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Regulation of the Proapoptotic ARTS Protein by Ubiquitin-mediated Degradation*

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ARTS is a mitochondrial protein that promotes apoptosis induced by a variety of proapoptotic stimulators. ARTS induces apoptosis, at least in part, through binding to and antagonizing IAPs (inhibitors of apoptosis proteins). As a result of ARTS binding to IAPs, caspase inhibition is removed and apoptosis can be executed. Here we show that high cellular levels of ARTS protein sensitize cells toward apoptosis. Accordingly, in healthy cells ARTS levels are kept low through constant ubiquitin-mediated degradation. Upon proapoptotic stimuli, the ubiquitination process is inhibited, resulting in increased levels of ARTS. Increased ARTS in turn leads to a decrease of Bcl-2 and Bcl-x_L protein levels, cytochrome *c* release from mitochondria and apoptosis.

Apoptosis serves as a major mechanism for the precise regulation of cell numbers and as a defense against unwanted and potentially dangerous cells (1–3). The main executioners of apoptosis are caspases, a family of cysteine proteases that is ubiquitously expressed as inactive proenzymes (4, 5). A central component in transmitting apoptotic signals is the mitochondrion (6). Several mitochondrial proteins directly involved in apoptosis are cytochrome *c*, Smac/Diablo, Omi/HTRA2r (7–9), and ARTS (10). ARTS is a proapoptotic protein that promotes apoptosis in response to various proapoptotic inducers (11). ARTS is a member of the septin protein family and contains a P-loop GTP-binding domain conserved in different classes of ATP/GTPases (12). Like other mitochondrial proapoptotic proteins such as Smac/Diablo and Omi/H2ra, ARTS induces apoptosis through binding to and antagonizing IAPs[1](inhibitors of apoptosis proteins) (11). However, the mechanism by which ARTS antagonizes IAPs seems to be distinct because ARTS does not contain the characteristic IAP-binding motif of the Reaper family proteins (13, 14). Upon apoptotic stimuli, ARTS is released from mitochondria, binds XIAP, and decreases its protein levels. As a result, caspase inhibition is removed and apoptosis can be executed (11). More recently, it has been shown that ARTS functions as a tumor suppressor in leukemia (15). Furthermore, inactivation of the *Sept4* gene in mouse, which encodes ARTS, reveals a critical function of this gene in late spermeogenesis (16). In particular, this study supports an important physiological role of ARTS in caspase regulation through IAP-inhibition.

The ubiquitin-proteasome pathway plays a central role in the regulation of essential cellular processes, including the regulation of apoptosis (17). In particular, many IAPs encode E3-ubiquitin ligases that ubiquitinate key apoptotic proteins including caspases, Bcl-2 family members, and IAPs themselves (18–21). Upon the induction of apoptosis, IAPs such as XIAP, c-IAP1, and DIAP1, auto-ubiquitinate and are rapidly degraded, thereby removing a major blockade of caspase activity (18, 19, 22).

In this study, we demonstrate that high levels of ARTS protein are sufficient to induce apoptosis and that elevated ARTS levels can sensitize cells toward apoptosis. This suggests that healthy cells may employ regulatory mechanisms that keep ARTS protein levels low for survival. Moreover, we show that the ARTS protein is tightly regulated through proteasome-mediated ubiquitination. Upon proapoptotic stimuli, inhibition of ubiquitination results in increased levels of ARTS. Elevated ARTS, in turn, leads to decreased levels of Bcl-2 and Bcl-x_L proteins and to cytochrome *c* release and apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents—Media, serum, antibiotics, and supplements were purchased from Biological Industries (Beit Haémek, Israel). TGF-β was a kind gift from Dr. Anita B. Roberts. Etoposide and staurosporine (STS) were purchased from Sigma. Cycloheximide and MG132 (carbobenzoxyl-Leu-Leu-leucinal) were purchased from Calbiochem. Monoclonal and polyclonal antibodies specifically aimed at the unique C′ terminus of ARTS were purchased from Sigma-Aldrich. All other reagents used were from Sigma-Aldrich.

Cell Lines and Transfections—COS-7 and HeLa cell lines were grown in Dulbecco's modified Eagle's medium, and SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium/F12 medium, supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100

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[1] The abbreviations used are: IAP, inhibitor of apoptosis; AMC, 7-amino-4-methylcoumarin; BOC, benzyloxycarbonyl-Asp(Ome)-fluoromethyl ketone; E3, ubiquitin-activating enzyme; IRES, internal ribosome entry site; MG132, carbobenzoxyl-Leu-Leu-leucinal; PBS, phosphate-buffered saline; Smac, second mitochondria-derived activator of caspase; STS, staurosporine; TGF-β, transforming growth factor β; TUNEL, TdT-mediated dUTP-biotin nick end labeling; XIAP, X-linked inhibitor of apoptosis protein; ATPγS, adenosine 5′-*O*-(thiotriphosphate); TMR, tetramethylrhodamine.

μg/ml), and glutamine (2 mM) at 37 °C in 5% CO_2 atmosphere. Cells were transiently transfected using the electroporation method (EasyjecT Plus, Equibio) and the Lipofectamine method (Invitrogen).

Plasmid Constructs—Vectors pEF1-AU5 and pEF1-AU5-ARTS were used for all of the transient transfection experiments (10). For the stable transfection we generated a new construct based on a pEF1-IRES vector (kindly provided by S. Hobbs), which was found to be efficient for stable expression of proteins in mammalian cells (23). This plasmid contains cDNA encoding AU5-tagged ARTS and puromycin resistance gene, spaced by an encephalomyocarditis internal ribosome entry site (IRES) termed pE ARTS. In this construct cDNA of ARTS was taken from pEF1-AU5-ARTS by XhoI digestion followed by insertion into XhoI sites of pEF1-IRES, downstream to the EF-1 promoter. Empty pEF1-IRES, which served as the control vector, is termed pE. Sequence and orientation were verified by sequencing reaction. Vector expressing green fluorescent protein (pEGFP-N1, Clontech) was used to visualize co-transfected cells.

Establishment of COS pE ARTS Stable Cell Line—COS-7 cells stably transfected with pE ARTS and pE vector using the Lipofectamine method resulted in stable cell lines termed COS pE ARTS and COS pE, respectively (23).

Apoptotic Stimulation—For apoptosis assays, 40 h after the transient transfection cells were treated with different agents: TGF-β (10 ng/ml) for 24 h in medium containing 1% fetal calf serum, etoposide 50 μg/ml for 16 h, or staurosporine (1–1.2 μM) for 0–8 h.

Cell Death Detection Assay—An apoptosis assay was performed according to the manufacturer's instructions. Briefly, an equal amount of cells was taken for each experiment (determined by cell count). The cells were lysed, and 20 μl were transferred to a 96-well plate precoated with anti-histone together with antibody to DNA. The binding reaction was performed at room temperature for 2 h. The wells were washed three times, and substrate was added for 10 min allowing a color reaction to develop. The color reaction levels were measured using a 405-nm filter. The color level was in direct correlation with the apoptosis level in each sample (Roche Applied Science).

