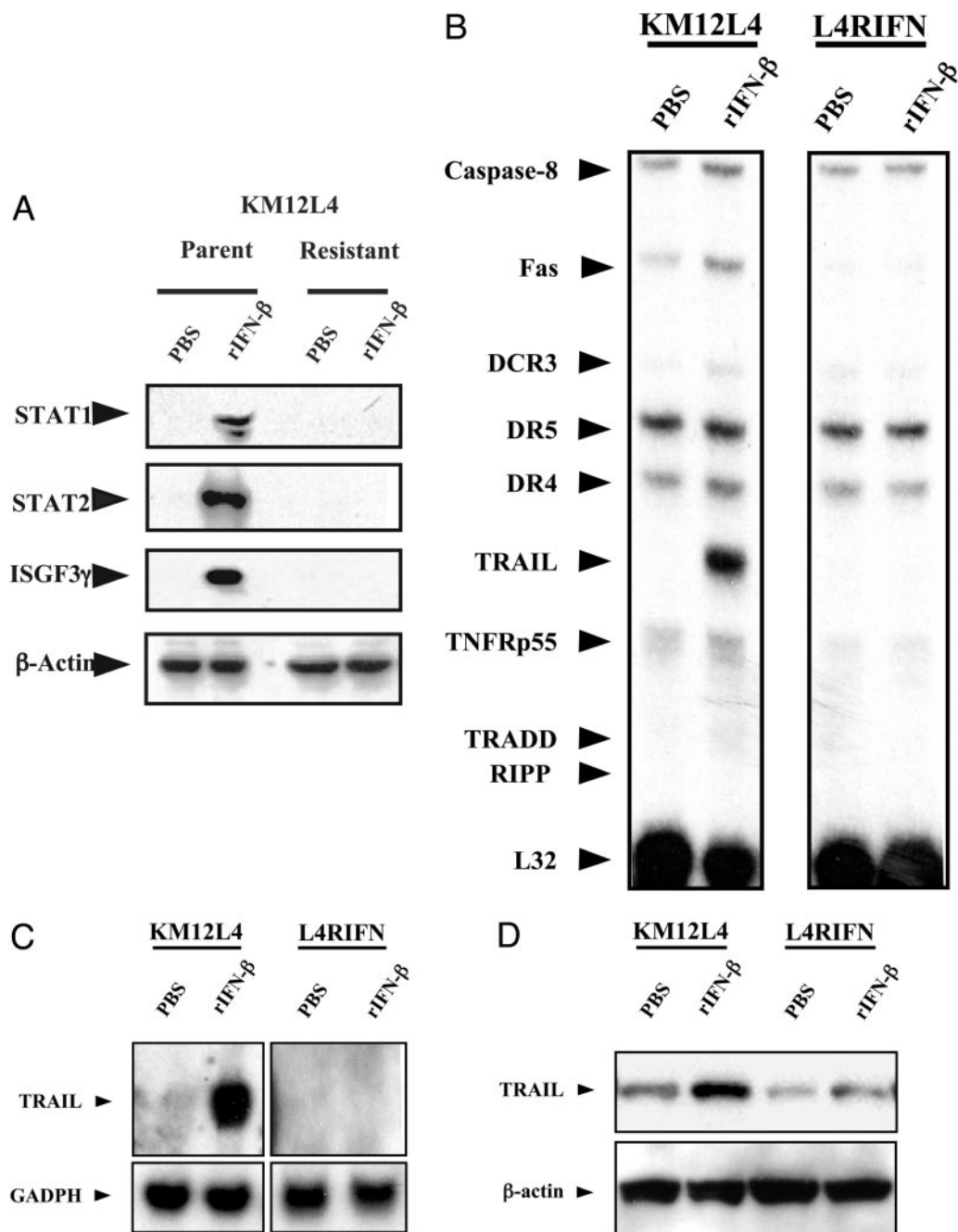


Fig. 2. IFN- $\beta$  treatment of KM12L4 cell line is associated with induction of apoptosis, the members of the ISGF-3 transcription complex, and TRAIL. **A**, proteins (50  $\mu$ g/lane) were resolved on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and assayed for Stat1, Stat2, and ISGF3 $\gamma$  protein expression. Equal loading of protein was confirmed by probing blots for  $\beta$ -actin expression. **B**, cell RNA was harvested and assayed by RPA for caspase-8, Fas, DR3, DR5, DR4, TRAIL, TNFRp55, TRADD, and RIPP mRNA expression. L32 and G<sub>3</sub>PDH mRNA expression were used as an internal control. **C**, Northern blot analysis for TRAIL (30  $\mu$ g of total RNA). Equal loading of RNA was confirmed by probing blots for G<sub>3</sub>PDH. **D**, Western blot analysis for TRAIL (50  $\mu$ g of protein/lane). Equal loading of protein was confirmed by probing blots for  $\beta$ -actin expression.

