XAF1 Mediates Tumor Necrosis Factor-α-induced Apoptosis and X-linked Inhibitor of Apoptosis Cleavage by Acting through the Mitochondrial Pathway*

Shawn L. Straszewski-Chavez†, Irene P. Visintin†, Natasha Karassina§, Georgyi Los¶, Peter Liston¶, Ruth Halaban** and Gil Mor***

From the †Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, the Departments of Obstetrics, Gynecology, and Reproductive Sciences and **Dermatology, Yale University School of Medicine, New Haven, Connecticut 06520, ‡Promega Corporation, Madison, Wisconsin 53711, and the §Department of Pediatrics, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Tumor necrosis factor-α (TNF-α) and Fas ligand induce apoptosis by interacting with their corresponding membrane-bound death receptors and activating caspases. Since both systems share several components of the intracellular apoptotic cascade and are expressed by first trimester trophoblasts, it is unknown how these cells remain resistant to Fas ligand while sensitive to TNF-α. XAF1 (X-linked inhibitor of apoptosis (XIAP)-associated factor 1) is a proapoptotic protein that antagonizes the caspase-inhibitory activity of XIAP. Here, we demonstrated that XAF1 functions as an alternative pathway for TNF-α-induced apoptosis by translocating to the mitochondria and promoting XIAP inactivation. In addition, we showed that the overexpression of XAF1 sensitized first trimester trophoblast cells to Fas-mediated apoptosis. Furthermore, we also determined that the differential expression of XAF1 in first and third trimester trophoblast cells was due to changes in XAF1 gene methylation. Our results establish a novel regulatory pathway controlling trophoblast cell survival and provide a molecular mechanism to explain trophoblast sensitivity to TNF-α and the increased number of apoptotic trophoblast cells observed near term. Aberrant XAF1 expression and/or localization may have consequences for normal pregnancy outcome.

Apoptosis, or programmed cell death, is an active process by which superfluous or dysfunctional cells are eliminated to maintain normal tissue function. Depending on the stimulus, apoptosis may be initiated intrinsically by the mitochondrial pathway or extrinsically by one of the members of the TNF death receptor family. The Fas/Fas ligand system represents a dominant negative form of XIAP, which interferes with both Fas and TNF-R1, the death receptor that mediates TNF-α-induced apoptosis (6, 7), share several components of the intracellular apoptotic cascade, it is unknown how first trimester trophoblast cells remain resistant to Fas but sensitive to TNF-α-induced apoptosis.

The central executioners of apoptosis are the caspases, a family of cysteine proteases that are subdivided into two groups. Whereas the initiator caspases initiate apoptosis by activating the downstream effector or executioner caspases, the effector caspases cleave numerous vital cellular proteins in order to affect the apoptotic cascade (8). Activation of the caspase cascade is tightly regulated by several endogenous intracellular inhibitors, which prevent further propagation of the death signal either at the “initiator” or “effector” level. Inhibitors of apoptosis (IAPs) are unique in that they are capable of inhibiting both initiator and effector caspases. To date, eight human IAPs have been identified, but X-linked inhibitor of apoptosis (XIAP) is the most potent and versatile member of the family (9). XIAP contains three tandem BIR domains and a C-terminal RING domain, which are known to differentially inhibit initiator and effector caspases (10). The BIR2 domain together with the linker region between the BIR1 and BIR2 domains of XIAP have been shown to inhibit the activation of “effector” caspase-3 and caspase-7 (11), whereas the inhibitory activity of “initiator” caspase-9 was localized to the BIR3-RING domain of XIAP (12). Upon certain apoptotic stimuli, caspases can cleave XIAP into two distinct fragments, an N-terminal fragment containing BIR1–2 and a second fragment containing BIR3-RING (13, 14). The BIR1–2 fragment has diminished ability to inhibit caspase-3 and is not easily detected, since it is susceptible to further caspase-mediated degradation. In contrast, the BIR3-RING fragment is more stable and retains the ability to inhibit caspase-9 but is unable to suppress Fas-induced apoptosis (13). It has been suggested that this XIAP cleavage product may represent a dominant negative form of XIAP, which interferes with receptor apoptotic pathways. Despite expressing both Fas and Fas ligand, trophoblast cells isolated from first trimester placentas are resistant to Fas-mediated apoptosis under normal conditions (1–3). However, in contrast to Fas ligand-induced apoptosis, first trimester trophoblast cells have been shown to be sensitive to TNF-α-mediated apoptosis (1, 2, 4, 5). Since both Fas and TNF-α are expressed by first trimester trophoblasts, it is unknown how first trimester trophoblast cells remain resistant to Fas but sensitive to TNF-α-induced apoptosis.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
XAF1 Translocates to Mitochondria

the function of the active, full-length form of XIAP by promoting caspase activation (14).

Our laboratory and others previously demonstrated that the full-length form of XIAP is highly expressed in trophoblast cells of first trimester placentas (3, 15) and that it protects first trimester trophoblast cells from Fas-mediated apoptosis (3). In addition, it has been shown that the expression of the full-length form of XIAP significantly decreases in the trophoblast layer of third trimester placentas (15), correlating with the concomitant increase in placental apoptosis (16, 17). Moreover, we demonstrated that the XIAP BIR3-RING cleavage fragment is primarily expressed in third trimester placentas and in isolated first trimester trophoblast cells undergoing apoptosis and that its appearance correlated with an increase in caspase-9 and caspase-3 activity (3). How the anti-caspase activity of XIAP is regulated in first trimester trophoblast cells and the factor(s) that can overcome the expression of XIAP and allow first trimester trophoblast cell apoptosis to occur are unknown.

The inhibition of XIAP and other IAP family members is mediated by a group of proteins that includes Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low propidium iodide), OMI/HtrA2 (high temperature requirement protein A2), and XAF1 (XIAP-associated factor 1). Although XAF1 is thought to be a proapoptotic nuclear protein (18), Smac/DIABLO and OMI/HtrA2 have been shown to be mitochondria-associated proteins that are released from mitochondria upon certain apoptotic stimuli to inhibit XIAP function (19–21), albeit by different mechanisms. Both Smac/DIABLO and OMI/HtrA2 bind to the BIR domains within XIAP, thereby displacing caspase-9 and caspase-3 and allowing caspase activation. However, OMI/HtrA2 is also a serine protease that has been shown to cleave XIAP, rendering it inactive (22, 23).

