Identification of X-linked Inhibitor of Apoptosis-associated Factor-1 as an Interferon-stimulated Gene That Augments TRAIL Apo2L-induced Apoptosis*

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In the course of gene array studies aimed at identifying IFN-stimulated genes associated with interferon β (IFN-β)-induced apoptosis, we identified X-linked inhibitor of apoptosis-associated factor-1 (XAF1) as a novel IFN-stimulated gene. XAF1 mRNA was up-regulated by IFN-α and IFN-β in all cells examined. However, IFNs induced high levels of XAF1 protein predominantly in cell lines sensitive to the proapoptotic effects of IFN-β. In apoptosis-resistant cells including WM164 melanoma, WM35 melanoma, U937 pro-monocytic leukemia, and HT1080 fibrosarcoma cells, XAF1 mRNA was strongly up-regulated but XAF1 protein was up-regulated only weakly or not at all. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a critical mediator of IFN-β-induced apoptosis, but most melanoma cell lines were resistant to recombinant TRAIL protein. For example, A375 melanoma cells were defective in TRAIL induction by IFN-β and were resistant to TRAIL-induced apoptosis. However, IFN-β pretreatment sensitized them to subsequent recombinant TRAIL-induced apoptosis. A375 cells expressing XAF1 constitutively were more sensitive to TRAIL-induced apoptosis compared with empty vector-transfected cells. The degree of sensitization by XAF1 was similar to that provided by IFN pretreatment and was correlated with the level of XAF1 expressed. Furthermore, the overexpression of the zinc-finger portion of XAF1 blocked IFN-dependent sensitization of A375 melanoma cells to the proapoptotic effects of TRAIL. These results suggested that IFN-dependent induction of XAF1 strongly influenced cellular sensitivity to the proapoptotic actions of TRAIL.

Interferons (IFNs)1 are synthesized and secreted by cells following exposure to virus or other stimuli and are an important component of the innate immune response where they act upon neighboring cells to inhibit the replication of various DNA and RNA viruses (1). All of the IFNs increase the abundance or affect the activities of specific effector proteins encoded by subsets of early response genes known collectively as IFN-stimulated genes (ISGs), the protein products of which mediate physiological responses to IFNs (2–6). Many ISGs were identified initially as differentially regulated mRNA species rather than on the basis of their biological functions (see Refs. 2 and 7). Others were identified as enzymes that showed increased activity in virus-treated or IFN-stimulated cells (2, 8). At least 100 ISGs induced by IFN-α or IFN-β have been identified to date (1, 8–10). IFNs also have potent antigrowth effects on most cell types, and the identities of the ISGs that regulate antiproliferative or proapoptotic effects have begun to emerge.

IFN-β but not IFN-α induced apoptosis in a broad range of solid tumor cell types (11). TRAIL was an important regulator of the IFN-dependent apoptotic process in melanoma (11) and myeloma cell lines (12) and in dendritic and natural killer cells (13–15). Although TRAIL was necessary for IFN-β-induced apoptosis in melanoma cells (11), exogenous recombinant TRAIL protein alone was a poor proapoptotic agent in melanoma cells (16), suggesting that other ISGs may contribute to the IFN-dependent apoptotic process.

Counteracting cellular apoptotic processes are factors involved in preventing unregulated cell suicide, the inhibitors of apoptosis (IAPs). First identified in baculoviruses where they function to prevent the death of infected host cells, at least six human IAPs have since been described, XIAP/hILP, NAIP, c-IAP1/HIAP-2, c-IAP2/HIAP-1, Survivin, and BRUCE (17). XIAP is expressed in all adult and fetal tissues with the exception of peripheral blood leukocytes (18). XIAP binds directly to caspases via one or more of its baculovirus inhibitory repeat domains and may function as a competitive inhibitor of caspase catalytic function (19, 20). The constitutive and induced expression of IAPs blocked caspase activity (19, 20). IAPs themselves were regulated by cellular proteins including Smac/Diablo, a mitochondrial protein that was released into the cytoplasm inhibiting IAP function and allowing the caspase-dependent cleavage of substrates (21–23). XIAP has recently been implicated in conferring resistance to TRAIL in melanoma cells (24), and the inhibition of XIAP activity may be an obligatory component of TRAIL-induced apoptosis (25).

