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Identification of a Novel Anti-apoptotic E3 Ubiquitin Ligase That Ubiquitinates Antagonists of Inhibitor of Apoptosis Proteins SMAC, HtrA2, and ARTS*

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Background: Mitochondrial proapoptotic proteins such as SMAC, HtrA2, and ARTS promote apoptosis by antagonizing the inhibitor of apoptosis proteins (IAPs).
Results: A novel anti-apoptotic E3 ubiquitin ligase, AREL1 was cloned and identified to interact with and ubiquitinate the IAP antagonists.
Conclusion: AREL1 inhibits apoptosis through ubiquitinating the cytosolic IAP antagonists.
Significance: AREL1-mediated degradation of IAP antagonists represents a new anti-apoptotic signaling pathway.

Identification of new anti-apoptotic genes is important for understanding the molecular mechanisms underlying apoptosis and tumorigenesis. The present study identified a novel anti-apoptotic gene named AREL1, which encodes a HECT (homologous to E6-AP carboxyl terminus) family E3 ubiquitin ligase. AREL1 interacted with and ubiquitinated IAP antagonists such as SMAC, HtrA2, and ARTS. However, AREL1 was cytosolic and did not localize to nuclei or mitochondria. The interactions between AREL1 and the IAP antagonists were specific for apoptosis-stimulated cells, in which the IAP antagonists were released into the cytosol from mitochondria. Furthermore, the ubiquitination and degradation of SMAC, HtrA2, and ARTS were significantly enhanced in AREL1-expressing cells following apoptotic stimulation, indicating that AREL1 binds to and ubiquitinates cytosolic but not mitochondria-associated forms of IAP antagonists. Furthermore, the anti-apoptotic role of AREL1-mediated degradation of SMAC, HtrA2, and ARTS was shown by simultaneous knockdown of three IAP antagonists, which caused the inhibition of caspase-3 cleavage, XIAP degradation, and induction of apoptosis. Therefore, the present study suggests that AREL1-mediated ubiquitination and degradation of cytosolic forms of three IAP antagonists plays an important role in the regulation of apoptosis.

Apoptosis, or programmed cell death, can be induced through two basic and distinct signaling pathways: intrinsic and extrinsic apoptosis signaling pathways. Both of these pathways culminate in proteolytic activation of the caspase protease family (1). Because proteolytic cleavage is irreversible and caspases can amplify proteolysis cascades, caspase activation is tightly regulated. The only known cellular caspase inhibitors are members of the inhibitor of apoptosis (IAP)[3] family that commonly contain baculoviral repeat (BIR) domains and, in some cases, a zinc RING finger domain (2, 3). Recent studies suggest that the X-linked inhibitor of apoptosis (XIAP) is a *bona fide* caspase inhibitor because it exhibits greater potency than other IAPs (4, 5). The anti-apoptotic functions of XIAP are apparent in cancer chemotherapy, as the frequent overexpression of XIAP in many human tumors confers chemoresistance, and knocking out XIAP restores chemosensitivity (6).

The caspase-inhibitory functions of XIAP are negatively regulated by endogenous inhibitors that were originally identified in *Drosophila* such as Reaper, Hid, and Grim, which commonly contain an N-terminal IAP-binding motif that is required for IAP binding (7). In mammals, SMAC and HtrA2 were identified as IAP-binding motif domain mitochondrial IAP antagonists that are released into the cytosol from the intermembrane space in mitochondria in response to apoptosis signals (8–12). However, recent studies have revealed that SMAC and HtrA2 target many proteins involved in various signaling pathways rather than regulation of the catalytic activity of caspases through XIAP (6). ARTS and XAF1 preferentially interact with XIAP. ARTS, which normally resides in mitochondria but moves to the cytosol upon apoptotic stimulation, does not contain an IAP-binding motif but preferentially binds to and promotes the ubiquitination and degradation of XIAP (13). XAF1 was identified as a nuclear protein that binds to XIAP, thereby sequestering XIAP in nuclear inclusions (14). In the present study, we identified a novel anti-apoptotic E3 ubiquitin ligase, AREL1,[4] which targets mitochon-

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[3] The abbreviations used are: IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; XAF1, XIAP-associated factor 1.
[4] The symbol (AREL1) and name (apoptosis-resistant E3 ubiquitin ligase) of the gene was approved by the HUGO Gene Nomenclature Committee. The mouse original clone cDNA sequence accession no. is HM024719.1 and GI 296410389 (http://www.ncbi.nlm.nih.gov/nuccore/HM024719).

drial pro-apoptotic proteins SMAC, HtrA2, and ARTS (but not XAF1), only when they are released into the cytosol upon apoptotic stimulation.

EXPERIMENTAL PROCEDURES

Functional cDNA Cloning Strategy—The osteosarcoma cell line SaOs-2 was transfected with a cDNA library constructed by cloning mouse testis cDNA into a λ pCEV29 expression vector to enhance DNA integration into chromosomes and decrease variations in cDNA expression (15). Approximately 2,000 stable transfectants were obtained from each 100 mm transfection plate after G418 selection, with a total of ~300,000 independent transfectants generated. The G418-resistant colonies, which reached a diameter of 1 mm, were then infected with a recombinant adenovirus encoding p53 (16). Surviving colonies were transferred to new dishes and cultured. Plasmid DNA was isolated from the genomic DNA of the surviving cells as described previously (15).