XAF1 was identified based on its ability to bind to XIAP and was proposed to cause the redistribution of XIAP from the cytoplasm to the nucleus, thereby inhibiting the anti-caspase activity of XIAP (9). Indeed, a subsequent study demonstrated that endogenous XAF1 localized to both the cytoplasm to the nucleus, thereby inhibiting the anti-caspase activity of XIAP (9). Since XIAP antagonizes XIAP function (9) and the inhibition of XIAP renders first trimester trophoblast cells sensitive to Fas-induced apoptosis, we hypothesized that XAF1 plays a role in sensitizing trophoblast cells to Fas-mediated apoptosis. Therefore, the aim of this study was to characterize XAF1 expression and function in trophoblast cells. We describe a novel role for XAF1 in TNF-α-induced apoptosis and how XAF1 expression is temporally regulated throughout normal pregnancy.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The agonistic anti-human Fas monoclonal antibody (mAb) (clone E059.1) was obtained from BD PharMingen (San Diego, CA), and the recombinant human TNF-α was from ProproTech, Inc. (Rocky Hill, NJ). The pancaspase inhibitor, benzoylcarbonyl-VAD-fluoromethyl ketone, was purchased from R&D Systems, Inc. (Minneapolis, MN), and actinomycin D was from Sigma. The NF-κB inhibitor, SN50, was obtained from BIOMOL (Plymouth Meeting, PA), and the OMI/HtrA2 protease inhibitor, ucf-101, was from Calbiochem. The mouse anti-XAF1 mAb (1:200) has been described previously (9), and the rabbit anti-XAF1 polyclonal antibody (IMG-379; 1:100) was purchased from Imgenex (San Diego, CA). Both the mouse anti-XIAP mAb (clone 28; 1:1000) and the mouse anti-Bax mAb (clone 3; 1:500) were obtained from BD Transduction Laboratories (San Diego, CA). The mouse anti-caspase-9 mAb (clone LAP6; 1:2000) was purchased from R&D Systems, the rabbit anti-caspase-3 polyclonal antibody (catalog number 9661; 1:5000) was from Cell Signaling Technology, Inc. (Beverly, MA), and the rabbit anti-cytochrome c polyclonal antibody (catalog number S2050; 1:10,000) was from BD Biosciences (San Diego, CA). The mouse anti-NF-κB p65 mAb (clone F-6; 1:2000) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the rabbit polyclonal antibody for β-actin (A2066; 1:10,000) was purchased from Sigma. Primary antibody signals were detected using either a horseradish peroxidase-conjugated horse anti-mouse, or a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) from Vector Laboratories (Burlingame, CA).

Placental Tissue—Term placentas (38–40 weeks gestational age) were obtained from clinically normal pregnancies following vaginal delivery. First trimester placentas (7–12 weeks gestational age) were obtained from normal pregnancies, voluntarily terminated for reasons unrelated to the present study. A signed, written consent form was obtained from each patient. The use of placental tissue specimens and consent forms was approved by the Yale University Human Investigation Committee. Tissue specimens were collected in cold, sterile phosphate-buffered saline and immediately transported to the laboratory for cell culture preparation.

First Trimester Primary Trophoblast Cell Isolation and Culture—Primary trophoblast cells were isolated from first trimester placentas as previously described (2, 3). Isolated first trimester trophoblast cells were cultured at 37 °C/5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% human serum (Gemini Bio-Products, Woodland, CA).

Cell Lines—The first trimester human trophoblast cell line, 3A, has been previously described (2, 3), whereas the TPA third trimester trophoblast cell line was purchased from the American Type Culture Collection (Manassas, VA). Both cell lines were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10 mm Heps, 0.1 mm minimal essential medium nonessential amino acids, 1 mm sodium pyruvate, and 100 units/ml penicillin/streptomycin (Invitrogen) and maintained at 37 °C/5% CO₂.
**HaloTag Labeling System**—Cells transiently expressing XAF1-HaloTag were labeled with 5 mM HaloTag tetramethyl rhodamine (TMR) ligand for 15 min according to the manufacturer’s instructions (Promega). Unbound ligand was removed by washing the cells twice with phosphate-buffered saline. Fluorescence intensities in cell lysates were measured at 535 and 600 nm using an automatic microplate reader (SpectraMax M5, Molecular Devices, Union City, CA). The expression of the fusion protein was determined by SDS-PAGE on a fluorescence imager (Typhoon 9400; Amersham Biosciences) and by Western blot analysis with an anti-XAF1 antibody. Live cells were visualized with a FV500 confocal microscope (Olympus) using a 545-nm green HeNe laser (6.0% transmittance, PMT gain = 600) and a TRITC filter set.

**Fluorescence-activated Cell Sorting Analysis**—Cells (2 × 10⁶) were detached with 0.05% trypsin-EDTA (Invitrogen) and centrifuged as previously described (3). The pelleted cells were washed twice with 5 ml of cold phosphate-buffered saline. After the final centrifugation, the cellular pellet was resuspended in 1 ml of cold phosphate-buffered saline and incubated on ice with 5 mg/ml Hoechst 33342 dye (Molecular Probes) and 1 mg/ml propidium iodide (Sigma) for 15 min. Hoechst 33342 dye stains the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells, whereas propidium iodide is only permeant to dead cells. The staining pattern that results

**Mitochondrial and Cytoplasmic Cell Fractionation**—Mitochondrial and cytoplasmic fractionation was performed by centrifugation using the ApoAlert cell fractionation kit (catalog number 630105; BD Biosciences) according to the manufacturer’s instructions. Briefly, the cells were pelleted by centrifugation, resuspended in 1 ml of ice-cold wash buffer, and centrifuged at 2,500 rpm for 5 min at 4 °C. Once the supernatant was removed, the cells were resuspended in 200 μl of ice-cold fractionation buffer mix containing protease inhibitor mixture and 1 mM dithiothreitol. The tube was incubated on ice for 10 min, and the cells were passed through a 21-gauge 1/2-needle syringe 25 times to homogenize the sample. The homogenate was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 2,750 rpm for 10 min at 4 °C. Following centrifugation, the supernatant was transferred to a new tube and centrifuged at 10,000 rpm for 25 min at 4 °C to separate the cytosolic and mitochondrial fractions. The supernatant containing the cytoplasmic extract was transferred to a new tube, whereas the remaining pellet, which is the mitochondrial fraction, was resuspended in 200 μl of the fractionation buffer mix. All extracts were stored at −80 °C until use. The expression of COX4, which is a mitochondria-specific protein that is retained on the inner mitochondrial membrane even in cells undergoing apoptosis, was analyzed in the mitochondrial and cytoplasmic fractions to ensure the integrity of the preparations.

**Immunohistochemistry**—First and third trimester placental samples were fixed with 4% paraformaldehyde and paraffin-embedded as previously described (26). Sections of placenta (5 μm) were deparaffinized, rehydrated, and masked with target retrieval solution (Dako, Carpinteria, CA) in a steamer at 95 °C for 30 min. XAF1 expression was evaluated using the rabbit anti-XAF1 polyclonal antibody (1:100). The slides were mounted with permanent aqueous mounting medium (ScyTek, Logan, UT) and visualized by light microscopy.

**Cell Viability Assay**—Cell viability was evaluated using the CellTiter 96 assay (Promega) according to the manufacturer’s instructions. In brief, 5 × 10³ cells were plated in triplicate wells of a 96-well microtiter plate (BD Biosciences) in a 100-μl volume per well. Following treatment, 20 μl of the CellTiter sub-strate, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium (MTS) was added to each well, and the plate was incubated at 37 °C/5% CO₂ for 1–4 h. Optical densities of the samples were measured at 490 nm using an automatic microplate reader (SpectraMax M5, Molecular Devices, Union City, CA). The values of the treated cells were compared with the values generated from the untreated control and reported as percentage viability.

**Plasmid Constructs**—The HA-tagged XAF1 open reading frame and 3′-untranslated region were subcloned from the pACT vector into EcoRI sites of the pCI-neo vector (Promega, Madison, WI) as previously described (9). The XAF1–15L-HaloTag fusion cassette was constructed by replacing the p65-coding region in a vector encoding p65–15L-pHT2 (Promega) described previously (57) with the XAF1 coding region. XAF1 was amplified using the primers 5′-CTGACATTCACTTTGCCTTTCCTCT-3′ and 5′-GTTATCCACCGTGTAGATGGATC-3′ and inserted into NheI and MluI sites of the p65–15L-pHT2 vector. The sequence of the XAF1–15L-HaloTag fusion construct was confirmed by DNA sequencing.