Caspase 9 Activity Assay—Apoptosis assay was performed according to the manufacturer's instructions. Briefly, an equal amount of cells was taken for each experiment (determined by cell count). The cells were lysed, and 20 μl were taken for further analysis. 10 μl of LEHD-AMC (a specific synthetic substrate for caspase 9 covalently attached to AMC molecule) was added, and after 2 h at 37 °C, the amount of free AMC was measured using a spectrophotometer at excitation filter 370–425 nm and emission filter 490–530 nm. The free AMC levels were in direct correlation to caspase 9 activity in each sample (R&D Systems).

Immunofluorescence Microscopy—For the Immunofluorescence microscopy, 24-h pretransfection 2×10^5 cells were seeded on cover slides (2×2 cm) covered with fibronectin inside 6-well plates. Cells were treated with different apoptotic stimulations at the indicated times and fixed with 4% paraformaldehyde in 1× PBS for 20 min at room temperature. Fixed cells were washed with 1× PBS and then permeabilized with 0.5% Triton-X in 1× PBS for 5 min. The cover slides were treated with primary antibody, diluted in 5% bovine serum albumin in 1× PBS for 2 h at room temperature. They were then washed three times with 1× PBS/0.1% Triton-X and incubated with fluorescein isothiocyanate-conjugated anti-mouse (Jackson ImmunoResearch) and Rhodamine^{TX}-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch) for 1 h at room temperature. The cells were washed three times with 1× PBS/0.1% Triton-X and mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Cells were examined with a laser-scanning microscope, Provis AX70 (Olympus), and counted in a blind manner, 200 cells each time. The assay was repeated independently three times.

Caspase 3 Activity Assay—Cells attached to cover slides were fixed and double probed with antibodies against ARTS or AU5 and against the active form of caspase 3. For each experiment, 200 ARTS/AU5-positive cells were counted, and the cells that were positive for active caspase 3 were considered as apoptotic cells.

In Situ TUNEL-TMR—In the apoptotic process, the DNA strands break in a unique fashion exposing a 3′-OH end and serve as a marker. Cells attached to cover slides were fixed and permeabilized. The apoptotic cells were labeled by enzymatic reaction between nucleotides attached to TMR and the DNA broken strands. The cells were then probed to antibody against ARTS/AU5. For each experiment, 200 ARTS/AU5-positive cells were counted, and the cells that were TMR-positive were considered as apoptotic cells (Roche Applied Science).

Cell Lysates—In all of the experiments cell lysates were prepared from floating dead cells and adherent cells harvested together.

40 h after the transfection, the cells were treated with different reagents according to the indicated assay. The cells were harvested by scraping the plate, washed twice with ice-cold 1× PBS, and lysed using radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate containing protease inhibitors) (mini complete, Roche Applied Science). The cells were allowed to remain on ice for 30 min followed by four cycles of freeze and thaw. The cell extract was centrifuged for 20 min at maximum speed (13,000 rpm) 4 °C, and the supernatant was collected. Protein concentration was determined using the BCA kit (Promega).

Subcellular Fractionation—Cells were harvested by scraping, washed twice in ice-cold PBS, and resuspended in 600 μl of homogenizing buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium-EGTA, and 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and mini complete protease mixture). After 30 min of incubation on ice, cells were homogenized in the same buffer using a glass homogenizer (Dounce tissue grinder, at least 80 strokes). Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifugation at $500 \times g$ for 5 min at 4 °C. The resulting supernatant was centrifuged at $10{,}000 \times g$ for 20 min at 4 °C to obtain a mitochondria-containing pellet. The remaining supernatant was considered as the cytosolic fraction. The mitochondria pellet was washed three times in homogenizing buffer, and 50 μl of TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 5 mM $CaCl_2$) containing protease inhibitors was added (24). Protein concentration was determined using a Micro-BCA kit (Pierce).

Western Blotting Analysis—Proteins were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 3% nonfat milk in 1× Tris-buffered saline containing 0.05% Tween for 1 h at room temperature, incubated with the primary antibody at room temperature for 3 h, and washed three times with 1% nonfat milk in 1× Tris-buffered saline containing 0.05% Tween followed by incubation with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The membrane was developed using the enhanced chemiluminescence detection system (Pierce). To quantify the protein levels, the blots were analyzed using a densitometer and compared with the level of a housekeeping gene (β-actin). Calculations were carried out using densitometry-analyzing software (Vilber Lourmat, Marne la Vallée, France).

Half-life Assay—COS-7 cells were transiently transfected with pEF1-AU5-ARTS and were plated in 100-mm plates. 40 h after the transfection, the cells were treated with 120 μg/ml cycloheximide for 0, 15, 30, 60, 90, 120, and 150 min. The cells were harvested, and 50 μg of protein lysate were separated on 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-AU5 monoclonal antibodies. The membrane was then stripped using the Re-prob kit (Chemicon) and probed with anti-actin antibodies. The protein levels were calculated as mentioned above, and the values of ARTS-AU5 were plotted against the values of actin. The value of ARTS/actin at time 0 was taken as 100%.

Pulse-Chase Analysis—COS-7 cells were transiently transfected with pEF1-AU5-ARTS and plated on 60-mm plates until they reached full confluency. The cells were washed twice with Hanks' solution at 37 °C, and 1 ml of "starvation medium" at 37 °C (without Met/Cys, containing dialyzed fetal calf serum and antibiotics) was added for 1 h. 10 μl of [^{35}S]Met and [^{35}S]Cys were added for 1 h followed by two washes with Hanks' solution at 37 °C. Complete medium (containing 300 μg/ml Met/Cys) was added to the cells for the indicated times. Cells were harvested by scraping in 1 ml of ice-cold 1× PBS and collected into an Eppendorf tube followed by centrifugation at 3000 rpm for 2 min at 4 °C. 200 μl of cold radioimmune precipitation assay buffer was added, and lysates were put on ice for 30 min with stirring. After centrifugation ($16{,}000 \times g$ for 15 min at 4 °C), equal amounts of protein were taken for immunoprecipitation with monoclonal anti-ARTS antibodies (Sigma).

Immunoprecipitation Assay—For each time point, an equal amount of protein was taken with 10 μl of anti-ARTS antibodies and placed on ice for 4 h. 25 μl of protein G-Sepharose beads (Amersham Biosciences; 20% slurry washed five times with 1× PBS) was added to the reaction and placed at 4 °C for 2 h with constant rotation. The beads were collected by spinning at maximum speed for 20 s, the supernatant was aspirated, and the beads were washed four times with radioimmune precipitation assay buffer. All of the washing steps were performed at 2000 rpm for 2 min at 4 °C. The bound proteins were separated from the beads by adding 20 μl of 1.5× loading sample buffer and boiling for 5 min at 100 °C. The proteins were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, and visualized by exposing the membrane to film sensitive to ^{35}S.