the sensitive KM12L4 cell line (Fig. 3A). As expected, no pro-caspase cleavage was observed after treatment of the IFN- $\beta$ -resistant L4RIFN cell line. We used a nonspecific caspase inhibitor to confirm the role of these caspases in the IFN- $\beta$ -induced apoptotic pathway. The cell-permeable general caspase inhibitor z-VAD-FMK blocked IFN- $\beta$ -induced apoptosis in the KM12L4 cells. The inhibition of apoptosis was dose dependent, and the effect peaked at 50  $\mu$ M as demonstrated by the apoptosis ELISA (Fig. 3B). *In situ* TUNEL staining demonstrated a qualitative decrease in apoptotic events with caspase inhibitor treatment (Fig. 3C).

**IFN- $\beta$  Treatment Induces TRAIL in Other Colorectal Cancer Cell Lines.** After demonstrating the induction of TRAIL in the sensitive colorectal cancer cell line KM12L4, we evaluated two other colorectal cell lines, SW620 and COLO205, for the induction of TRAIL after IFN- $\beta$  treatment. Both cell lines are sensitive to the cytostatic and cytotoxic effects of IFN- $\beta$  determined by the trypan blue exclusion method and apoptosis ELISA (data not shown). As demonstrated in Fig. 4, Northern analyses demonstrated a significant induction of TRAIL mRNA in both cell lines after IFN- $\beta$  treatment. These data indicate that the IFN- $\beta$  induction of TRAIL is not specific to the KM12L4 cell line.