**Cell Transfection**—Cells were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions using 2 μg of plasmid DNA at a transfection reagent/plasmid DNA ratio of 3:1. Transient transfections were performed overnight (16–24 h) at 37 °C/5% CO₂, and cells were allowed to recover in medium with 10% fetal bovine serum for 1–2 days before use. To ensure the integrity of the preparations, the nuclear and cytoplasmic extracts were collected, and the remaining insoluble pellet, which contains the nuclei, was resuspended in 1 ml of ice-cold wash buffer, and centrifuged at 13,000 rpm for 10 min at 4 °C, the supernatant containing the cytoplasmic fraction was collected, and the remaining insoluble pellet, which contains the nuclei, was resuspended in 50–100 μl of ice-cold NER buffer containing 0.2 mg/ml phenylmethylsulfonyl fluoride and the protease inhibitor mixture (Roche Applied Science) was added to the cellular pellet based on the amount of packed cell volume, vortexed, and incubated on ice for 10 min. 5.5–11 μl of ice-cold CER II was added to the tube to isolate the cytoplasmic extract, vortexed, and incubated on ice for 1 min. Following centrifugation at 13,000 rpm for 10 min at 4 °C, the supernatant containing the cytoplasmic extract was collected, and the remaining insoluble pellet, which contains the nuclei, was resuspended in 50–100 μl of ice-cold CER I buffer containing 0.2 mg/ml phenylmethylsulfonyl fluoride and the protease inhibitor mixture. The tube was vortexed and incubated on ice for 10 min four times for a total of 40 min. Following centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatant containing the nuclear extract was collected. All extracts were stored at −80 °C until use. To ensure the integrity of the preparations, the nuclear and cytoplasmic fractions were analyzed for the expression of DNA topoisomerase I, a nucleus-specific protein.
from the simultaneous use of both of these dyes makes it possible to distinguish between apoptotic and dead cells by flow cytometry. Unstained cells served as a measure of background fluorescence. The samples were analyzed using a Vantage fluorescence-activated cell sorter (BD Biosciences) with 488 nm/UV dual excitation. Propidium iodide staining was detected in the FL-2 channel, and Hoechst staining was detected in the SSc-W channel. Data were analyzed using CellQuest software (BD Biosciences).

RNA Isolation and Reverse Transcription-PCR—Total RNA was isolated from cells using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer’s instructions. Reverse transcription was performed using 5 μg of total RNA and the First Strand cDNA synthesis kit from Amersham Biosciences. Half of the reverse transcription reaction was amplified using the XAF1-specific primers 5′-GAGCACCAGCAG- GTTGGGTG-3′ and 5′-AATCATTTGGTTGCAATTAT-3′ as previously described (24). Thirty cycles of PCR were performed at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

Western Blot Analysis—The cells were lysed in 1% Nonidet P-40 and 0.1% SDS in the presence of 0.2 mg/ml phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Roche Applied Science). 20 μg of total cellular protein was loaded per lane and separated under reducing conditions by SDS-PAGE as previously described (3). The blots were developed using the enhanced chemiluminescence system (PerkinElmer Life Sciences).

Caspase Activity Assay—Caspase activity was measured using the Caspase Glo assay (Promega) according to the manufacturer’s instructions. In brief, 10 μg of total cellular protein from cell lysates was added to the proluminescent LEHD (caspase-9) or DEVD (caspase-3) substrate at a 1:1 ratio in a 100-ml volume and incubated at room temperature for 1 h in the dark. Following incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). All samples were assayed in triplicate. Luminescence was expressed as relative light units (RLU) and is proportional to the amount of caspase activity present in the sample.

XAF1 Promoter Methylation—The methylation status of the XAF1 promoter was assessed by bisulfite sequencing. This procedure takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite DNA modification, which deaminates unmethylated cytosine, converting it to uracil, but spares methylated cytosine (27). Genomic DNA was isolated, and a total of 2 μg was bisulfite-modified and PCR-amplified with the forward and reverse primers GTTTTGGTTTTGTAGAAA and AAAACCATTTCTACTCCCTCTCAA, respectively, which bind in the non-CpG dinucleotide region flanking the ATG start site. Reactions were hot started at 94 °C for 5 min, after which 1 unit of Platinum Taq polymerase (Invitrogen) was added, followed by 40 cycles of 94 °C for 30 s, optimal annealing temperature of 49.8 °C for 30 s, extension at 72 °C for 30 s, and finally 7 min at 72 °C. The amplified 186-bp PCR products were loaded onto 1% agarose gels, stained with ethidium bromide, visualized under UV illumination, gel-purified, and sequenced by Applied Biosystems 3730 capillary instruments employing fluorescence-labeled dideoxynucleotides at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Statistical Analysis—The data are represented as the average ± S.D. and analyzed for statistical significance (p < 0.05) using one-way analysis of variance (ANOVA) with the Bonferroni correction. All experiments were repeated three times with similar results.

RESULTS

XAF1 Is Only Expressed in Third Trimester Placentas and Correlates with the Activation Status of XIAP in Vivo—Our initial objective was to determine whether XAF1 was expressed in placental tissues throughout normal pregnancy. Therefore, the expression of XAF1 was evaluated in first and third trimester placentas by Western blot analysis. As Fig. 1A demonstrates, XAF1 (34 kDa) was expressed in third trimester placentas, but no XAF1 expression could be detected in first trimester placentas. To determine whether the differential expression of XAF1 in placental tissues correlated with the activation status of XIAP, XIAP expression was also evaluated by Western blot. Consistent with XAF1 expression, high levels of the active form (45 kDa) of XIAP were observed in first trimester placentas, whereas the inactive XIAP fragment (30 kDa) was primarily detected in third trimester placentas (Fig. 1A).

XAF1 Localizes to both the Nucleus and Cytoplasm of Syncytiotrophoblasts in Third Trimester Placentas—In order to determine the placental cell type(s) that XAF1 localized to, the expression of XAF1 was evaluated in first and third trimester placentas by immunohistochemistry. Analogous to the above findings, XAF1 immunoreactivity was observed in third trimester placentas (n = 4; Fig. 1B, 2), but not first trimester placentas (n = 8; Fig. 1B, 1).

FIGURE 1. XAF1 is differentially expressed in first and third trimester placentas. A, Western blot analysis of XAF1 expression and the activation status of XIAP in first trimester and third placentas obtained from normal pregnancies. The active form of XIAP was primarily expressed in first trimester placentas, whereas the inactive fragment of XIAP was predominantly detected in third trimester placentas. B, XAF1 expression was also evaluated in first trimester (1) and third (2) placental sections (magnification ×20) by immunohistochemistry. Sections incubated with a rabbit IgG antibody served as a negative control (3). Note that XAF1 localized to both the nucleus and cytoplasm of syncytiotrophoblasts in third trimester placentas.
IgG1 isotype control (Fig. 1B, 3). Interestingly, XAF1 primarily localized to the syncytiotrophoblast layer of third trimester placentas, and XAF1 expression was observed in the nucleus as well as the cytoplasm of syncytiotrophoblasts, suggesting that XAF1 is not confined to the nucleus of trophoblast cells.