* This work was supported in part by National Institutes of Health Grant 1R01CA90837 (to D. W. L.) and CA90914 (to E. C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: IFN, interferon; ISG, IFN-stimulated gene; IAP, inhibitors of apoptosis; XAF1, X-linked inhibitor of apoptosis-associated factor-1; HA, hemagglutinin; ZF, zinc finger; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Received for publication, May 16, 2002, and in revised form, May 22, 2002
Published, JBC Papers in Press, May 23, 2002, DOI 10.1074/jbc.M204851200
XAF1 demonstrated that XAF1 blocked the inhibitory activity of XIAP for caspase-3, and co-expression of XAF1 and XIAP inhibited XIAP-dependent caspase-3 suppression (27). XAF1 was implicated as a tumor suppressor based on the observation that expression was lower in tumor cell lines compared with some normal tissues and that transient expression of XAF1 sensitized tumor cells to the proapoptotic effects of etoposide (26, 27).

Several recent studies have demonstrated that IFN can sensitize cells to the proapoptotic effects of recombinant TRAIL (28, 29). Here we report the identification of XAF1 as a novel type I IFN-induced protein that correlates with the cellular sensitivity to IFN-α/β-induced apoptosis and contributes to IFN-α/β-dependent sensitization of cells to TRAIL-induced apoptosis.

**MATERIALS AND METHODS**

**XAF1 Plasmids and Transfections**—The full-length XAF1 cDNA expression vector pcDNAhaX20 has been described previously (27) and encodes XAF1 as a fusion protein with the influenza hemagglutinin (HA) epitope. A 300-bp portion of the XAF1 cDNA was cloned into pcDNA3.1 in an antisense orientation to serve as a control in stable transfection experiments. Stable transfections were performed by electroporation, and stable transfectants were selected in 500 μg/ml G418. An expression plasmid encoding the XAF1 zinc-finger region (amino acids 1–178) expressed in-frame with a N-terminal HA epitope was created by PCR amplification of the XAF1 zinc-finger domain followed by ligation into plasmid pcDNAha from which the full-length XAF1 cDNA had been excised.

**Assessment of XAF1 mRNA Induction**—RNA was isolated from cells by using Trizol reagent (Invitrogen) according to manufacturer specifications. XAF1 mRNA was detected by RT-PCR using XAF1-specific primers. PCR amplification of GAPDH was used to control for differences in RNA concentration and experimental variability. Cells were serum-starved overnight (0.1% serum) and then cultured in 10% serum for 8 h or were serum-deprived for 24 h prior to RNA isolation (serum withdrawal). Total cellular RNA was isolated, and 20 μg from each sample was separated on a 1% denaturing agarose gel and transferred to nylon membrane. XAF1 was detected by hybridization with a full-length XAF1 cDNA probe. The blot was then stripped and reprobed with GAPDH to control for RNA loading.

**Assessment of XAF1 protein induction in different melanoma cell lines.** A, melanoma cell lines with varying sensitivities to the pro-apoptotic effects of IFNs were left untreated or treated for 8 h with IFN-α (500 units/ml) or IFN-β (500 units/ml), recombinant human tumor necrosis factor-α (50 ng/ml), recombinant human TRAIL (50 ng/ml), or camptothecin (5 μM). Alternatively, cells were serum-starved overnight (0.1% serum) and then cultured in 10% serum for 8 h or were serum-deprived for 24 h prior to RNA isolation (serum withdrawal). Total cellular RNA was isolated, and 20 μg from each sample was separated on a denaturing agarose gel and transferred to nylon membrane. XAF1 was detected by hybridization with a full-length XAF1 cDNA probe. The blot was then stripped and reprobed with GAPDH to control for RNA loading.

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**Fig. 1.** Induction of XAF1 in WM9 melanoma cells. A, WM9 cells were left untreated or treated with 500 units/ml IFN-α2 or IFN-β for 2, 8, 24, and 48 h. Total cellular RNA was isolated by using Trizol reagent, and 20 μg of RNA from each sample was separated on a denaturing agarose gel and transferred to nylon membrane. XAF1 was detected by hybridization with a full-length XAF1 DNA probe. The blot was then stripped and reprobed with GAPDH to control for RNA loading. B, induction of XAF1 protein in WM9 melanoma cells. Cell lysates were isolated from WM9 cells left untreated or treated with IFN-α or IFN-β for 24 or 72 h.