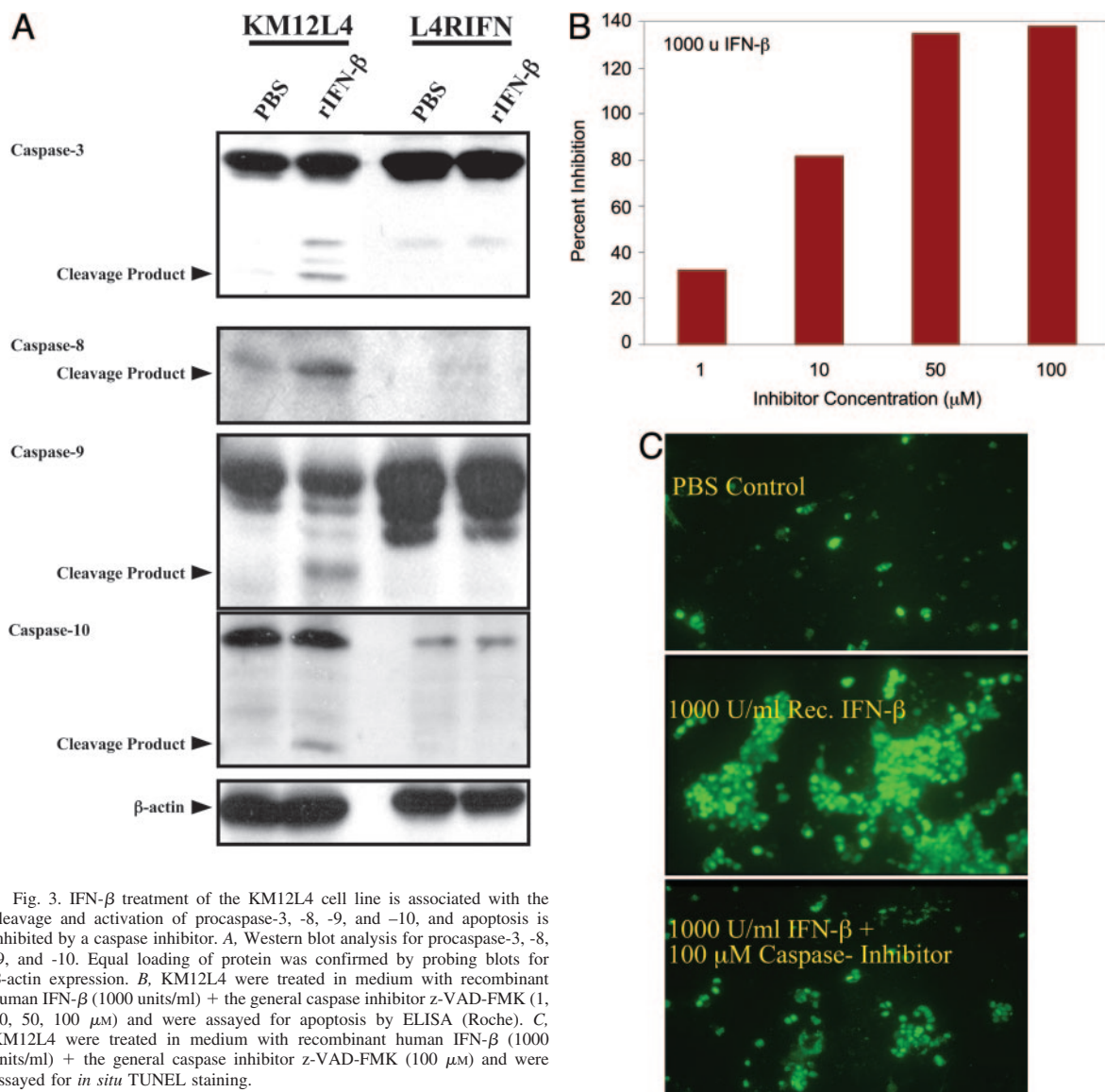


Fig. 3. IFN- $\beta$  treatment of the KM12L4 cell line is associated with the cleavage and activation of procaspase-3, -8, -9, and -10, and apoptosis is inhibited by a caspase inhibitor. *A*, Western blot analysis for procaspase-3, -8, -9, and -10. Equal loading of protein was confirmed by probing blots for  $\beta$ -actin expression. *B*, KM12L4 were treated in medium with recombinant human IFN- $\beta$  (1000 units/ml) + the general caspase inhibitor z-VAD-FMK (1, 10, 50, 100  $\mu$ M) and were assayed for apoptosis by ELISA (Roche). *C*, KM12L4 were treated in medium with recombinant human IFN- $\beta$  (1000 units/ml) + the general caspase inhibitor z-VAD-FMK (100  $\mu$ M) and were assayed for *in situ* TUNEL staining.

**IFN- $\beta$ -induced Apoptosis and Induction of TRAIL Is Dependent on Stat1.** In an effort to delineate the role of Stat1 in IFN- $\beta$ -induced apoptosis, we evaluated the response to IFN- $\beta$  in a fibrosarcoma cell line, 2ftgh, and a single deletion clone of this cell line that lacks Stat1 expression, U3A (kindly provided by Dr. George Stark).

Treatment of the parent cell line resulted in significant cell death and was associated with induction of apoptosis as measured by cell counts, *in situ* TUNEL staining, and caspase-3 activity. The Stat1-deficient U3A cell line was resistant to the cytotoxic effects of IFN- $\beta$  (Fig. 5).

To evaluate the role of Stat1 in IFN- $\beta$  induction of TRAIL, we performed RPA analyses of the 2ftgh and U3A cell lines after IFN- $\beta$  treatment. As demonstrated in Fig. 5D, an increase in TRAIL mRNA was observed after IFN- $\beta$  treatment in the parent cell line. However the Stat1-deficient U3A cells did not demonstrate a similar induction of TRAIL after IFN- $\beta$  treatment. These data indicate that IFN- $\beta$ -induction of the proapoptotic mediator TRAIL is dependent on Stat1 function.