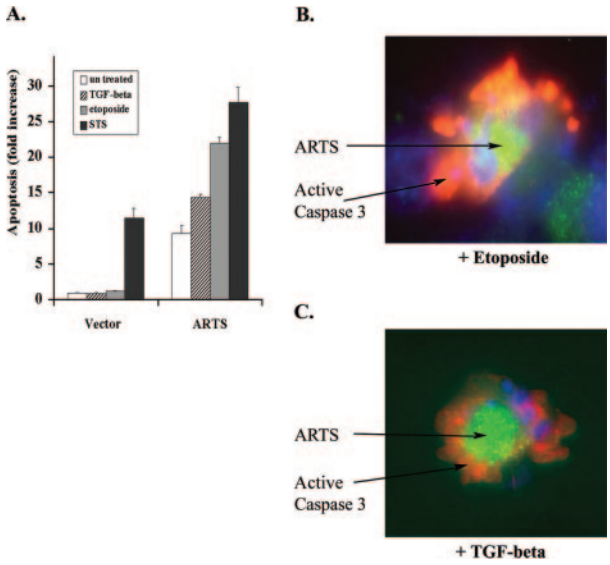

FIG. 1. **ARTS sensitizes cells to apoptosis in response to a variety of apoptotic stimuli through activation of caspase 3.** *A*, COS-7 cells were transiently transfected with either control vector or AU5-ARTS construct and treated with various apoptotic stimuli including: TGF-β (10 ng/ml for 24 h), etoposide (50 μM for 16 h), and STS (1.2 μM for 4 h). Apoptosis was measured using anti-caspase 3 active antibodies or TUNEL-positive staining in cells expressing ARTS. The results are presented as -fold increase relative to results obtained in nontreated vector-transfected cells (calculated as 1). Cells treated with etoposide (*B*) or TGF-β (*C*) show typical morphological changes associated with apoptosis. *B*, immunofluorescence staining of etoposide-treated HeLa cells expressing AU5-ARTS. Morphological changes include membrane blebbing, cytoplasmic staining with anti-active caspase 3 (*red*), DNA condensation (*blue*), and ARTS translocation to the nucleus (*green*). *C*, COS-7 cell after TGF-β treatment exhibit similar morphological changes.

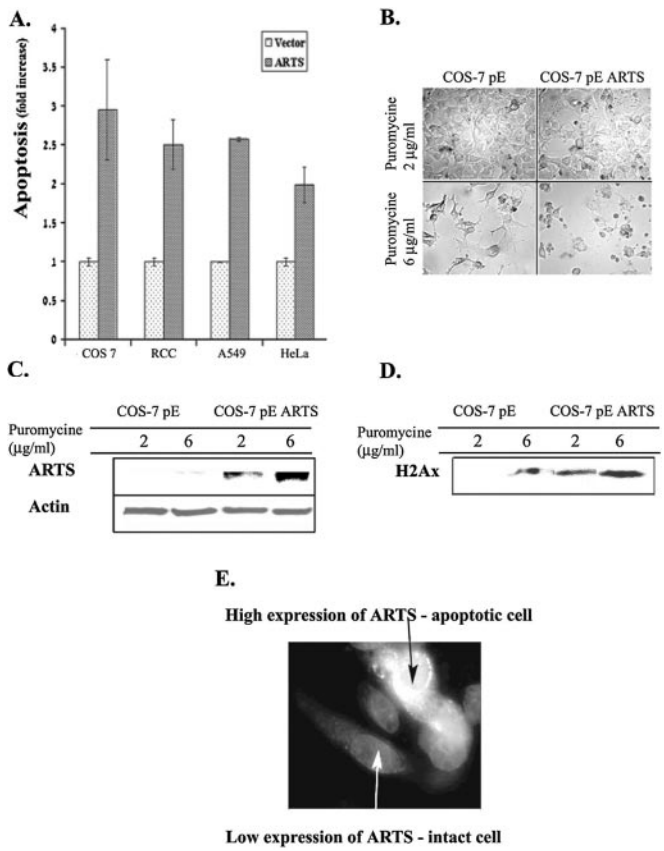

FIG. 2. **High protein levels of ARTS are sufficient to induce apoptosis in various tumor cells.** *A*, four different cell lines, COS-7, RCC, A549, and HeLa, were transfected with either control vector or AU5-ARTS constructs. Cells were treated with TGF-β, and apoptosis levels were determined using a cell death detection kit (Roche Applied Science) for COS-7 and RCC cell lines or the caspase 9 activity assay kit (R&D Systems) for A549 and HeLa cell lines. High levels of apoptosis were found in all cells transfected with AU5-ARTS. Results are presented as -fold increase relative to results obtained in nontreated vector transfected cells (calculated as 1). *B*, COS-7 cells stably transfected with pE control vector or pE-ARTS were incubated with medium containing 2 or 6 μg/ml puromycin. The higher dose of puromycin resulted in cell death only in pE-ARTS cells and not in pE control cells. *C*, ARTS expression is in direct correlation with increased puromycin levels. COS-7 cells stably transfected with pE control vector or pE-ARTS, were incubated with media containing 2 or 6 μg/ml puromycin. Cells stably expressing pE-ARTS show three times higher levels of ARTS when grown in the presence of higher levels of puromycin (6 μg/ml) as compared with cells grown in the presence of low levels of puromycin (2 μg/ml). ARTS expression in control pE cells treated with puromycin was below detection. *D*, apoptosis rate increases with elevation of ARTS levels. COS-7 cells stably transfected with pE control vector or pE-ARTS were incubated with medium containing 2 or 6 μg/ml puromycin. In control pE cells, the H2Ax apoptotic marker is seen only when treated with 6 μg/ml. Apoptosis is evident in pE-ARTS cells even with treatment of 2 μg/ml puromycin and increases with 6 μg/ml treatment. *E*, COS-7 cells stably transfected with pE-ARTS show different ARTS expression levels. Cells that show high levels of ARTS exhibit typical morphological changes associated with apoptosis. Cells that show mild or low expression levels of ARTS appear intact.

Ubiquitination in Vivo—COS-7 cells were transiently transfected with pEF1-AU5-ARTS and plated on 100-mm plates. 40 h after the transfection, half of the plates were treated with 20 μg/ml MG132 (Calbiochem) for 0, 0.5, 1, 1.5, 2, 3, and 4 h. The cells were harvested and lysed as described above. The protein levels were calculated as mentioned above, and the values were plotted against the values of actin. The value of ARTS/actin at time 0 was taken as 1.

"Hot Lysis" Immunoprecipitation Assay—COS-7 cells were transiently transfected with pEF1-AU5-ARTS and treated with MG132 (20 μM) for 6 h. The medium was aspirated and the cells washed three times with 1× PBS (37 °C). The cells were scraped in 500 μl of HLB buffer (1% SDS, 1 mM EDTA in 1× PBS) at 37 °C, boiled for 5 min, and cooled on ice for 3 min. The cells were homogenized by passing through a 25-gauge needle (3–5 times), boiled for 3 min and cooled on ice, vortexed, and centrifuged for 5 min (maximum speed) at room temperature. The supernatant was transferred into clean Eppendorf tubes, and 1 volume of IMS buffer (2% SDS, 0.5% sodium deoxycholate, 1% bovine serum albumin, 1 mM EDTA with protease inhibitors; mini complete, Roche Applied Science) and 10 μl of monoclonal anti-ARTS antibodies (Sigma) were added. The mixture was incubated for 2 h on ice and added to 25 μl of A/G Sepharose mix (50% slurry; Amersham Biosciences). The mix was incubated for 2 h at 4 °C and spun down (20 s at 14,000 rpm). After two washes with IM buffer (1% Triton X 100, 1% SDS, 0.5% sodium deoxycholate, 1% bovine serum albumin, 1 mM EDTA in 1× PBS) and two washes with 0.1× PBS, the proteins were eluted by adding 15 μl of sample 1× buffer and boiled for 5 min.