**Fig. 2.** Induction of XAF1 mRNA by various stimuli. WM9 cells were left untreated or treated for 8 h with IFN-β (500 units/ml) or IFN-α2 (50 units/ml), recombinant human tumor necrosis factor-α (50 ng/ml), recombinant human TRAIL (50 ng/ml), or camptothecin (5 μM). Alternatively, cells were serum-starved overnight (0.1% serum) and then cultured in 10% serum for 8 h or were serum-deprived for 24 h prior to RNA isolation (serum withdrawal). Total cellular RNA was isolated, and 20 μg from each sample was separated on a denaturing agarose gel and transferred to nylon membrane. XAF1 was detected by hybridization with a full-length XAF1 cDNA probe. The blot was then stripped and reprobed with GAPDH to control for RNA loading.

**Fig. 3.** Induction of XAF1 mRNA and protein in different melanoma cell lines. A, melanoma cell lines with varying sensitivities to the pro-apoptotic effects of IFNs were left untreated or treated with 500 units/ml IFN-α2 or IFN-β for 8 h. Total cellular RNA was isolated and analyzed by RT-PCR using XAF1-specific primers. PCR amplification of GAPDH was used to control for differences in RNA concentration and experimental variability. B, cell lysates were isolated from the indicated melanoma cell lines that were left untreated or treated with IFN-β for 24 or 48 h. Total cellular protein (15 μg) was separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. A monoclonal XAF1 antibody was used to detect XAF1 protein, after which the blot was stripped and reprobed with a polyclonal XIAP antibody.
cellular RNA (2 μg) was reverse-transcribed with Moloney murine leukemia virus RT (Promega) and random hexamer primers (42 °C for 60 min). One-tenth of the RT reaction was subjected to PCR analysis using XAF1-specific primers (5′-GGACCAAGCAGGTGGTG-3′ and 5′-AATCTATTTGTGTCAATAT-3′). PCR products were separated on 1% (w/v) agarose gels and visualized by staining with ethidium bromide. Gel images were captured on a digital camera, and the images inverted to give a dark band on a light background for presentation.

**Immunoblotting**—XAF1 protein was detected in total cell lysate lysis buffer (50 μM Tris-Cl, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin, aprotinin, and pepstatin) by probing Western blots with an XAF1-specific monoclonal antibody. This antibody was generated against full-length GST-XAF1 following standard techniques and recognized a reactive band at ~35 kDa when used at a 1:500 dilution in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 1 mM EDTA) containing 0.1% (v/v) Tween 20 and 1% (v/v) bovine serum albumin. Immunoreactive bands were visualized by using a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad) followed by enhanced chemiluminescence (PerkinElmer Life Sciences). XIAP was detected by using commercially available polyclonal antisera (Cell Signaling Technologies, Inc.), and actin was detected by using monoclonal antibodies (Sigma).

**Apoptosis and Cell Growth Analyses**—Cells undergoing apoptosis were identified by using Annexin V staining (BD PharMingen) followed by fluorescence-activated cell sorting (FACS). The cells were left untreated or were treated with IFN-β for 72 h. After that time, the cells were harvested and stained with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Bio-Rad) followed by enhanced chemiluminescence (PerkinElmer Life Sciences). XIAP was detected by using commercially available polyclonal antisera (Cell Signaling Technologies, Inc.), and actin was detected by using monoclonal antibodies (Sigma).

**Cell Fractionation Studies**—A375 melanoma cells were left untreated or were treated with 500 units/ml IFN-β for 24 h and then fractionated into cytoplasmic, nuclear, and mitochondrial fractions as described previously (30). The nuclei were isolated from cells by incubation in nuclear extraction buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM Hepes, pH 7.4, 1% Triton X-100) until cell membranes were completely disrupted as determined by trypan blue staining. The lysates were centrifuged at 2000 × g for 10 min and washed twice in a nuclear wash (320 mM sucrose, 5 mM MgCl₂, 10 mM Hepes, pH 7.4). The nuclei were then solubilized in lysis buffer (as described above) on ice for 15 min and then clarified by centrifugation at 14,000 × g for 10 min.