**Overexpression of c-FLIP in the KM12L4 Cell Line Results in Attenuation of the IFN- $\beta$ -induced Cell Death.** To evaluate the contributions of the cell-surface death signaling pathway, we evaluated a stable clone of the KM12L4 cell line overexpressing c-FLIP (KM12FLIP). The generation of this clone is described in "Materials and Methods." Fig. 6A demonstrates confirmation of overexpression of c-FLIP protein. As demonstrated by cell survival analysis in Fig. 6B, the KM12FLIP clone was resistant to IFN- $\beta$ -induced apoptosis compared with a clone containing control plasmid (KM12pcDNA)

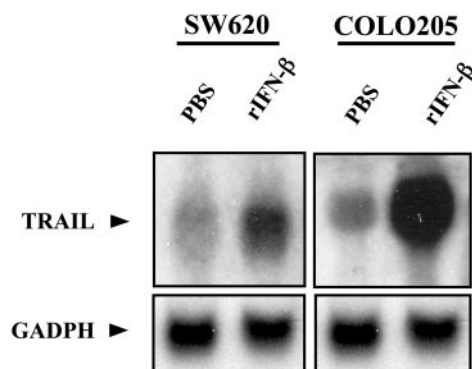


Fig. 4. IFN- $\beta$  induces TRAIL in the SW620 and COLO205 colorectal cancer cell lines. Northern blot analysis for TRAIL (30  $\mu$ g of total RNA). Equal loading of RNA was confirmed by probing blots for GAPDH.