In Vitro Ubiquitination Assay—In vitro conjugation of ARTS was carried out in a total volume of 12.5 μl containing 50 μg of whole cell lysate proteins, 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mg of ubiquitin, 0.5 μg of Ub-Al, and 2 mM ATPγS. For depletion of ATP, 0.5 μg of hexokinase and 20 mM 2-deoxy-D-glucose were added. The reaction mixtures were carried out at 37 °C for 50 min. Reactions were terminated by the addition of sample buffer and resolved by SDS-PAGE.

RESULTS

ARTS Promotes Apoptosis Induced by Various Proapoptotic Stimuli—ARTS was initially found to be important for TGF-β induced apoptosis (10). To test whether ARTS promotes apoptosis in response to other proapoptotic inducers, COS-7 cells transiently transfected with either ARTS expression vector or empty control plasmids were treated with TGF-β, etoposide, or STS. In the absence of transfected ARTS, COS-7 cells were basically resistant to both TGF-β and etoposide. Cells overexpressing ARTS showed a 9-fold increase of "background" apoptosis (*i.e.* without any additional apoptotic stimuli), and these cells showed even higher levels of apoptosis upon treatment with apoptotic inducers (Fig. 1*A*). Similar results were obtained for a variety of tumor cell lines (Fig. 2*A*). Over-expres-

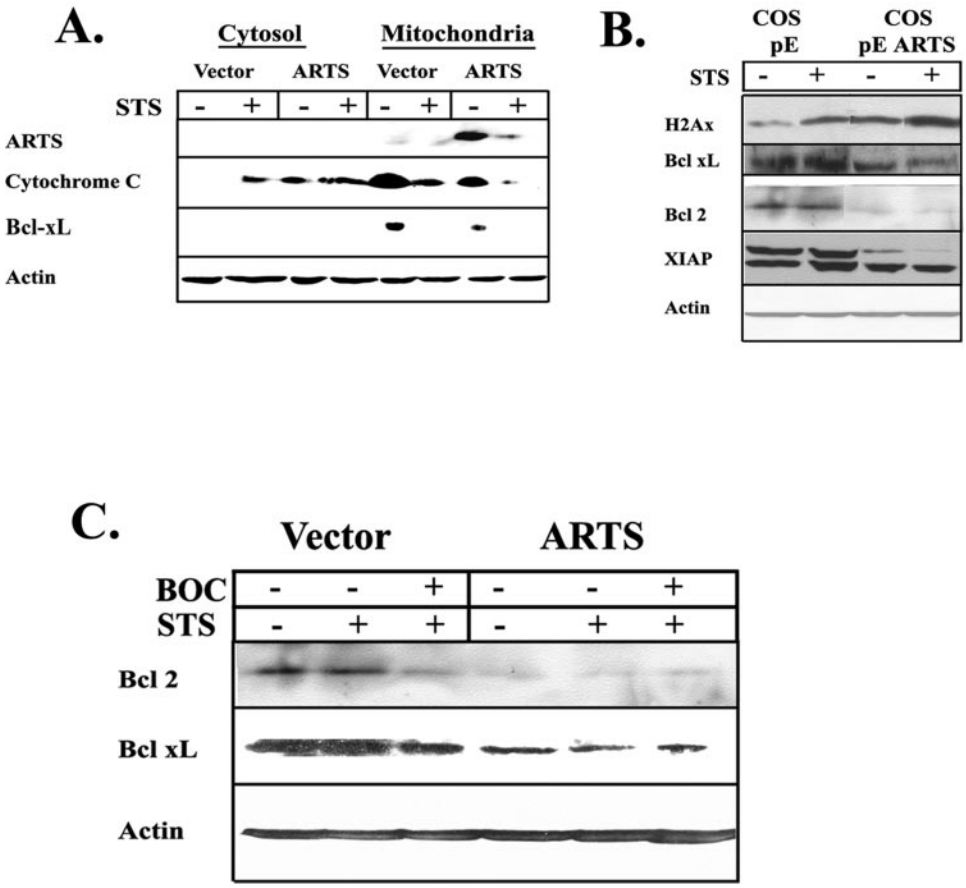

FIG. 3. **ARTS-induced apoptosis is accompanied by release of cytochrome *c* and down-regulation of Bcl-2 and Bcl-x_L.** *A*, Western blot analysis of cytosolic and mitochondrial fractions of COS-7 cells transiently transfected with AU5-ARTS or AU5 empty vector with or without treatment of 1.2 μM STS. ARTS over-expression alone is sufficient to induce cytochrome *c* release to the cytosol and Bcl-x_L down-regulation. *B*, Western blot analysis of stably transfected COS pE-ARTS and COS pE cell lines with and without 1.2 μM STS. pE-ARTS cells exhibit lower levels of the anti-apoptotic proteins Bcl-2, Bcl-x_L, and XIAP as compared with pE control cells. Apoptosis levels (as evaluated by H2Ax, apoptotic marker staining) in the nontreated pE-ARTS cells are similar to those shown in the STS-treated control cells. A much higher apoptosis rate is revealed in STS-treated pE-ARTS cells. *C*, ARTS over-expression causes Bcl-2 and Bcl-x_L down-regulation in a caspase-independent manner. COS-7 cells transiently transfected with AU5-ARTS or AU5 empty vector were incubated with 40 μM BOC for 2 h prior to STS induction. Western blot analysis revealed that ARTS over-expression results in Bcl-2 and Bcl-x_L down-regulation even without STS induction. The addition of BOC to these cells had no impact on down-regulation of Bcl-2 and Bcl-x_L.

sion of ARTS in RCC, A549, and HeLa cells all caused significantly increased apoptosis. Therefore, over-expression of ARTS increases the susceptibility of cells toward apoptotic inducers, and high levels of ARTS are sufficient to induce cell death in a variety of cultured cell lines. ARTS was originally found to promote apoptosis induced by TGF-β (10). Here we show that elevated levels of ARTS promote apoptosis in response to other apoptotic inducers, including etoposide and STS.

We have previously shown that ARTS promotes caspase activation in response to TGF-β treatment and that the ARTS protein translocates from mitochondria to the nucleus under these conditions (Ref. 10 and Fig. 1*C*). Likewise, etoposide treatment of HeLa cells over-expressing ARTS resulted in caspase 3 activation and translocation of ARTS to the nucleus (Fig. 1*B*). Virtually identical results were obtained for arabinoside-C and STS (data not shown). Thus, the apoptotic response of cells over-expressing ARTS was overall very similar for TGF-β, etoposide, and STS. In all cases, ARTS promoted the activation of caspase 9 and caspase 3, the release of cytochrome *c* from the mitochondria, and translocation of ARTS to the nucleus. We conclude that different apoptotic stimuli converge to activate a common pathway in which ARTS is released from mitochondria and promotes caspase activation and cell death.