**XAF1 Induces Trophoblast Cell Apoptosis**—Since XAF1 expression was only observed in trophoblast cells of third trimester placentas, which are characterized by a greater incidence of apoptosis (16, 17), we hypothesized that XAF1 may regulate trophoblast survival. Therefore, our next aim was to evaluate the effect of XAF1 expression in trophoblast cells in vitro. In order to accomplish this, 3A first trimester trophoblast cell line and 7-week primary trophoblast cells were transiently transfected with pCI-XAF1, and cell viability was observed in the 3A cells and the primary trophoblast cells, respectively (p < 0.001), following transfection with pCI-XAF1. In contrast, no change in cell viability could be detected in trophoblast cells transfected with the empty pCI vector. To determine if this decrease in cell viability was due to apoptosis, first trimester trophoblast cells transiently expressing XAF1 were stained with propidium iodide and the Hoechst 33342 dye, and fluorescent intensities were analyzed by flow cytometry. The percentage of double positive cells increased from 17.99% in the vector control to 59.67% in the XAF1-transfected cells (Fig. 2B), further suggesting that XAF1 was inducing trophoblast cell apoptosis.

**XAF1 Expression Correlates with XIAP Degradation in Vitro**—Our next objective was to determine the effect of XAF1 expression on the activation status of XIAP and the caspase cascade in trophoblast cells. As Fig. 2C indicates, first trimester trophoblast cells normally do not express XAF1, which correlates with the expression of the active form of XIAP (45 kDa) as well as the lack of caspase-9 and caspase-3 activation. However, when 3A trophoblast cells were transiently transfected with pCI-XAF1 at expression levels similar to native third trimester trophoblasts (see Fig. 7G, c), a decrease in the active
XAF1 Translocates to Mitochondria

**FIGURE 3.** XAF1 renders first trimester trophoblast cells sensitive to Fas-mediated apoptosis. First trimester trophoblast cells (A), which stably expressed XAF1 (XAF1), were incubated in the absence and presence of a 500 ng/ml concentration of an agonistic anti-Fas (α-Fas) mAb for 24 h. A, cell viability was determined using a colorimetric assay. The bar graph shows percentage cell viability relative to the untreated control (NT). B, caspase-3 activity was measured using a luminescent assay. The bar graph shows caspase activity expressed as RLU. *, p < 0.001. C, XAF1 expression and the activation status of XIAP were evaluated by Western blot analysis. Note the decrease in the active form of XIAP and the increase in the inactive XIAP fragment in XAF1-transfected trophoblast cells treated with α-Fas. NT, no treatment. The figure is representative of at least three independent experiments.

form of XIAP (45 kDa), followed by the appearance of the inactive form of XIAP (30 kDa) was observed. Consistent with this increase in XIAP degradation, the active forms of caspase-9 (36 kDa) and caspase-3 (17/19 kDa) were detected in XAF1-transfected trophoblast cells. This result was further confirmed by a caspase-9 and caspase-3 activity assay, as evidenced by the 10-fold increase in LEHDase activity (Fig. 2D) and DEVDase activity (Fig. 2E), respectively, following XAF1 transfection (p < 0.001). In contrast, no change in the activation status of XIAP and the caspase cascade was observed with the vector control.

XAF1 Confers Sensitivity to Fas-mediated Apoptosis—After determining that XAF1 induced XIAP inactivation and trophoblast apoptosis, we next sought to determine whether XAF1 plays a role in sensitizing trophoblast cells to Fas-mediated apoptosis. Similar to previous studies (1–3), first trimester trophoblast cells exhibited no change in cell viability (Fig. 3A) or caspase-3 activity (Fig. 3B) following treatment with an agonistic anti-Fas mAb for 24 h. However, in 3A trophoblast cells, which stably expressed XAF1 (34 kDa), a decrease in cell viability was observed (Fig. 3A), whereas degradation of the active form (45 kDa) and an increase in the inactive fragment (30 kDa) of XIAP was detected upon Fas stimulation (Fig. 3C). Moreover, the inactivation of XIAP correlated with a 2-fold increase in caspase-3 activity in the untreated and a 5-fold increase in the anti-Fas mAB-treated XAF1-expressing trophoblast cells (p < 0.001; Fig. 3B), as measured by DEVDase activity, suggesting that XAF1 confers sensitivity to Fas-mediated apoptosis by inhibiting XIAP function.

TNF-α Up-regulates XAF1 Expression and Induces XIAP Inactivation and the Activation of Caspase-9 and -3—Analogous to Fas-mediated apoptosis, TNF-α induces apoptosis in several cell types by binding to its membranal TNF death receptor, resulting in death-inducing signaling complex (DISC) formation and the activation of caspase-8 (28). Although first trimester trophoblast cells are Fas-resistant, they are sensitive to TNF-α-induced apoptosis (1, 2, 4, 5). In addition, TNF-α has been shown to render first trimester trophoblast cells sensitive to Fas-mediated apoptosis (2). Since the activation of the classical TNF death receptor pathway has been shown to be inhibited in first trimester trophoblast cells due to the expression of endogenous intracellular inhibitors, such as FLIP and XIAP (1–3), and XIAP has the potential to regulate both death receptor pathways, this suggests that TNF-α induced trophoblast apoptosis by activating an alternative pathway. Therefore, we sought to determine whether TNF-α caused trophoblast cells to undergo apoptosis by regulating the expression of XAF1. Indeed, XAF1 expression (34 kDa) increased in a dose-dependent manner in both 10-week primary trophoblast cells (Fig. 4A) and the 3A trophoblast cell line (Fig. 4B) following treatment with TNF-α. A similar increase in XAF1 expression was detected in first trimester trophoblast cells over time, with peak XAF1 expression observed at 48 h of TNF-α treatment (Fig. 4B). The expression of XAF1 correlated with XIAP inactivation, as evidenced by the dose- and time-dependent increase in the inactive form of XIAP (30 kDa). In addition, a significant increase (p < 0.001) in caspase-9 (LEHDase; Fig. 4C) as well as caspase-3 activity (DEVDSase; Fig. 4D) was observed over time following TNF-α treatment. Moreover, we determined that TNF-α-induced XAF1 expression was at the level of transcription, since XAF1 mRNA expression (320 bp) was detected in 3A trophoblast cells by reverse transcription-PCR within 6–9 h of TNF-α treatment, and this effect was inhibited following the addition of the transcriptional inhibitor, actinomycin D (Fig. 4E).

TNF-α Induces XAF1 Expression through NF-κB Activation—Our next objective was to determine the intracellular pathway by which TNF-α induced the expression of XAF1. At least two alternative TNF signaling pathways have been identified, one of which results in the activation of NF-κB (29). Therefore, we next sought to determine whether NF-κB played a role in TNF-α-induced XAF1 expression. Following treatment with TNF-α
of TNF-α treatment, an increase in the expression of the active form of NF-κB (65 kDa) was observed in the nuclear fraction of 3A trophoblast cells, and this increase in NF-κB p65 expression was sustained until 180 min of TNF-α treatment (Fig. 4F).

To verify that the increase in nuclear NF-κB expression was not due to cytoplasmic contamination, the expression of the nucleus-specific protein, topoisomerase I, was also evaluated in the cytoplasmic and nuclear fractions. As Fig. 4F illustrates, topoisomerase I expression (100 kDa) was detected in the nuclear fraction of first trimester trophoblast cells but not the cytoplasmic fraction, ensuring the integrity of the cell fractionation preparations.