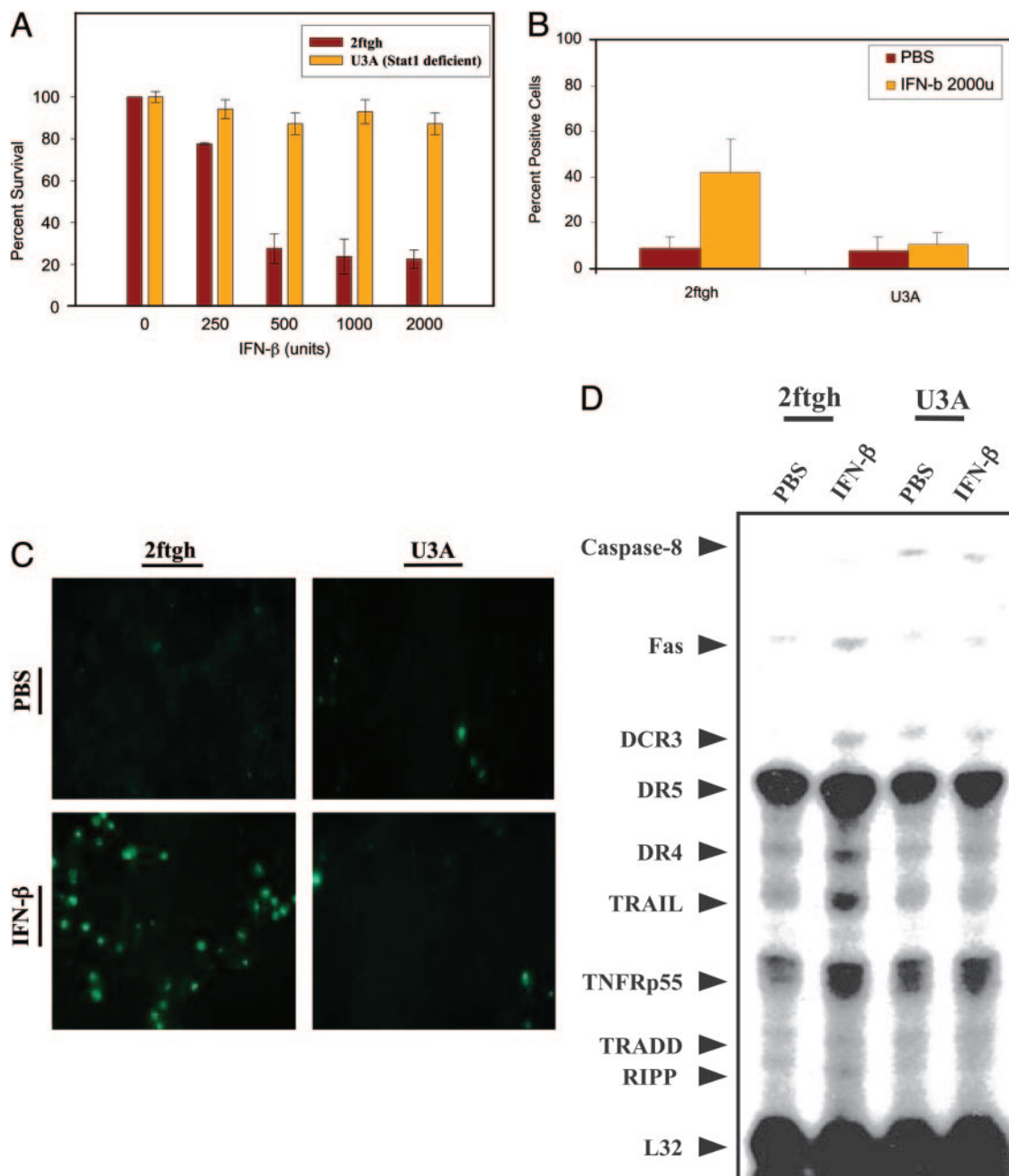


Fig. 5. IFN- $\beta$  induction of TRAIL and apoptosis is Stat1 dependent. Fibrosarcoma cell line 2ftgh and a single deletion clone of 2ftgh that lacks Stat1 expression, U3A, were treated with recombinant human IFN- $\beta$  (2000 units/ml) for 48 h and were examined. **A**, cell viability was then determined by trypan blue exclusion method. Columns, cell survival percentage compared with the PBS control groups. **B**, apoptosis was evaluated by flow cytometric analysis of caspase-3 activity. Data presented as percentage positive cells; error bars, SD. **C**, *in situ* TUNEL staining of treated cells. Apoptosis was demonstrated by *in situ* TUNEL staining and analysis with fluorescent microscopy. **D**, induction of TRAIL mRNA by RPA analysis. Cell RNA was harvested and assayed by RPA for caspase-8, Fas, DR3, DR5, DR4, TRAIL, TNFRp55, TRADD, and RIPP mRNA expression. L32 and GAPDH mRNA expression were used as an internal control.

and the parent cell line. Although c-FLIP can interfere with multiple cell-surface death ligands, we evaluated the effect of recombinant TRAIL protein on these cells. Fig. 6C demonstrates that the KM12FLIP clone was resistant to TRAIL treatment compared with the KM12pcDNA clone and the parent cell line

**IFN- $\beta$  Treatment Increases the Activity of the TRAIL Promoter.** Wang *et al.* (14) have reported the characterization of the human TRAIL promoter. This promoter encompasses the ~1.6-kb segment 5' upstream of the start codon. We have evaluated this promoter sequence with DNASTAR software and have identified a putative ISRE sequence at -655 relative to the start codon.

To determine whether IFN- $\beta$  treatment resulted in inducible activity of the TRAIL promoter, a plasmid containing the ~1.6-kb 5'-flanking region of the TRAIL gene cloned upstream of the luciferase reporter gene (1523pGL2m, kindly provided by Dr. Mark Evers, University of Texas-Galveston, Galveston, TX) was transfected into the KM12L4- and IFN- $\beta$ -resistant cell lines. Cells were then treated with rIFN- $\beta$  (1000 units) or PBS, and luciferase activity was measured. A >3-fold increase in promoter activity was observed in KM12L4 cells transfected with the 1523pGL2m plasmid after IFN- $\beta$  treatment (Fig. 7). Although basal promoter activity was similar, no significant induction of promoter activity