ARTS Can Induce Apoptosis in Stably Transfected COS-7 Cells—To further investigate the effect of ARTS protein levels on apoptosis, we established a stably transfected COS-7 cell line expressing ARTS under a puromycin-inducible promoter (COS-7 pE ARTS; see "Materials and Methods"). As a control, we also established a COS-7 cell line stably transfected with empty pE vector (COS-7 pE cells). Treatment of COS-7 pE ARTS cells with low levels of puromycin (2 μg/ml) resulted in a modest increase of ARTS protein, but these levels were not sufficient to induce a significant increase of apoptosis compared with controls (Fig. 2, *B* and *C*). On the other hand, elevated levels of puromycin (6 μg/ml) resulted in higher ARTS protein levels (Fig. 2*C*) and prominent cell death (Fig. 2*B*). Immunofluorescence assays were used to confirm that cells expressing high levels of ARTS exhibited condensed nuclear DNA typical for apoptosis, whereas cells with low ARTS levels showed morphologically intact nuclei (Fig. 2*E*). Similarly, an increase in levels of the phosphorylated histone H2Ax, an apoptotic marker (25), was seen in response to induction of ARTS in COS-7 pE ARTS cells (Fig. 2*D*). Stably transfected COS-7 pE ARTS cells treated with 2 or 6 μg/ml puromycin eventually acquired the ability to grow, but they displayed high steady state levels of background apoptosis (Fig. 2*D*). These results

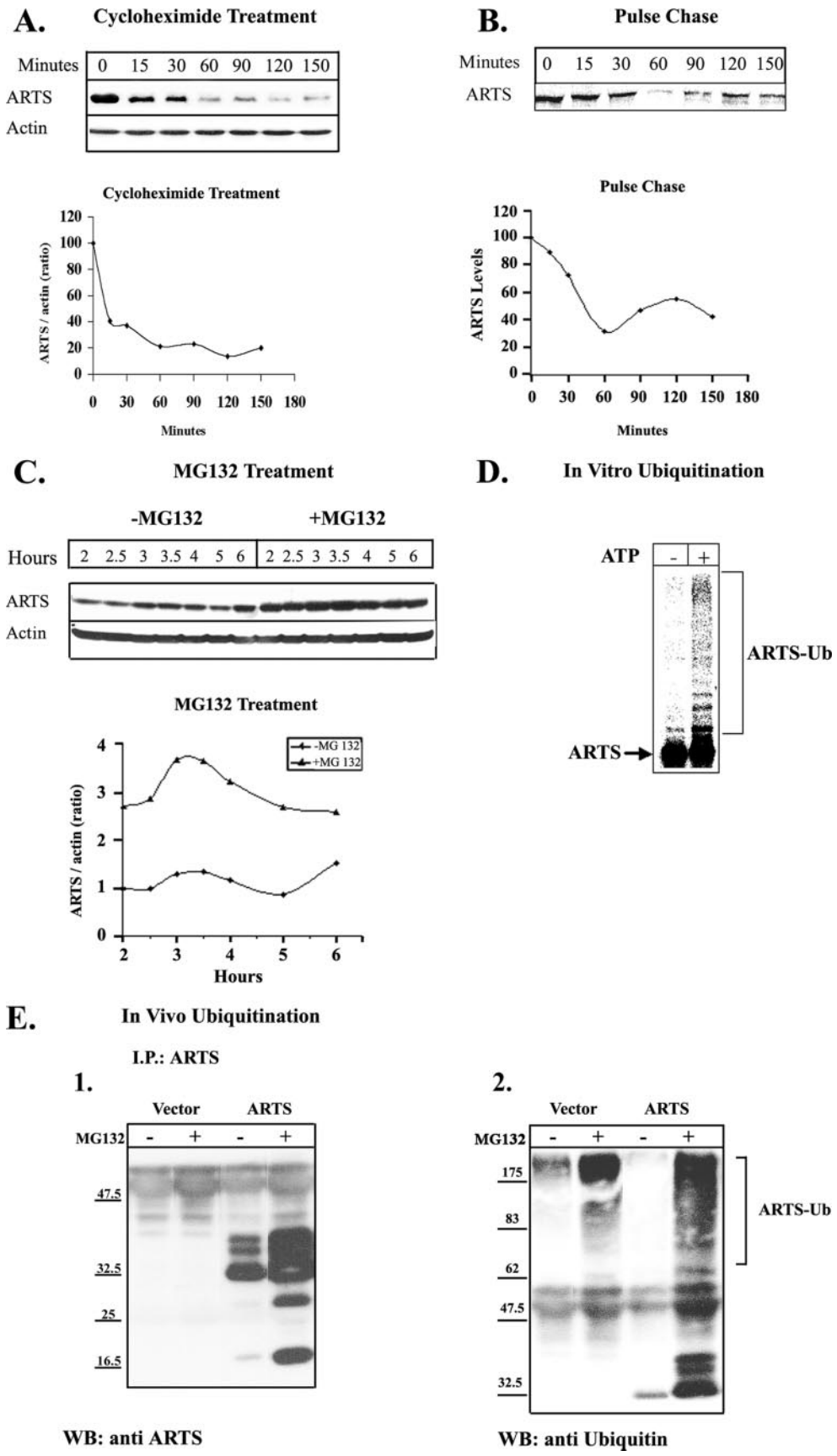

FIG. 4. **ARTS protein levels are tightly regulated through ubiquitin mediated degradation.** *A*, ARTS half-life is ~30 min. COS-7 cells were transiently transfected with AU5-ARTS and treated with 120 μg/ml cycloheximide. Western blot analysis was performed using anti-AU5 antibodies. The membrane was stripped and reblotted with anti-actin. The graph represents ARTS/actin ratio levels as measured by densitometry. ARTS levels at time 0 were calculated as 100%. *B*, pulse-chase assay. COS-7 cells were transiently transfected with AU5-ARTS and labeled with ^{35}S for 60 min. The cells were harvested, lysed, and subjected to immunoprecipitation using monoclonal anti-ARTS antibodies. The graph

provide additional support for the idea that ARTS sensitizes cells toward apoptosis.

High Levels of ARTS Lead to a Decrease in Bcl-2 and Bcl-x_L Levels, Cytochrome c Release from Mitochondria, and Apoptosis—The release of cytochrome *c* from mitochondria to the cytosol is an event often considered as a commitment of the cell toward apoptotic death (6, 26, 27). We have shown that ARTS is also released from mitochondria to the cytosol upon proapoptotic induction, and its release appears to precede that of cytochrome *c* (11, 28). To explore whether ARTS over-expression can stimulate cytochrome *c* release, we transiently transfected COS-7 cells with ARTS and used cell fractionation to assess the distribution of cytochrome *c* (Fig. 3*A*). As expected, cytochrome *c* was detected in the cytosol upon treatment with STS in both untransfected control cells and cells over-expressing ARTS, and a corresponding decrease of cytochrome *c* levels was seen in the mitochondrial fraction. Significantly, over-expression of ARTS was sufficient to trigger the release of cytochrome *c* from mitochondria to the cytosol without any proapoptotic stimulus (Fig. 3*A*). Next we asked whether over-expression of ARTS had any effect on the levels of Bcl-2 and Bcl-x_L. Bcl-2 family proteins have been shown to control the release of cytochrome *c* from mitochondria (29–31), and down-regulation of Bcl-2 and Bcl-x_L, two anti-apoptotic mitochondrial Bcl-2 family members, has been associated with apoptosis (32, 33). We found that over-expression of ARTS by transient transfection of COS-7 cells led to reduced levels of Bcl-x_L in mitochondria (Fig. 3*A*). Further support for the idea that ARTS levels influence Bcl-2 and Bcl-x_L protein levels came from analyses of COS pE ARTS cells. In these stably transfected cells, both Bcl-x_L and Bcl-2 levels were much lower compared with COS pE control cells (Fig. 3*B*). Likewise, consistent with previous findings using transient transfection assays (11), XIAP levels were significantly reduced in COS pE ARTS cells, and the apoptotic marker H2Ax was increased compared with control COS pE cells (Fig. 3*B*). Next we considered the possibility that the observed decrease of Bcl-2 and Bcl-x_L was merely the consequence of caspase activation and apoptosis. If so, the decrease in Bcl-2 and Bcl-x_L levels in response to over-expression of ARTS should be blocked by caspase inhibitors. COS-7 cells transiently transfected with ARTS were treated with the wide range caspase inhibitor BOC, and Bcl-2 and Bcl-x_L levels were examined in these cells before and after induction of apoptosis (Fig. 3*C*). Whereas control cells had easily detectable levels of Bcl-2 and Bcl-x_L, COS-7 cells over-expressing ARTS had again significantly lower levels of both proteins. Importantly, this decrease was not at all blocked by BOC. This suggests that the reduction of Bcl-2 and Bcl-x_L by ARTS is not simply a consequence of caspase activity and/or apoptosis, but rather it appears to be mediated through a more direct effect of ARTS on these proteins.