In order to confirm a role for NF-κB in TNF-α-induced XAF1 expression, 3A trophoblast cells were incubated with TNF-α in the absence and presence of the NF-κB inhibitor, SN50, which prevents the nuclear translocation of NF-κB. Indeed, treatment with SN50 inhibited the activation of NF-κB, as evidenced by the decrease in the translocation of the p65 form to the nucleus (data not shown). Moreover, a decrease in XAF1 expression (34 kDa) was also observed in SN50- and TNF-α-treated trophoblast cells in comparison with TNF-α treatment alone (Fig. 4G), suggesting that NF-κB activated the transcription of XAF1 following treatment with TNF-α.

**XAF1 Translocates to Mitochondria**

Since XAF1 was proposed to be a nuclear protein (9) and XIAP inactivation and the activation of the caspase cascade is thought to occur in the cytoplasm (13, 14), we next sought to determine how XAF1 might be mediating its proapoptotic effects. In order to establish the subcellular compartment that XAF1 localized to, a XAF1-HaloTag fusion cassette was constructed as described under “Experimental Procedures.” Following transient transfection with the XAF1-HaloTag fusion construct for 24 h, 3A trophoblast cells were allowed to recover for 24–48 h and then incubated with the HaloTag TMR ligand.

for 15–180 min, first trimester trophoblast cells were separated into cytoplasmic and nuclear fractions, and the expression of NF-κB was evaluated by Western blot analysis. Within 15 min
Live cells were visualized by confocal microscopy 48 or 72 h after transfection using a HaloTag TMR ligand filter set. As shown in Fig. 5A, XAF1 was predominantly expressed in the cytoplasm, with a small amount of XAF1 expression detected in the nuclei of first trimester trophoblast cells. This observation was confirmed when the cytoplasmic and nuclear fractions of first trimester trophoblast cells were separated by centrifugation, and the expression of the XAF1-HaloTag fusion protein (64 kDa) was evaluated by SDS-PAGE analysis using either a fluorescence imager (Fig. 5B, left, upper band) or by Western blot using an anti-XAF1 antibody (Fig. 5B, right). The majority of XAF1 expression was detected in the cytoplasmic fraction rather than the nuclear fraction of first trimester trophoblast cells, with higher XAF1 expression levels observed 48 h following transfection.

In order to further characterize the subcellular compartment that XAF1 localized to, first trimester trophoblast cells were separated into cytoplasmic and mitochondrial fractions following transient transfection with the XAF1-HaloTag fusion construct for 24 h. As shown in Fig. 5C, the fluorescent intensity of the XAF1-HaloTag fusion protein peaked at 48 h post-transfection in both the cytoplasmic and mitochondrial fractions. Moreover, the amount of fluorescence detected in each fraction correlated with the level of XAF1-HaloTag fusion protein expression (64 kDa), with the highest expression observed at 48 h post-transfection in the cytoplasmic and mitochondrial fractions of first trimester trophoblast cells (Fig. 5D).

The specificity of these findings was confirmed by transiently transfecting first trimester trophoblast cells with pcI-XAF1 (XAF1), separating into mitochondrial and cytoplasmic fractions and evaluating the expression of XAF1 by Western blot analysis. As shown with the XAF1-HaloTag fusion protein, XAF1 (34 kDa), localized to the mitochondrial fraction of 3A trophoblast cells (Fig. 5E). To ensure that the localization of XAF1 to mitochondria was not due to nuclear or cytoplasmic contamination, the expression of topoisomerase I and COX4, which is a mitochondria-specific protein that is retained on the inner mitochondrial membrane even in cells undergoing apoptosis, was also evaluated in the cytoplasmic and mitochondrial fractions. As Fig. 5E demonstrates, topoisomerase I expression (100 kDa) was not detected, and COX4 expression (17 kDa) was only observed in the mitochondrial fraction of first trimester trophoblast cells, verifying the localization of XAF1 to mitochondria.

FIGURE 5. XAF1 localizes to mitochondria. First trimester trophoblast cells (3A) were transiently transfected with a XAF1-HaloTag fusion construct and labeled with the HaloTag TMR ligand. A, live cells were visualized by confocal microscopy 48 (left) or 72 (right) h (H) post-transfection using a HaloTag TMR ligand filter set. 1, fluorescent image; 2, light image; 3, composite image. Note that XAF1 primarily localized to the cytoplasm of 3A cells. B, the nuclear and cytoplasmic fractions were separated by centrifugation, proteins were resolved by SDS-PAGE, and the expression of the XAF1-HaloTag fusion protein was analyzed on a fluorescence imager (left) and by Western blot using an anti-XAF1 antibody (right). XAF1 expression levels were higher in the cytoplasmic fraction of 3A cells at 48 h post-transfection. C, 3A cells were transiently transfected with the XAF1-HaloTag fusion construct, separated into cytoplasmic and mitochondrial fractions by centrifugation, and labeled with the HaloTag TMR ligand. The amount of fluorescence was measured 24, 48, and 72 h post-transfection. D, the expression of the XAF1-HaloTag fusion protein was evaluated by Western blot analysis 24, 48, and 72 h post-transfection. E, 3A cells were transiently transfected with pcI-XAF1 (XAF1), the cytoplasmic (cyt.) and mitochondrial (mit.) fractions were separated by centrifugation, and the expression of XAF1 was evaluated by Western blot using COX4 and topoisomerase I (Topo I) as fractionation controls. XAF1 was predominantly detected in the mitochondrial fraction of 3A cells. NT, no treatment; + C, positive control (untransfected 3A whole cell lysate).
induced XIAP cleavage was dependent on caspase activation. In order to accomplish this, 3A trophoblast cells were transiently transfected with XAF1 in the absence and presence of the pancaspase inhibitor, benzoxycarbonyl-VAD-fluoromethyl ketone, and caspase-3 activation was measured by DEVDase activity. Although caspase-3 activity was significantly abrogated in the presence of benzoxycarbonyl-VAD-fluoromethyl ketone (p < 0.001; Fig. 7A), the cleavage product of XIAP (30 kDa) was still observed following XAF1 transfection (Fig. 7B), suggesting that XAF1-induced XIAP cleavage was independent of caspase activation.

OMI/HtrA2 is a mitochondrial protein with serine protease activity, which has also been shown to be associated with the cleavage of XIAP (22, 23). Therefore, we evaluated whether the translocation of XAF1 to mitochondria and the XIAP cleavage observed following XAF1 transfection or TNF-α treatment might be related to OMI/HtrA2 activity. As Fig. 7C illustrates, however, the use of the OMI/HtrA2 proteolytic inhibitor, ucf-101 (32), did not prevent the cleavage of XIAP (30 kDa) but instead induced a significant increase (p < 0.001) in caspase-3 activity (Fig. 7D) as determined by DEVDase activity following XAF1 transfection. In addition, no difference in the expression or translocation of the active, mature form of OMI/HtrA2 (36 kDa) (33) was detected in either the cytoplasmic or mitochondrial fractions of XAF1 transfected first trimester trophoblast cells (Fig. 7E) or following treatment with TNF-α (data not shown). This suggests that OMI/HtrA2 was not responsible for XAF1-induced XIAP cleavage and that the cleavage of XIAP was mediated by other proapoptotic factor(s) that may also be released from mitochondria upon XAF1 translocation.