**RESULTS**

**Identification of XAF1 as an IFN-induced Protein**—Three independent Affymetrix oligonucleotide array studies assessing genes induced by IFNs in melanoma cells established XAF1 as an ISG whose expression was regulated by both IFN-α and IFN-β. To confirm the gene array results, Northern blot analyses were performed to examine XAF1 mRNA expression in WM9 melanoma cells. XAF1 mRNA was not detected in untreated cells but was induced following treatment with IFN-β and IFN-α (Fig. 1A). The induction of XAF1 protein by both IFNs was confirmed in WM9 cell lysates by using a XAF1-specific monoclonal antibody. XAF1 protein was undetectable in untreated cells but was induced within 24 h of IFN stimulation and sustained through 72 h (Fig. 1B). Compared with IFN-α, XAF1 protein was induced to a substantially greater extent by IFN-β (Fig. 1B). Although less potently, XAF1 mRNA was also up-regulated by IFN-γ and tumor necrosis factor-α (Fig. 2). No other stimulus including epidermal growth factor, serum addition or withdrawal, camptothecin, TRAIL, or interleukin-6 induced XAF1 mRNA above basal levels (Fig. 2 and data not shown).

RT-PCR analyses were performed to assess basal and IFN-induced XAF1 expression in various tumor cell lines. In all melanoma cell lines, XAF1 mRNA was either absent or present constitutively at low levels and was induced by both IFN-α
and IFN-β with the exception of WM164 cells in which only IFN-β induced XAF1 mRNA (Fig. 3A). When XAF1 protein was assessed, all of the apoptosis-sensitive melanoma lines including WM9, WM3211, FEMX, and Guilliams exhibited strong up-regulation of the XAF1 protein that was sustained for at least 48 h (Fig. 3B) (Table I and data not shown). In contrast, apoptosis-resistant WM35 cells exhibited minimal XAF1 protein induction by IFN-β (Fig. 3B) that was detected only following long exposure with higher concentrations of antibody and cell extract (data not shown). Similarly, apoptosis-resistant WM164 and SKMEL28 melanoma cells displayed weak or transient induction (Fig. 3B and Table I). XAF1 protein was induced strongly in A375 melanoma cells (Fig. 3B). A375 cells were shown previously to be resistant to IFN-β-induced apoptosis because of the lack of TRAIL induction by IFN-β (11). Thus, the induction of both XAF1 and TRAIL, but not either one independently, correlated with IFN-β-induced apoptosis in melanoma cell lines (Table I). Although the loss of apoptotic protease activating factor 1 has been implicated in melanoma cell resistance to chemotherapeutic agents, all of the melanoma cell lines used in these studies expressed apoptotic protease activating factor 1 protein (data not shown). The XAF1 protein expression was assessed in a panel of tumor cell lines representing different tumor types. OVCAR3 ovarian carcinoma, U266 myeloma, and ACHN renal cell carcinoma exhibited potent up-regulation of XAF1 protein and TRAIL induction and underwent IFN-β-induced apoptosis. In contrast, the tumor lines resistant to IFN-β-induced apoptosis including HeLa cervical carcinoma, K562 chronic myelogenous leukemia, HT080 fibrosarcoma, and others exhibited IFN-dependent XAF1 mRNA up-regulation, but protein induction was weak or undetectable (Table I).

XAF1 Augmentation of TRAIL-induced Apoptosis—To determine whether XAF1 overexpression could mimic IFN-dependent sensitization of cells to the cytotoxic effects of TRAIL, a XAF1 expression plasmid was transfected into WM9, A375 melanoma, and HeLa cervical carcinoma cells and stable transfectants selected in G418. Each cell line was also stably transfected in parallel with pcDNA3.1 empty vector and separately with a XAF1 antisense plasmid. The antisense plasmid included only 300 bp of the XAF1-coding region and did not influence endogenous XAF1 protein levels (data not shown) but served as an additional negative control. The XAF1 expression plasmid encoded an influenza HAXAF1 fusion protein to allow the distinction of the exogenously expressed protein from the endogenous protein.

A375 cells formed G418-resistant stable colonies following transfection with all three of the aforementioned plasmids. In contrast, WM9 cells readily formed stable colonies with the empty vector or antisense controls but few colonies with the XAF1 sense expression plasmid (Fig. 4A). Most of the colonies that formed within the XAF1 sense-transfected cultures did not progress beyond the 10-cell stage at which point they underwent a death process resembling apoptosis (Fig. 4A). Similar observations were made with HeLa where few XAF1 stable transfectants survived G418 selection. XAF1 sense plasmid-transfected WM9 or HeLa cells that survived G418 selection did not express constitutively the XAF1 protein (Fig. 4B).