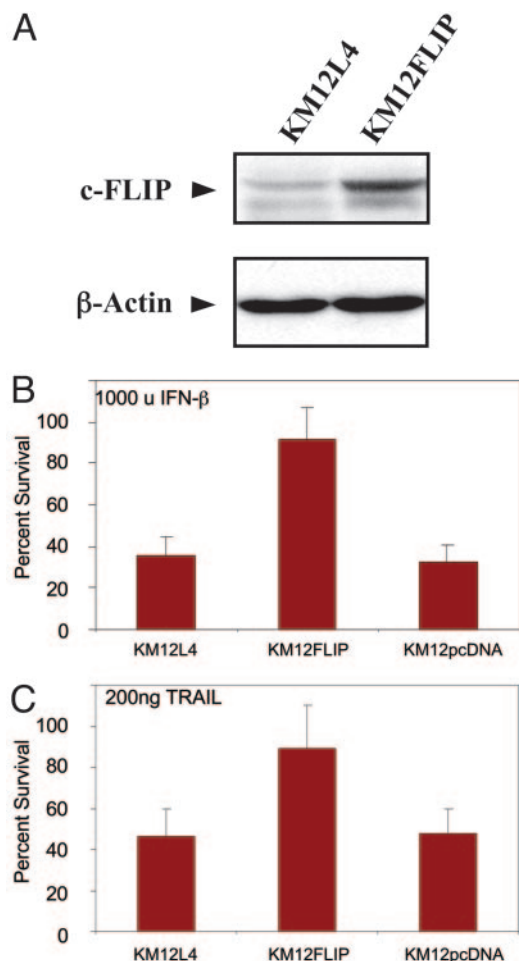


Fig. 6. Overexpression of c-FLIP in the KM12L4 cell line results in attenuation of the IFN- $\beta$ -induced cell death. *A*, total protein (50  $\mu$ g/lane) from the KM12L4, KM12FLIP, and KM12Bcl-X<sub>L</sub> were assayed for c-FLIP protein expression. Equal loading of protein was confirmed by probing blots for  $\beta$ -actin expression. *B*, the KM12L4, KM12FLIP, and KM12pcDNA cell line and clones were treated with IFN- $\beta$ . Cell viability was then determined by trypan blue exclusion method. *Columns*, cell survival percentage compared with the PBS control groups; *error bars*, SD. *C*, the KM12L4, KM12FLIP, and KM12pcDNA cell line and clones were treated with TRAIL. Cell viability was then determined by trypan blue exclusion method. *Columns*, cell survival percentage compared with the PBS control groups; *error bars*, SD.

was observed after IFN- $\beta$  treatment of the IFN-resistant KM12L4RIFN1. The pGL2m vector was used as a control. These data further support the role of IFN- $\beta$  in TRAIL regulation and is consistent with the hypothesis that IFN- $\beta$ -induced apoptosis is mediated by TRAIL.

## DISCUSSION

Type I IFNs engender many pleiotropic effects, including cytostatic and cytotoxic activity against human tumor cells via the type I receptor. Interaction with their receptor results in phosphorylation of Jak1 and Tyk2 receptor-associated tyrosine kinases, which in turn phosphorylate and activate the Stat family of proteins including Stat1 and Stat2 (3, 15). Classically, Stat1 along with Stat2 and ISGF3 $\gamma$  (p48) form the ISGF3 complex transcription factor. This transcription factor binds to the ISRE, which results in transcriptional induction of the ISGs (3).

The cell death receptor signaling pathway involves binding of ligands to cell-surface death receptors, recruitment of the adapter protein FADD/Mort-1, and activation of caspase-8 and -10 (9, 16–19). These ligands and their receptors are present on colorectal cancer

cells and are important mediators of apoptosis in these cells (20). TRAIL is a member of the TNF family, which is expressed in most adult human tissues and is capable of killing transformed but not normal cells through binding to two proapoptotic receptors, DR4 and KILLER/DR5 (21–29). Normal cells also express TRAIL receptors, but they appear to be protected from TRAIL-mediated killing through competition for ligand binding by two decoy receptors, TRID (24, 27, 30–33) and TRUNDD (30, 34, 35). c-FLIP interrupts the cell-surface apoptotic pathway by binding FADD (36). The end result of the cell-surface signaling pathway is widespread proteolysis and endonucleolytic cleavage of chromosomal DNA, events that characterize the apoptotic phenotype.

Little is known about the induction of apoptosis by type I IFNs in human cancer cells. However, with increasing knowledge of programmed cell death pathways, more evidence has accumulated concerning this process. Chin *et al.* (37, 38) have demonstrated the induction of caspase-1 and p21 via the Stat pathway by IFN- $\gamma$ . IFN- $\gamma$  has also been shown to up-regulate Fas and Fas ligand expression via Stat1 (39). Ossina *et al.* (40) demonstrated induction of Bak by IFN- $\gamma$ ; however, a role in the apoptotic process was not delineated. Shin *et al.* (41) demonstrated the induction of Fas and TRAIL in hepatocellular carcinoma cell lines after IFN- $\gamma$  treatment. Less is known of the mechanisms of induction of apoptosis by type I IFNs. IFN- $\beta$  engenders biological responses via a different receptor and pathway from IFN- $\gamma$ , and, therefore, the process of IFN- $\beta$ -induced apoptosis may be distinct. Takizawa *et al.* observed up-regulation of Fas after treatment with IFN- $\beta$  (42). Kito *et al.* demonstrated induction of apoptosis and activation of caspase-8 after IFN- $\beta$  exposure (43). More recently, Kayagaki *et al.* (10) have demonstrated induction of TRAIL by IFN- $\beta$  in human peripheral blood T lymphocytes. They demonstrated that the induction of this death receptor ligand could result in T-cell-mediated tumoricidal activity. Others have reported the induction of TRAIL in monocytes and dendritic cells and TRAIL-dependent tumor cell killing after IFN- $\beta$  treatment (11, 44). Balachandran (9) reported the type I IFN potentiation of virus-induced apoptosis through the activation of the cell-surface death receptor pathway of FADD and caspase-8. Although these observations clearly implicate TRAIL and the cell-surface apoptotic pathways in cells of hematopoietic origin and cell-mediated immunity, the role of