**XAF1 Mitochondrial Localization Induces Bax Translocation and Cytochrome c Release**—Since the mitochondrial translocation of the proapoptotic Bcl-2 family member, Bax, and the release of cytochrome c from mitochondria are associated with the activation of the mitochondrial pathway (34–36), our next objective was to determine whether the translocation of XAF1 to mitochondria had any effect on Bax and cytochrome c expression and translocation. Therefore, the expression and cellular localization of cytochrome c and Bax were evaluated by Western blot analysis 8–48 h after XAF1 transfection and cell fractionation. As shown in Fig. 7E, within 24 h of transfection, the expression of XAF1 (34 kDa) was detected in the cytoplasmic fraction of 3A trophoblast cells. A similar expression pattern was observed in the mitochondrial fraction, with XAF1 expression peaking between 24 and 48 h post-transfection. This peak in XAF1 expression was associated with a concomitant increase (p < 0.001) in caspase-3 (DEVDase) activity in the cytoplasmic fraction of 3A cells (Fig. 7F). Furthermore, the localization of XAF1 correlated with the mitochondrial translocation of Bax and the release of cytochrome c from mitochondria, evidenced by the time-dependent increase in the expression of Bax (21 kDa) in the mitochondrial fraction and cytochrome c expression (15 kDa) in the cytoplasmic fraction of first trimester trophoblast cells, respectively (Fig. 7E). Interestingly, an increase in Bax expression was detected in the cytoplasmic fraction, suggesting that XAF1 might also effect Bax transcription. A similar increase in Bax expression and mitochondrial translocation as well as cytochrome c release from
FIGURE 7. The translocation of XAF1 to mitochondria induces Bax translocation and cytochrome c release. First trimester trophoblast cells (3A) were transiently transfected with pci-XAF1 (XAF1) in the absence and presence of a pancaspase inhibitor (panCI) using the empty pci vector (Vector) as a control. A, caspase-3 activity was measured using a luminescent assay. B, the expression of XAF1 and the activation status of XIAP were evaluated by Western blot analysis. C, first trimester trophoblast cells (3A) were transiently transfected with pci-XAF1 in the absence and presence of the OMI/HtrA2 inhibitor, ucf-101. XAF1 expression and the activation status of XIAP were analyzed by Western blot. NT, no treatment; +C, positive control (untreated 3A cells). D, bar graph shows caspase-3 activity expressed as RLU. E, Western blot analysis of XAF1, Bax, cytochrome c (Cyt-c), and OMI expression in 3A cells following pci-XAF1 transient transfection. The cytoplasmic and mitochondrial fractions were separated by centrifugation using COX4 as a fractionation control. Note the time-dependent increase in the expression of XAF1 and Bax in both the cytoplasmic and mitochondrial fractions of 3A cells. An increase in cytochrome c expression was also observed in the cytoplasmic fraction of 3A cells. F, caspase-3 activity was measured in the cytoplasmic fraction of XAF1-transfected 3A cells using a luminescent assay. The bar graph shows caspase activity expressed as RLU. G, the methylation status of XAF1 in first and third trimester trophoblast cells. Top, schematic representation of the amplified XAF1 promoter region. Shown are chromatograms of first trimester (3A) (a) and third trimester (TPA) (b) trophoblast cells. The ovals and rectangles indicate CG and TA base pairs corresponding to methylated and unmethylated sequences, respectively. C, Western blot analysis of XAF1 expression in 3A and TPA trophoblast cells. Note that XAF1 is expressed in TPA cells but not in 3A cells. 3A trophoblast cells transfected with XAF1 were used as positive control (+C). H, model for XAF1 function. The effect of XAF1 is observed at two levels: 1) nucleus (induces Bax expression) and 2) mitochondria (translocates to and promotes cytochrome c release, Bax activation, and the release of an unknown protein that induces the cleavage of XIAP, resulting in caspase-9 and caspase-3 activation).
mitochondria was observed following TNF-α treatment (data not shown). In contrast, XAF1 mitochondrial localization following either XAF1 transfection or TNF-α treatment had no effect on Bcl-2 expression or translocation (data not shown), which suggests that Bax might be a specific target for XAF1.

The XAF1 Promoter Is Hypermethylated in First Trimester Trophoblast Cells—Our next objective was to determine the mechanism mediating the differential expression of XAF1 between first and third trimester trophoblast cells. DNA methylation is a mechanism by which genes can be silenced and is important for the epigenetic regulation of placental development (37). Therefore, we sought to determine if the lack of XAF1 expression in first trimester trophoblasts was due to the methylation of the XAF1 promoter. To accomplish this, we sequenced bisulfate-modified genomic DNA that was isolated from the 3A first trimester trophoblast cell line and the third trimester trophoblast cell line, TPA. As shown in Fig. 7G, a, the XAF1 promoter in the region of amplification contained 8 CpGs (top; represented by hollow ovals) that were all hypermethylated in the first trimester trophoblast cell line, 3A. In contrast, only unmethylated TG pairs (bottom; represented by hollow rectangles) were observed in this region of the XAF1 promoter in the TPA third trimester trophoblast cell line (Fig. 7G, b). This correlated with the expression of XAF1 in these cells, as evidenced by the expression of the XAF1 protein in TPA cells, but not in 3A cells (Fig. 7G, c).

DISCUSSION

In this study, we characterized the role of XAF1 in the regulation of XIAP function and demonstrate that XAF1 mediates TNF-α-induced apoptosis in first trimester trophoblast cells. Our findings suggest that XAF1 antagonizes the anti-caspase activity of XIAP by a novel mechanism. Furthermore, we show that although XAF1 normally localizes to the nucleus, where it may have transcription-dependent functions, such as the
induction of Bax expression, XAF1 can translocate to mitochondria and activate the mitochondrial pathway.

XIAP and several other IAP family members have been shown to be expressed by the trophoblast throughout normal pregnancy (3, 38–40) and are thought to play a critical role in regulating trophoblast cell survival (41). The function of the IAP family is inhibited by a group of proteins that includes Smac/DIABLO, OMI/HtrA2, and XAF1, albeit by different mechanisms (9, 19–23). XAF1 was originally identified based on its ability to bind to XIAP and demonstrated to be underexpressed in several cancer cell lines and primary carcinomas (18, 42–46), which correlated with relatively high levels of XIAP expression in these cells (18, 46). In addition, the overexpression of XAF1 has been shown to reverse the XIAP-mediated inhibition of caspase-3 activity, and antisense-induced depletion of XAF1 was demonstrated to increase resistance to apoptosis only in cells that expressed endogenous XAF1 (9). Therefore, it is known that XAF1 antagonizes the anti-caspase activity of XIAP; however, the mechanism by which it does so was unclear.

Using a breast cancer cell model, we previously demonstrated that the transfection of chromosome 17p13.2, which contains XAF1 (18), renders these cells sensitive to Fas-induced apoptosis (25). Since XAF1 antagonizes XIAP function (9) and the inhibition of XIAP renders first trimester trophoblast cells sensitive to Fas-induced apoptosis (3), we hypothesized that XAF1 may play a role in the regulation of Fas-mediated apoptosis. Indeed, both transient and stable expression of XAF1 induced XIAP inactivation and the activation of the caspase cascade and conferred first trimester trophoblast cell sensitivity to Fas stimulation.