ARTS Is a Highly Labile Protein with a Very Short Half-life in Healthy Cells—Besides sensitizing cells toward apoptosis, high level expression of ARTS was sufficient to induce apoptosis in the absence of any further proapoptotic stimulus in many cell lines (Fig. 2*A*). Presumably, high level expression of ARTS can overcome regulatory safeguards that normally prevent unwanted induction of apoptosis by endogenous ARTS. Therefore, keeping ARTS protein at low levels in living cells would become a necessary task to prevent the cell from undergoing apoptotic death. Consistent with this idea, we found that ARTS levels are strictly controlled through the ubiquitin-proteasome-mediated pathway.

In healthy cells, ARTS localizes to mitochondria and is virtually nondetectable in the cytoplasm. Upon apoptotic stimuli, over-expressed ARTS can sometimes be detected in the cytosol (11). We considered the possibility that ARTS is a labile protein in the cytosol and that its cytosolic levels may be regulated via ubiquitin-mediated protein degradation. Therefore, we determined the half-life ($t_{½}$) of ARTS in cycloheximide-treated COS-7 cells over-expressing ARTS (Fig. 4). A dramatic reduction (60%) in ARTS levels was detected 30 min after cycloheximide treatment (Fig. 4*A*). Pulse-chase experiments confirmed these results and indicated that ARTS has a $t_{½}$ of about 30 min (Fig. 4*B*). Thus, ARTS is a very short-lived protein in healthy cells.

ARTS Protein Levels Are Regulated through Ubiquitin-mediated Protein Degradation—Short-lived proteins are typically degraded by the proteasome (34–36). To further examine the mechanism involved in the regulation of ARTS protein levels, we pretreated the cells with MG132, a potent proteasome inhibitor. Upon MG132 treatment, ARTS levels were increased almost 3-fold after 2 h and almost 4-fold after 3 h of treatment. (4C). This suggests that ARTS is degraded by the proteasome. Next, we investigated whether ARTS is ubiquitinated *in vitro* (Fig. 4*D*) and *in vivo* (Fig. 4*E*). In this *in vitro* ubiquitination assay we used extracts from nontreated HeLa cells. HeLa cells contain high levels of the ubiquitin-proteasome component and are commonly used for these assays. We found that ARTS is ubiquitinated in this system (Fig. 4*D*). To confirm the relevance of these results *in vivo*, we pretreated HeLa cells over-expressing ARTS with MG132 for 6 h (see "Materials and Methods") to allow accumulation of potential polyubiquitinated forms of ARTS. ARTS was immunoprecipitated from lysates of these cells, and Western blot analyses with antibodies against ARTS and ubiquitin were performed (Fig. 4*E*). These experiments revealed a strong increase in ARTS levels following treatment of cells with proteasome inhibitor (Fig. 4*E*, *1*). Furthermore, both endogenous and over-expressed ARTS underwent constant ubiquitination, as revealed by the appearance of high levels of the ARTS-ubiquitin-conjugated forms (Fig. 4*E*, *2*). These results suggest that cellular levels of ARTS are regulated through ubiquitin-mediated proteasome degradation.

Moreover, in healthy cells virtually all ARTS protein appears to reside within mitochondria. Given this localization, the short half-life of ARTS is very surprising, because proteins inside mitochondria are thought to be protected from ubiquitination (17, 37, 38). One possible explanation for our findings is that there is constantly some "leakage" of ARTS protein from mitochondria into the cytosol. According to this model, steady-state

represents ARTS levels as measured by densitometry. ARTS levels at time 0 were calculated as 100%. *C*, ARTS levels are regulated through the ubiquitin-proteasome pathway. COS-7 cells transiently transfected with AU5-ARTS and treated with MG132 (proteasome inhibitor). The time intervals represent the harvest times (after MG132 treatment). Western blot analysis was performed using anti-AU5 antibodies. The graph represents ARTS/actin ratio levels as measured by densitometry. ARTS/actin levels at time 0 were calculated as 1. Treatment of ARTS transfected COS-7 cells with MG132 proteasome inhibitor resulted in a 2.8-fold increases in ARTS protein levels. *D*, *in vitro* ubiquitination of ARTS. ARTS protein, translated and labeled with ^{35}S was produced using the *in vitro* transcription and translation kit (Promega). This protein was used as a substrate in an *in vitro* ubiquitination assay performed in the presence of 50 μg of total lysate from HeLa cells with or without ATP. The ARTS-ubiquitin conjugates formed are indicated, demonstrating that ARTS is a target for the ubiquitin system in living cells. *E*, *in vivo* ubiquitination of ARTS. HeLa cells were transiently transfected with AU5-ARTS or AU5 empty vector and treated with 20 μM MG132 for 6 h. The cells were harvested and subjected to immunoprecipitation (*I.P.*) using anti-ARTS monoclonal antibodies. Western blot analysis was performed with anti-ARTS antibodies (*panel 1*) to confirm ARTS expression. The membrane was stripped and reblotted (*WB*, Western blot) with anti-ubiquitinantibodies (*panel 2*). In living cells, ARTS is conjugated to ubiquitin, forming high molecular weight ARTS-ubiquitin conjugates.