An analysis of haXAF1 protein expression in individual A375 clones selected from the stably transfected population demonstrated that fewer than 50% of the clones expressed XAF1 constitutively (Fig. 5A and data not shown). Thus, three clones with varying levels of constitutive haXAF1 protein expression were selected for further analysis. A375/XAF1 clone 7 expressed high constitutive levels of HA-tagged XAF1, whereas clone 1 exhibited lower constitutive expression (Fig. 5A, upper band). Clone 2 was resistant to G418 but did not express haXAF1 constitutively (Fig. 5A). Most of the three clones exhibited normal up-regulation of endogenous XAF1 in response to IFN-β (Fig. 5A, lower band) and TRAIL receptor (DR4 and DR5) expression levels were not significantly different among the three clonal lines (data not shown).

The sensitivity of the haXAF1-expressing clones to TRAIL-induced apoptosis was initially examined by using Annexin V staining. Unlike empty vector-transfected cells, XAF1-transfected clones 1 and 7 expressing medium and high levels of XAF1, respectively, displayed potent sensitivity to TRAIL as a single agent (Fig. 5B). Clone 2, which did not express XAF1 protein constitutively, had a phenotype identical to untransfected or empty vector-transfected A375 (Fig. 5B). All of the three clones exhibited IFN-β-dependent sensitization to TRAIL-induced apoptosis, and this process was enhanced in clones 1 and 7 (Fig. 5B). These apoptosis studies were extended to include an assessment of TRAIL-dependent cell killing in the
XAF1 Augments TRAIL-induced Apoptosis

A. IFN-β

B. TRAIL (ng/ml)

C. SRB Staining

Fig. 5. XAF1 augments TRAIL-induced apoptosis. A, clones of A375 melanoma cells stably transfected with an XAF1 expression plasmid, pCDNAbAX-20, or pCDN3.1 empty vector were left untreated with IFN-β for 24 h, and lysates separated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. A monoclonal XAF1 antibody was used to detect endogenous XAF1 protein and the HA-tagged XAF1 protein encoded by pCDNAbAX-20. B, augmentation of TRAIL-induced apoptosis. Stably transfected clones 1, 2, and 7 were left untreated or treated with recombinant human TRAIL for 24 h. C, augmentation of TRAIL-induced cell killing. A375 XAF clones were left untreated or treated in duplicate with increasing concentrations of recombinant human TRAIL for 24 h. Afterward, the cells were fixed in 10% trichloroacetic acid and stained with 0.4% (w/v) sulforhodamine B to assess cell density.

XAF1-transfected clones. Whereas clone 2 was resistant to TRAIL at doses <100 ng/ml, clones 1 and 7 were both sensitive to TRAIL at doses as low as 20 ng/ml, which killed >80% of cells within 24 h (Fig. 5C).

A direct implication of XAF1 function in IFN-dependent sensitization of cells to TRAIL-induced apoptosis was obtained by overexpressing the zinc-finger region of XAF1 (amino acids 1–178). This region has been implicated in XAF1 self-association, whereas the C-terminal region has been implicated in other biological functions. It was hypothesized that overexpression of the zinc-finger domain would inhibit downstream XAF1 functions that depend on XAF1 self-association or interaction with other proteins. Indeed, A375 melanoma cell clones constitutively expressing the XAF1 ZF domain, exhibited dramatically reduced (Fig. 6, ZF Cl.2) or complete ablation (ZF Cl.1 and ZF Cl.5) of IFN-dependent sensitization to TRAIL-induced apoptosis that was observed in parental A375 cells. The degree of inhibition was correlated with the level of XAF1 ZF domain expression (Fig. 6A) and was apparent in both cell growth assays (Fig. 6B) and Annexin V apoptosis assays (Fig. 6C). The overexpression of XAF1 fragments not encompassing the zinc-finger domain had no effect on IFN-induced sensitization to TRAIL (data not shown).

Endogenous XAF1 protein was up-regulated normally in response to IFN-β treatment in all of the ZF XAF1 expressing clones examined (Fig. 6A).