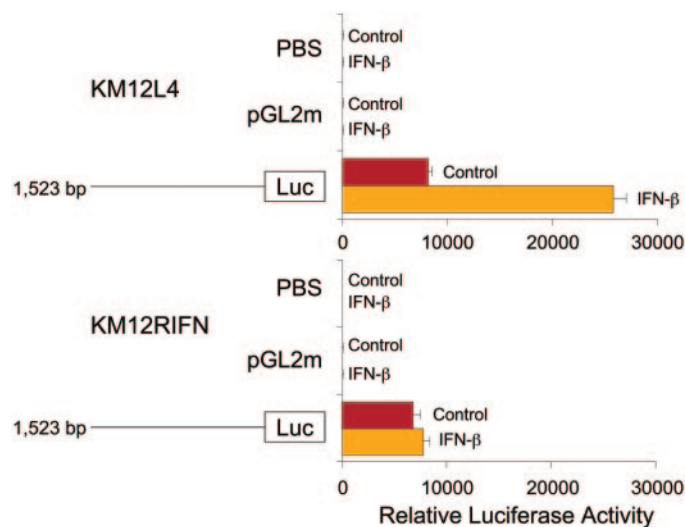


Fig. 7. TRAIL promoter activity in KM12L4 cells is induced after IFN- $\beta$  treatment. KM12L4 cells were transiently transfected with the TRAIL promoter Luciferase (*Luc*) plasmid and were treated with IFN- $\beta$  (1000 units). Luciferase activity was measured 24 h after treatment ( $n = 4$ ).



TRAIL in IFN-induced apoptosis in epithelial cancers remains unclear.

On the basis of these observations, we hypothesized that the apoptotic response in human colorectal cancer cells after IFN- $\beta$  treatment may be mediated by TRAIL. In the present study, we demonstrate the induction of apoptosis in human colorectal cancer cell line KM12L4 is associated with the activation of the Jak-Stat signaling pathway. The resistant clone of this cell line, L4RIFN, did not demonstrate activation of the Jak-Stat pathway, indicating that this pathway may be crucial for the induction of apoptosis. The importance of the Jak-Stat pathway in IFN- $\beta$ -induced apoptosis is supported by the observation that the Stat1-deficient U3A cells were resistant to IFN- $\beta$ -induced apoptosis.

The induction of apoptosis by IFN- $\beta$  was associated with the induction of the proapoptotic mediator TRAIL and activation of caspase-3, -8, -9, and -10, indicating activation of the cell-surface apoptotic pathway. The absence of induction of TRAIL in the resistant cell line indicates a correlation between induction of this proapoptotic mediator and apoptosis in these cells. The analysis of the 2ftgh- and Stat1-deficient U3A cells indicates Stat1 is a crucial mediator of IFN- $\beta$ -induced apoptosis. Additionally, Stat1 appears necessary as a signal mediator for induction of TRAIL. These data suggest a connection between the Jak-Stat signaling pathway and induction of TRAIL. The resistance to IFN- $\beta$  cytotoxic effects of the KM12L4 clone that overexpressed cFLIP demonstrates the importance of the cell-surface apoptotic pathway in this process and further supports the role of TRAIL. Activation of the TRAIL promoter after IFN- $\beta$  treatment in these cells was also observed.

Our results suggest that IFN- $\beta$ -induced apoptosis is associated with the Stat1-dependent transcriptional and translational up-regulation of TRAIL. The analysis of the TRAIL promoter suggests the mechanism of induction of TRAIL may occur via direct transcriptional activation of this gene by the ISGF3 transcription factor or via an intermediary factor complex. The identification of possible transcriptional factors or translational signals responsible for the induction of proapoptotic mediators may provide targets for additional cancer therapies. The induction and activation of the cell-surface pathway by IFN- $\beta$  represents a novel pathway for this agent to engender its effect and warrants further investigation.

## ACKNOWLEDGMENTS

We thank Dr. George Stark for his support and discussions related to this work.

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