XIAP expression has been shown to decrease in the trophoblast with gestational age (15), correlating with a concomitant increase in trophoblast apoptosis (16, 17). Therefore, if XAF1 regulates XIAP activity and trophoblast survival during pregnancy, a correlation between placental expression of XAF1 and XIAP and the incidence of placental apoptosis should be observed in vivo. Consistent with this notion, we determined that XAF1 was only expressed in term placentas, which correlated with the activation status of XIAP in placental tissue samples. This suggests that the expression of XIAP may be developmentally regulated during pregnancy. Epigenetic regulation is critical for the development and function of the placenta (37), and our findings that XAF1 is hypermethylated in first trimester trophoblast cells suggest that the methylation of proapoptotic genes is a mechanism that prevents trophoblast cell death.

Since apoptosis increases in the placenta with gestational age (16, 17) or due to elevated levels of TNF-α at the maternal-fetal interface, we postulate that XAF1 is demethylated, resulting in the induction of XAF1 expression and an increase in trophoblast apoptosis. Current studies are aimed at determining whether XAF1 is unmethylated in placentas from complicated pregnancies, such as preeclampsia, intrauterine growth restriction (IUGR) and preterm labor, which are characterized by a greater incidence of trophoblast apoptosis (47–52).

Since TNF-α secretion has been shown to be higher in the placental bed of patients with severe preeclampsia (53) and the expression of TNF-α increases in normal placentas with gestational age (54, 55), it is thought that the increase in trophoblast apoptosis may be mediated by the production of proinflammatory cytokines, such as TNF-α. Several groups, including our own, demonstrated that first trimester trophoblast cells are sensitive to TNF-α-mediated apoptosis (1, 2, 4, 5); however, the mechanism by which TNF-α induces trophoblast cell apoptosis was unclear. Due to the expression of XIAP and other endogenous intracellular inhibitors, the activation of the classical TNF death receptor pathway has been shown to be inhibited in first trimester trophoblast cells (1–3). Our findings that TNF-α treatment induced XAF1 expression, followed by XIAP cleavage and the activation of the caspase cascade, provide an alternative pathway by which TNF-α mediates apoptosis. In addition, we demonstrated that TNF-α induces the expression of XAF1 at the transcription level, since the induction of XAF1 expression was not detected in the presence of a transcriptional inhibitor. Moreover, NF-κB appears to be associated with TNF-α-induced XAF1 expression, as evidenced by the decrease in XAF1 expression following the addition of an NF-κB inhibitor. However, the induction of XAF1 expression was not completely inhibited in the presence of the NF-κB inhibitor, suggesting that additional transcription factors might be involved in the regulation of XAF1 expression.

Interestingly, we determined that XAF1 was expressed in both the nucleus and the cytoplasm of trophoblast cells, whereas XIAP expression was only detected in the cytoplasm. This observation is in contradiction to the results obtained from a previous study, which proposed that XAF1 mediates its proapoptotic effects by sequestering XIAP to the nucleus (9). In accordance with our findings, a more recent study also did not find any XIAP expression in the nuclear fraction of interferon-β-treated cells (24), confirming that the inhibition of XIAP by XAF1 does not occur in the nucleus. This suggests that the nuclear redistribution of XIAP detected in the initial study (9) may have been a consequence of XAF1 overexpression and that the mechanism by which XAF1 induces apoptosis may be more complex than originally thought. Since XIAP inactivation and the activation of caspase-3 were also observed in the cytoplasmic fraction of first trimester trophoblast cells, this suggests that XAF1 inhibits the anti-caspase activity of XIAP by translocating to the cytoplasm rather than sequestering XIAP to the nucleus. Moreover, we show that by translocating to the mitochondria, XAF1 has specific effects within the cytoplasm and may regulate other antiapoptotic or proapoptotic factors, such as Bax and cytochrome c. This is supported by a recent study demonstrating an increase in cytochrome c release in XAF1-inducible cells that was inhibited by Bcl-2 overexpression (56). The use of the XAF1-HaloTag fusion construct in the present study allowed us to monitor the intracellular distribution of XAF1 without disrupting cell integrity. Using this system, we were able to localize XAF1 to the mitochondrial fraction by detecting the levels of fluorescence emitted by the expressed fusion protein, which was confirmed by Western blot. Interestingly, we also identified a putative MTS site in the N terminus of XAF1; however, given that XAF1 was not detected in the mitochondria of untreated cells, this suggests that XAF1 requires a stimulus, such as TNF-α, for its mitochondrial targeting.
Since XAF1 localizes to mitochondria and XIAP expression was not observed in the mitochondrial fraction of first trimester trophoblast cells following TNF-α treatment, this suggests that the inhibition of XIAP function by XAF1 is not the result of direct protein-protein interactions. However, Xia et al. (56) recently showed no significant increase in apoptosis in XIAP-negative fibroblasts following either Xaf1 transfection or TNF-α treatment, suggesting that XIAP is required for the pro-apoptotic effect of XAF1. Interestingly, the same study demonstrated that XAF1 sensitized these cells to TNF-α-induced apoptosis. A role for XAF1 in the mitochondrial pathway separate from its XIAP-antagonizing function is also supported by our findings that the XIAP cleavage observed in XAF1-expressing cells was not dependent on caspase activation.

Following either XAF1 transfection or TNF-α treatment, an increase in the p30 cleaved form of XIAP was observed in first trimester trophoblast cells, and the appearance of this XIAP cleavage fragment correlated with an increase in caspase-9 and caspase-3 activity. This suggests that the proapoptotic effect of XAF1 depends on the cleavage of XIAP. Although XAF1 has been shown to bind to XIAP (9), XAF1 is not known to be catalytic (18), suggesting that XAF1 induces XIAP cleavage indirectly. To date, only OMI/HtrA2 and caspases, in particular caspase-8 and caspase-3, have been demonstrated to cleave XIAP (56), which suggests that XAF1 can also induce apoptosis without binding to XIAP. A role for XAF1 in the inhibition of XIAP function by XAF1 is not the result of protein-protein interactions. However, Xia et al. (56)