Subcellular Localization of XAF1—XAF1 has been reported to reside in the nucleus based on the results obtained by using XAF1-green fluorescent protein fusion proteins (26). To determine the localization of the endogenous XAF1 protein following IFN stimulation, cell fractionation studies were performed. Cells were left untreated or treated with IFN-β for 24 h, lysed, and separated into cytoplasmic, nuclear, and mitochondrial fractions (30). Equivalent protein amounts from each fraction were assessed for XAF1 by Western blot analysis. XAF1 was localized to both the cytoplasm and the nucleus but was absent from the mitochondria (Fig. 7). Stimulation with IFN-β plus TRAIL did not alter XAF1 distribution (data not shown). The IFN-β-dependent induction of XAF1 did not alter the subcellular localization of XIAP, which remained predominantly cytoplasmic (Fig. 7).

DISCUSSION

IFNs have potent antiviral and antiproliferative activities and have been the most effective cytokine for treating human malignancies. Despite the >20 years that IFNs have been known to be effective as antitumor proteins (31), little progress has been made in dissecting the roles of the >100 IFN-stimulated gene products in inducing the cytotoxic effects of IFNs. Most ISGs are induced by IFNs in all cell types. However, substantial variability has been identified in the overall sensitivity of tumor cells to the antiproliferative or apoptotic effects of IFNs (11 and references therein). A better understanding of the molecular determinants of tumor cell sensitivity to IFNs could lead to increased therapeutic potential.

Through gene expression-profiling studies in WM9 melanoma cells that are preferentially sensitive to IFN-β compared with IFN-α2, TRAIL was identified as an ISG that was necessary but not sufficient to mediate IFN-β-induced apoptosis (11). The neutralization of TRAIL activity blocked IFN-induced ap-
optosis (11), but most of the cell lines examined did not apoptose in response to TRAIL as a single agent (Figs. 5 and 6) (29). Furthermore, TRAIL induction by IFN correlated poorly with IFN-induced apoptosis. TRAIL was induced in many cell lines that did not undergo apoptosis (29). Thus, additional factors either present constitutively or IFN-induced were postulated to be required to sensitize cells to IFN or synergize with TRAIL to stimulate an apoptotic response.

Additional gene profiling experiments implicated XAF1 as a potent ISG induced in a variety of cell types including melanoma, chronic myelogenous leukemia and endothelial cells.6

6 D. W. Leaman, M. Chawla-Sarkar, and E. C. Borden, unpublished observations.

Northern blot and RT-PCR analyses confirmed that XAF1 was a potent ISG induced at the transcriptional level by IFNs (Fig. 1). XAF1 mRNA was induced by IFN in nearly all of the cell lines examined and therefore did not correlate with IFN-induced apoptosis. However, XAF1 protein induction by IFN-β was strongly correlated with the proapoptotic effects of IFN-β. XAF1 protein was induced strongly in all of the cell lines that underwent IFN-β-induced apoptosis but was not in resistant cells except where the other components of the apoptotic machinery were defective (Table I). Two clear examples of this correlation were WM35 and WM9 melanoma cells. WM9 were highly sensitive to the proapoptotic effects of IFN-β, and XAF1 protein was strongly up-regulated by IFN-β. In contrast, WM35 cells were resistant to IFN-β-induced apoptosis, and

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**Fig. 6.** XAF1 zinc-finger domain overexpression inhibits IFN-dependent sensitization of cells to TRAIL-induced apoptosis. A, A375 melanoma parental cells and A375 clones stably transfected with an expression plasmid encoding the truncated XAF1 N-terminal zinc-finger domain (amino acids 1–178) were left untreated or were treated with IFN-β for 24 h. After that time, cell lysates were isolated and assessed for the expression of endogenous XAF1 and the truncated ZF XAF1 proteins by immunoblotting with a XAF1 monoclonal antibody. B, The ZF XAF1 clonal lines evaluated in A were assessed for sensitivity to the antigrowth effects of IFN-β (1000 units/ml for 48 h), TRAIL (50 ng/ml for 24 h), or IFN-β followed by TRAIL for 24 h. After treatment, the cells were fixed, stained with 0.4% (w/v) sulforhodamine B, and the relative cell numbers were assessed as described under “Materials and Methods.” Data from a representative experiment with duplicate samples represent the average spectrophotometric values derived from cell monolayers subjected to the indicated treatments relative to the control (untreated) values for each clone. C, the induction of apoptosis in parental A375 cells and the ZF XAF1-expressing clones was assessed by Annexin V staining followed by FACS analysis as described in Fig. 5B. The results from a single representative experiment are shown (of three replicates).
although XAF1 mRNA was up-regulated by IFN, little or no XAF1 protein was detected (Fig. 3). Nevertheless, XAF1 induction alone was clearly insufficient to activate apoptosis in A375 cells that lacked TRAIL induction by IFN but exhibited potent XAF1 protein up-regulation by IFN (Table I).