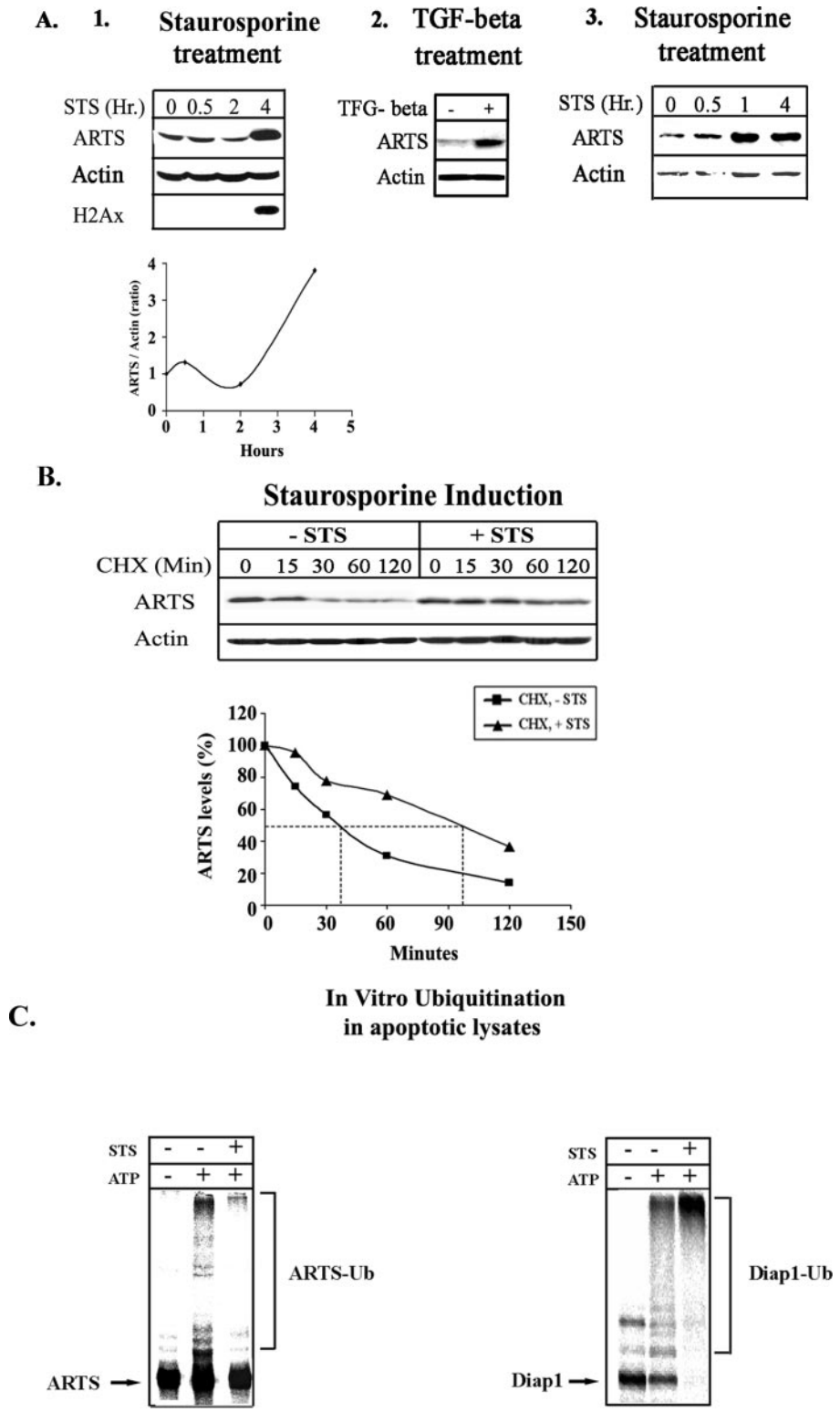

FIG. 5. **ARTS levels are elevated in response to apoptotic induction via inhibition of ubiquitin-mediated degradation.** *A*, ARTS levels are up-regulated during apoptosis. *1*, COS-7 cells were transiently transfected with AU5-ARTS and treated with 1.2 μM STS. Western blot analysis was performed using monoclonal anti-ARTS antibodies. The membrane was stripped and reblotted with anti-H2Ax used as apoptotic marker and anti-actin. The graph presents ARTS/actin ratio levels as measured by densitometry. ARTS/actin levels at time 0 were calculated as 1. Following 4 h of STS induction, ARTS levels were strongly up-regulated, and apoptosis was demonstrated by the appearance of H2Ax apoptotic marker. *2*, COS-7 cells were transiently transfected with AU5-ARTS and treated with 10 ng/ml TGF-β for 24 h. Western blot analysis was performed using monoclonal anti-ARTS antibodies. The membrane was stripped and reblotted with anti-actin. *3*, SH-SY5Y cells were treated with 1.2 μM STS. Western blot analysis was performed using monoclonal anti-ARTS antibodies. The membrane was stripped and reblotted with anti-actin. *B*, ARTS protein gains higher stability during apoptosis. COS-7 cells were transiently transfected with AU5-ARTS and treated/not treated with 1.2 μM STS prior to cycloheximide (*CHX*) treatment (120 μg/ml). Western blot analysis was performed using anti-AU5 antibodies. The membrane was stripped and reblotted with anti-actin. The graph represents ARTS/actin levels as measured by densitometry. ARTS levels at time 0 were calculated as 100%. ARTS half-life in living cells was found to be ~30 min (*lower line*), but under apoptotic conditions the half-life of ARTS protein was tripled to more than 90 min (*upper line*). *C*, ARTS *in vitro* ubiquitination is inhibited

levels of ARTS in healthy cells would be maintained by balancing the mitochondrial import of newly synthesized protein with its efflux and immediate ubiquitination/degradation of ARTS in the cytosol. Alternatively, not all ARTS protein may be imported into mitochondria. Consistent with either model, ARTS protein is virtually nondetectable in the cytoplasm of healthy cells, suggesting that it has an extremely short half-life in the cytosol.

ARTS Levels Are Increased during Apoptosis through Inhibition of Ubiquitination—Under apoptotic conditions ARTS cytosolic levels are elevated, and ARTS can be found in a complex with XIAP (11). We investigated the possibility that ARTS protein levels are increased in cells stimulated to undergo apoptosis. For this purpose, we used STS to induce apoptosis in COS-7 and in cells transiently transfected with ARTS (Fig. 5*A*). After 4 h of treatment with STS, the apoptotic marker H2Ax became detectable, and ARTS levels were increased 4-fold at this time (Fig. 5*A*, *1*). Significant increase in ARTS levels were also detected when cells were treated with TGF-β for 24 h (Fig. 5*A*, *2*). Moreover, a strong elevation in ARTS endogenous levels is shown in SH-SY5Y cells treated with STS for as briefly as 1 h (Fig. 5*A*, *3*). This further indicates that both endogenous as well as exogenous ARTS protein levels are increased in response to apoptotic induction. Next we examined whether the half-life of ARTS changes in response to STS treatment. For this purpose, we treated COS-7 cells with cycloheximide to determine the half-life ($t_{1/2}$) of ARTS under apoptotic and non-apoptotic conditions (Fig. 5*B*). As previously shown (Fig. 4*A*), the half-life of ARTS was ~30 min under non-apoptotic conditions. However, STS treatment of cells prolonged the half-life of ARTS to ~90 min (Fig. 5*B*). Therefore, the increased level of ARTS protein in response to the induction of apoptosis appears to be caused, at least in part, by increased stability of ARTS.

To investigate the mechanism underlying increased ARTS protein stability under apoptotic conditions, we tested the ability of HeLa cell extracts prepared from cells with and without STS to ubiquitinate ARTS *in vitro* (Fig. 5*C*). Whereas extracts from nontreated cells produced efficient conjugation of ARTS, extracts from STS-treated cells had hardly any activity above background controls. A parallel experiment using DIAP1 as a ubiquitination substrate gave rise to strong polyubiquitination of this protein, demonstrating that extracts from STS-treated cells had not generally lost their conjugation activity (Fig. 5*C*). These results suggest that in response to proapoptotic stimuli, ARTS levels are increased, at least in part, through inhibition of ARTS ubiquitination and degradation.