**REFERENCES**

1. Payne, S. G., Smith, S. C., Davidge, S. T., Baker, P. N., and Guilbert, L. J. (1999) Biol. Reprod. 60, 1144–1150
2. Aschkenazi, S., Straszewski, S., Verwer, K. M., Foellmer, H., Rutherford, T., and Mor, G. (2002) Biol. Reprod. 66, 1853–1861
3. Straszewski-Chavez, S. L., Abrahams, V. M., Funai, E. F., and Mor, G. (2004) Mol. Hum. Reprod. 10, 33–41
4. Yui, J., Garcia-Lloret, M., Wegmann, T. G., and Guibert, L. J. (1994) Placenta 15, 819–835
5. Knoff, M., Mosl, B., Bauer, S., Griesinger, G., and Husslein, P. (2000) Placenta 21, 525–535
6. Yui, J., Hemmings, D., Garcia-Lloret, M., and Guibert, L. J. (1996) Biol. Reprod. 55, 400–409
7. Rasmussen, C. A., Pace, J. L., Banerjee, S., Phillips, T. A., and Hunt, J. S. (1999) Placenta 20, 213–222
8. Fischer, U., Janicke, R. U., and Schulze-Osthoff, K. (2003) Cell Death Differ. 10, 76–100
9. Liston, P., Fong, W. G., Kelly, N. L., Toji, S., Miyazaki, T., Conte, D., Tamai, K., Craig, C. G., McBurney, M. W., and Korneluk, R. G. (2001) Nat. Cell Biol. 3, 128–133
10. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) Nature 388, 300–304
11. Takahashi, R., Deveraux, Q., Tamam, L. Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) J. Biol. Chem. 273, 7787–7790
12. Sun, C., Cai, M., Meadows, R. P., Xu, N., Gunasekera, A. H., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) J. Biol. Chem. 275, 33777–33781
13. Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999) EMBO J. 18, 5242–5251
14. Johnson, D. E., Gastman, B. R., Wieckowski, E., Wang, G. Q., Amosco, A., Delach, S. M., and Rabinowich, H. (2000) Cancer Res. 60, 1818–1823
15. Gruslin, A., Qiu, Q., and Tsang, B. K. (2001) Biol. Reprod. 64, 1264–1272
16. Smith, S. C., Baker, P. N., and Symonds, E. M. (1997) Ann. J. Obstet. Gynecol. 177, 57–65
17. Smith, S. C., Leung, T. N., To, K. F., and Baker, P. N. (2000) Ann. J. Obstet. Gynecol. 183, 697–699
18. Fong, W. G., Liston, P., Rajcan-Separovic, E., St. Jean, M., Craig, C., and Korneluk, R. G. (2000) Genomics 70, 113–122
19. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) Mol. Cell 6, 855–861
20. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Cell 102, 43–53
21. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Cell 102, 33–42
22. Yang, Q. H., Church-Hajduk, R., Ren, J., Newton, M. L., and Du, C. (2003) Genes Dev. 17, 1487–1496
23. Srinivasula, S. M., Gupta, S., Datta, P., Zhang, Z., Hegde, R., Cheong, N., Fernandes-Alnemri, T., and Alnemri, E. S. (2003) J. Biol. Chem. 278, 31469–31472
24. Leaman, D. W., Chawla-Sarkar, M., Vyas, K., Heheman, M., Tamai, K., Toji, S., and Borden, E. C. (2002) J. Biol. Chem. 277, 28504–28511
25. Lareef, M. H., Tahin, Q., Song, J., Russo, I. H., Mihaela, D., Slater, C. M., Balsara, B., Testa, J. R., Broccoli, D., Grobelny, J. V., Mor, G., Cuthbert, A., and Russo, J. (2004) Mol. Carcinog. 39, 234–246
26. Mor, G., Gutierrez, L. S., Eliza, M., Kayaoglu, F., and Arici, A. (1998) Am. J. Reprod. Immunol. 40, 89–94
27. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Mollay, P. L., and Paul, C. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1827–1831
28. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997) EMBO J. 16, 2794–2804
29. Gaur, U., and Aggarwal, B. B. (2003) Biochem. Pharmacol. 66, 1403–1408
30. Claros, M. G., and Vincens, P. (1996) Eur. J. Biochem. 241, 779–786
31. Kamsteeg, M., Rutherford, T., Sapi, E., Hanczaruk, B., Shahabi, S., Flick, M., Brown, D., and Mor, G. (2003) Oncogene 22, 2611–2620
32. Cilenti, L., Lee, Y., Hess, S., Srinivasula, S., Park, K. M., Junqueira, D., Davis, H., Bonventre, J. V., Alnemri, E. S., and Zervos, A. S. (2003) J. Biol. Chem. 278, 11489–11494
33. Seong, Y. M., Choi, J. Y., Park, H. J., Kim, K. J., Ahn, S. G., Seong, G. H., Kim, I. K., Kang, S., and Rhim, H. (2004) J. Biol. Chem. 279, 37588–37596
34. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14681–14686
35. Sharpe, J. C., Arnould, D., and Youle, R. J. (2004) Biochim. Biophys. Acta 1644, 107–111

Acknowledgments—We thank Elaine Cheng, Paulomini B. Aldo, and Thomas Taylor for technical assistance.
XAF1 Translocates to Mitochondria

36. Phillips, T. A., Ni, J., and Hunt, J. S. (2001) Placenta 22, 663–672
37. Arima, T., Hata, K., Tanaka, S., Kusumi, M., Li, E., Kato, K., Shiota, K., Sasaki, H., and Wake, N. (2006) Dev. Biol., 297, 361–373
38. Lehner, R., Bobak, J., Kim, N. W., Shroyer, A. L., and Shroyer, K. R. (2001) Obstet. Gynecol. 97, 965–970
39. Shiozaki, A., Kataoka, K., Fujimura, M., Yuki, H., Sakai, M., and Saito, S. (2003) Placenta 24, 65–76
40. Ka, H., and Hunt, J. S. (2003) Am. J. Pathol. 163, 413–422
41. Straszewski-Chavez, S. L., Abrahams, V. M., and Mor, G. (2005) Endocr. Rev. 26, 877–897
42. Ma, T. L., Ni, P. H., Zhong, J., Tan, J. H., Qiao, M. M., and Jiang, S. H. (2005) Chin. J. Dig. Dis. 6, 10–14
43. Yin, W., Cheepala, S., and Clifford, J. L. (2006) Biochem. Biophys. Res. Commun. 339, 1148–1154
44. Wang, J., He, H., Yu, L., Xia, H. H., Lin, M. C., Gu, Q., Li, M., Zou, B., An, X., Jiang, B., Kung, H. F., and Wong, B. C. (2006) J. Biol. Chem. 281, 2451–2459
45. Ng, K. C., Campos, E. I., Martinka, M., and Li, G. (2004) J. Invest. Dermatol. 123, 1127–1134
46. Byun, D. S., Cho, K., Ryu, B. K., Lee, M. G., Kang, M. J., Kim, H. R., and Chi, S. G. (2003) Cancer Res. 63, 7068–7075
47. Allaire, A. D., Ballenger, K. A., Wells, S. R., McMahon, M. J., and Lessey, B. A. (2000) Obstet. Gynecol. 96, 271–276
48. Smith, S. C., Baker, P. N., and Symonds, E. M. (1997) Am. J. Obstet. Gynecol. 177, 1395–1401
49. Crocker, I. P., Cooper, S., Ong, S. C., and Baker, P. N. (2003) Am. J. Pathol. 162, 637–643
50. Ishihara, N., Matsuoka, H., Murakoshi, H., Laoag-Fernandez, J. B., Samoto, T., and Maruo, T. (2002) Am. J. Obstet. Gynecol. 186, 158–166
51. DiFederico, E., Genbacev, O., and Fisher, S. J. (1999) Am. J. Pathol. 155, 293–301
52. Genbacev, O., DiFederico, E., McMaster, M., and Fisher, S. J. (1999) Hum. Reprod. 14, Suppl. 2, 59–66
53. Pijnenborg, R., McLaughlin, P. J., Vercruysse, L., Hanssens, M., Johnson, P. M., Keith, J. C., Jr., and Van Assche, F. A. (1998) Placenta 19, 231–239
54. Chen, H. L., Yang, Y. P., Hu, X. L., Yelavarthi, K. K., Fishback, J. L., and Hunt, J. S. (1991) Am. J. Pathol. 139, 327–335
55. Yelavarthi, K. K., and Hunt, J. S. (1993) Am. J. Pathol. 143, 1131–1141
56. Xia, Y., Novak, R., Lewis, J., Duckett, C. S., and Phillips, A. C. (2006) Mol. Cell Biochem. 286, 67–76
57. Schmid, J. A., Birbach, A., Hofer-Warbinek, R., Pengg, M., Burner, U., Furtmüller, P. G., Binder, B. R., and de Martin, R. (2000) J. Biol. Chem. 275, 17035–17042