By using A375 clones stably expressing haXAF1 protein at different constitutive levels, the amount of XAF1 expression was correlated with cellular sensitivity to TRAIL. Clones that constitutively expressed ectopic haXAF1 protein at levels comparable to those observed with the endogenous protein following IFN-treatment such as clone 7 were as sensitive to TRAIL as a single agent as the cells pretreated with IFN (Fig. 5B). Clones resistant to G418 but expressing no exogenous XAF1 behaved identically to parental A375 cells. These data, combined with the observation that apoptosis-resistant cell lines such as WM35 melanoma and HT1080 fibrosarcoma cells expressed TRAIL but little or no XAF1 protein in response to IFN-β stimulation, suggest that both TRAIL and XAF1 are required for cells to undergo apoptosis in response to IFN-β as a single agent.

The mechanisms underlying the proapoptotic functions of XAF1 are unknown, although its interaction with XIAP probably via the zinc-finger region appears to be critical. The overexpression of the zinc-finger domain blocked IFN-β-dependent sensitization of cells to the proapoptotic effects of TRAIL (Fig. 6). This overexpression may block the dimerization of the endogenous XAF1 protein or inhibit its interaction with downstream substrates such as XIAP. The XIAP-like negative effect was dependent upon the level of ZF XAF1 expression (Fig. 6). These data support the hypothesis that XAF1 induction by IFN-β contributes to the sensitization of cells to the proapoptotic effects of TRAIL.

IAPs have been increasingly recognized as critical regulators of cellular apoptotic processes (19, 20). Although initially thought to play a primary role in mediating NFκB-dependent anti-apoptotic function, constitutive IAP activity has been recognized as an important determinant of cellular viability. Inhibitors of IAP activity, particularly Smac/Diablo, have been viewed as essential for normal function of apoptotic caspase cascades (21–23, 25). Although XAF1 has little structural similarity with Smac/Diablo, it may have a similar physiological function once induced by IFNs (20). A recent report suggested that XAF1 functions by sequestering XIAP to the nucleus, thereby inactivating this critical IAP and allowing caspase cascades to proceed unabated (27). Our own cell fractionation studies were partially consistent with this possibility, because XAF1 was localized in both the cytoplasmic and nuclear fractions (Fig. 7). However, unlike the previous studies performed with XAF/green fluorescent protein and XIAP/green fluorescent protein fusion proteins (27), no re-distribution of XIAP occurred in cells overexpressing XAF1 in response to IFN-β (Fig. 7). Thus, additional studies are needed to address the mechanism of XAF1-dependent inhibition of XIAP.

In summary, IFN-induced apoptosis appeared to require the induction of at least two proteins, TRAIL and XAF1. Whereas TRAIL induction was previously shown to be necessary for IFN-induced apoptosis based on antibody neutralization experiments (11), it was also found to be insufficient as a single agent to induce apoptosis in most melanoma cell lines (16, 28, 29). XAF1 protein was more directly correlated with IFN-induced apoptosis, because it was induced only in cells that exhibited IFN-induced apoptosis (Table I). More importantly, the overexpression of XAF1 was shown here to augment sensitivity to TRAIL-induced cytotoxicity, and a truncated XAF1 could inhibit IFN-dependent sensitization of cells to TRAIL. Thus, IFN-induced apoptosis may minimally require the induction of TRAIL to initiate caspase-dependent apoptotic cascades and induction of XAF1 to inhibit IAP function, thereby allowing caspase cleavage events to proceed unabated. Whether other IFN-induced proteins are also required for IFN-regulated apoptosis and whether IFN-induced XAF1 plays important regulatory functions in other ligand-dependent apoptotic responses remain to be determined.

Acknowledgments—We thank Dr. A. Almasan for helpful discussions, the W. M. Keck Foundation for support of the Cleveland Clinic fluorescent-activated cell sorting facility, and Dr. M. Herlyn for the “WM” cell lines.

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