DISCUSSION

In this study, we examined the role and regulation of the proapoptotic ARTS protein during apoptosis. ARTS is an unusual septin protein family member that is localized within mitochondria in living cells (10). In response to proapoptotic stimuli, ARTS is released from mitochondria into the cytosol where it can bind to and inhibit IAPs such as XIAP (11). It is thought that ARTS induces apoptosis, at least in part, by promoting the degradation of IAPs and blocking their ability to inhibit caspases (11, 28). According to this model, healthy cells can express ARTS but are not killed, because the protein is sequestered away into mitochondria where it cannot interact with its apoptotic targets, the IAPs, which reside in the cytosol (28). Therefore, a critical step during ARTS-induced apoptosis appears to be the accumulation of ARTS protein in the cytosol. To investigate this further, we examined the consequences of elevated ARTS protein levels in different apoptotic paradigms and on different cell death proteins. One main conclusion from these experiments is that ARTS protein levels are tightly regulated via ubiquitin-mediated protein degradation and also that ubiquitination of ARTS is blocked in response to proapoptotic stimuli.

ARTS was originally found to promote apoptosis induced by TGF-β (10). Here we show that elevated levels of ARTS promote apoptosis in response to other apoptotic inducers, including etoposide and STS. Furthermore, the apoptotic response of cells over-expressing ARTS was overall very similar for TGF-β, etoposide, and STS. In all cases, ARTS promoted the activation of caspase 9 and caspase 3, the release of cytochrome *c* from the mitochondria, and translocation of ARTS to the nucleus. In addition, we also observed down-regulation of Bcl-2 and Bcl-x_L protein levels (Fig. 3) (see below). Therefore, ARTS can promote apoptosis in response to different signals, indicating that it plays a general role in the core apoptotic pathway.

In addition to sensitizing cells toward apoptosis, high level expression of ARTS was sufficient to induce apoptosis in the absence of any further proapoptotic stimulus in many cell lines (Fig. 2*A*). Presumably, high level expression of ARTS can overcome regulatory safeguards that normally prevent unwanted induction of apoptosis by endogenous ARTS. Therefore, keeping ARTS protein at low levels in living cells would become a necessary task to prevent the cell from undergoing apoptotic death. Consistent with this idea, we found that ARTS levels are strictly controlled through the ubiquitin-proteasome-mediated pathway (Figs. 4 and 5). In living cells, ARTS is a highly labile protein with a half-life of only ~30 min. Moreover, in healthy cells virtually all ARTS protein appears to reside within mitochondria. Given this localization, the short half-life of ARTS is very surprising, because proteins inside mitochondria are thought to be protected from ubiquitination (17, 37, 38). One possible explanation for our findings is that there is constantly some leakage of ARTS protein from mitochondria into the cytosol. According to this model, steady-state levels of ARTS in healthy cells would be maintained by balancing the mitochondrial import of newly synthesized protein with its efflux and immediate ubiquitination/degradation of ARTS in the cytosol. Alternatively, not all ARTS protein may be imported into mitochondria. Consistent with either model, ARTS protein is virtually nondetectable in the cytoplasm of healthy cells, suggesting that it has an extremely short half-life in the cytosol. On the other hand, following apoptotic induction increased levels of ARTS accumulate in the cytosol and also in the nucleus. We previously have shown that ARTS translocates to the nucleus following apoptotic induction (10). Moreover, we have shown that ARTS co-localizes with XIAP in the nucleus during apoptosis. In addition, immunofluorescence studies to visualize ARTS and XIAP clearly show that both proteins co-localize in the cytosol during the early phase of apoptosis (11). Therefore, we conclude that stabilized ARTS is found both in the cytosol and the nucleus and that the increase in its levels seems to result, at least in part, from inhibiting the ubiquitination of ARTS protein (Fig. 5).

Although it is possible that there is also increased release of ARTS from mitochondria in response to apoptotic stimuli, sta-

under apoptotic conditions. ^{35}S-Labeled ARTS was used as substrate in an *in vitro* ubiquitination assay in the presence of 25 μg of total protein lysates from non-apoptotic HeLa (*right panel*) or apoptotic HeLa cells (following 4 h of 2 μM STS induction) (*left panel*). ^{35}S-Labeled DIAP1 was used as positive control. Under apoptotic conditions ARTS was unable to form the ARTS-ubiquitin conjugates. Under the same experimental conditions DIAP1 was hyper-ubiquitinated. Thus, the increase in ARTS protein levels during apoptosis is caused at least in part by a specific inhibition of ARTS-ubiquitin-mediated degradation.

bilization of ARTS in the cytosol may be the primary mechanism for reaching levels that are sufficient to trigger the induction of apoptosis. Indeed, in apoptotic cells the half-life of ARTS was almost tripled as compared with its half-life under non-apoptotic conditions (Fig. 5*B*). In this context, it is interesting to note that other IAP antagonists, including Reaper and Smac/Diablo, are also regulated through ubiquitin-mediated degradation (21, 39, 40). In addition, IAPs themselves auto-ubiquitinate and are degraded in response to apoptotic stimuli (18), and Reaper can directly stimulate the E3 self-conjugating activity of IAPs in doomed cells (22, 40). Furthermore, ubiquitin-mediated degradation of Smac and p53 is inhibited during apoptosis (41, 42). Significantly, XIAP and cIAP1/2 are E3 ubiquitin ligases for Smac/Diablo (21, 39). Because ARTS can bind to IAPs, it is conceivable that this protein is also ubiquitinated by IAPs, but this possibility has not yet been critically tested.

Finally, it has been reported that caspase activation can inhibit proteasome function during apoptosis (41). Therefore, a series of complex changes in the pattern of ubiquitin-mediated protein degradation appear to be a key event in the regulation of apoptosis, and the observed inhibition of ARTS degradation during apoptosis may be a part of this general process. Yet, because the release of ARTS from mitochondria and its accumulation in the cytosol appears to be a caspase-independent event (11), we conclude that inhibition of ARTS ubiquitination and degradation is not simply the consequence of apoptosis but rather a part of its induction.

Interestingly, we also found that high levels of ARTS are associated with significantly reduced levels of Bcl-2 and Bcl-x_L (Fig. 3). One possibility is that reduction in Bcl-2 and Bcl-x_L levels is a downstream event resulting from ARTS-induced caspase activation. Indeed, caspase activity can promote the cleavage of Bcl-2 family members (42, 43). Yet, no increase in Bcl-2 and Bcl-x_L levels was observed in ARTS-transfected cells following treatment with STS in the presence of a potent caspase inhibitor (Fig. 3*C*). Thus, the effect of ARTS on Bcl-2 and Bcl-x_L levels is not mediated simply through caspase activation. Although the mechanism by which ARTS down-regulates Bcl-2 proteins remains to be elucidated, our findings are consistent with the idea that this phenomenon contributes to ARTS-mediated cell killing.

High levels of Bcl-2 family members, including Bcl-2 and Bcl-x_L, can protect cancer cells from apoptosis (44–46). Interestingly, ARTS protein expression is frequently lost in childhood leukemia, and ARTS appears to function as a tumor suppressor protein in ALL (15). Consequently, there appears to be an inverse correlation between the levels of ARTS and anti-apoptotic Bcl-2 family proteins in hematopoietic malignancies. Furthermore, a similar inverse correlation was seen in malignant astrocytoma patients (47). Therefore, it will be very interesting to investigate further the connection between the regulation of ARTS and Bcl-2 protein levels and its functional consequences for the induction of apoptosis and cancer.

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Regulation of the Proapoptotic ARTS Protein by Ubiquitin-mediated Degradation

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