Cell Culture and DNA Transfection—The pcDNA6-V5/His vector (Invitrogen) was used for the transient expression of cDNAs. Transfection efficiency was monitored by cotransfection with a *Renilla* luciferase vector (pRL-CMV; Promega). The retroviral vectors for the expression of cDNAs or shRNAs were constructed using pBABE-puro. Two mutant constructs that lacked the ability to form a ubiquitin-thioester complex, AREL1^{C790A} and AREL1- H^{C790A}, were generated using a QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing the entire coding region. siRNA oligonucleotides corresponding to the sequences of AREL1 (5′-AATTGGTCCCTGAGAACCTTT-3′), HtrA2 (5′-GGGGAGUUUGUUGUUGCCAdTdT-3′), SMAC (5′-GUCAGAGAGAGGAGUCCUU-3′), and ARTS (5′-CGTAGTGATGGGACACCATTT-3′) were generated and used for transfection with Lipofectamine RNAiMAX (Invitrogen). Scrambled siRNA was obtained from Proligo LLC.

Yeast Two-hybrid Screen—The yeast cell-expressing LexA-HECT (amino acids 454–823 of AREL1) was transformed with the mouse brain cDNA library fused to the GAL4-AD. The yeast two-hybrid system, Matchmaker LexA two hybrid system (Clontech), was used to detect AREL1-interacting proteins. Positive clones were initially selected and then assayed for β-galactosidase activity using a filter assay. Positive clones were identified by using the polymerase chain reaction followed by sequence analysis.

Western Blot Analysis and Immunoprecipitation—Western blot and immunoprecipitation analyses were performed as described previously (17) using the following antibodies: anti-XIAP (BD Pharmingen); anti-cleaved caspase-3 and anti-survivin (Cell Signaling Technology); anti-procaspase 3, anti-Hsp60, anti-HtrA2, anti-SMAC/DIABLO, anti-β-actin, anti-γ-tubulin, and anti-ubiquitin (Santa Cruz Biotechnology); anti-V5 (Invitrogen); and anti-FLAG (Sigma). Polyclonal rabbit anti-AREL1 antibodies were generated against a synthetic peptide that encompassed amino acid residues 796–807 by Abfrontier and amino acids 521–534 by Zymed Laboratories, Inc.. Primary antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat secondary antibodies in combination with an enhanced chemiluminescence system (Amersham Biosciences).

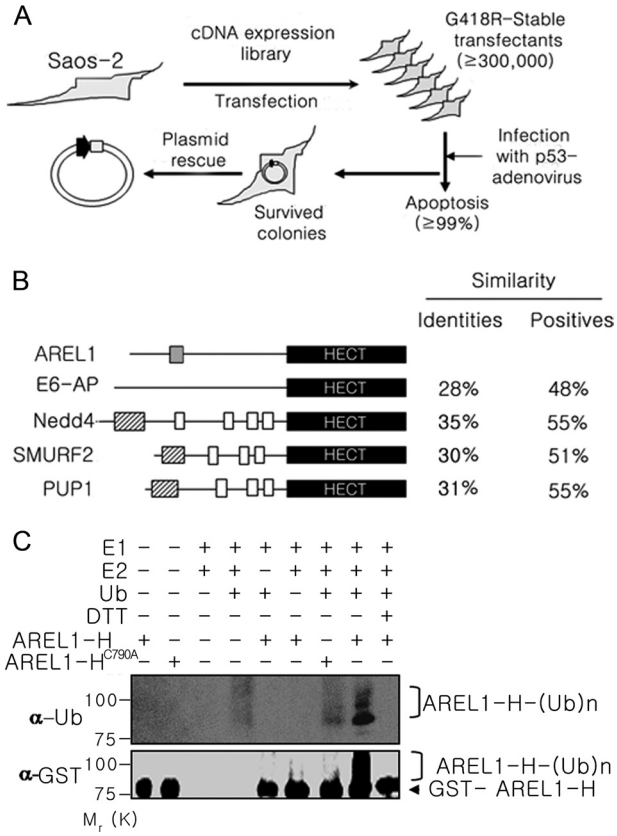

FIGURE 1. **Functional cDNA cloning of a novel anti-apoptotic E3 ubiquitin ligase.** *A*, functional cDNA cloning strategy for isolating cDNAs whose expression confers resistance to p53-induced apoptosis. *B*, schematic comparison of the HECT domain of AREL1 with that of known HECT family E3 ubiquitin (*Ub*) ligases. The HECT, filamin, WW, and C2 (Ca^{2+}-binding motif) domains are indicated by *black*, *shaded*, *striped*, and *open boxes*, respectively. The *right side* of the diagram shows the percentage of amino acids that are identical or similar (referred to as "positive") between the HECT domain of AREL1 and each of the known E3 ubiquitin ligase HECT domains. *C*, *in vitro* E3 ubiquitin ligase activity of AREL1-HECT proteins. GST-HECT proteins were incubated in the presence of E1, E2, ubiquitin, and ATP. The positions of the ubiquitin adducts on the GST-HECT protein are indicated.

In Vitro Ubiquitination Assay—For the ubiquitin binding assays, 5 μg of purified GST-AREL1-H protein was added to ubiquitin binding mixtures that contained 80 ng of E1 (Calbiochem), 500 ng of E2 (GST-UbcH5a, Calbiochem), and 5 μg of ubiquitin (Sigma) in a ubiquitination buffer (50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM creatine phosphate, and 15 units of creatine phosphokinase). For the *in vitro* ubiquitination of XIAP antagonists by AREL1, 1 μg each of purified SMAC or HtrA2 (BD Biosciences) was added to ubiquitination reaction mixtures containing 0.5 μg of GST-AREL1-H. Reactions were incubated for 90 min at 30 °C, terminated by the addition of SDS sample buffer, and resolved by SDS-PAGE.

Apoptosis Assays—Apoptotic sensitivity was determined by two methods. Cell viability was assessed using a tetrazolium salt (WST-8)-based colorimetric assay from the Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) (18) or by a trypan blue and propidium iodide exclusion assay (19).

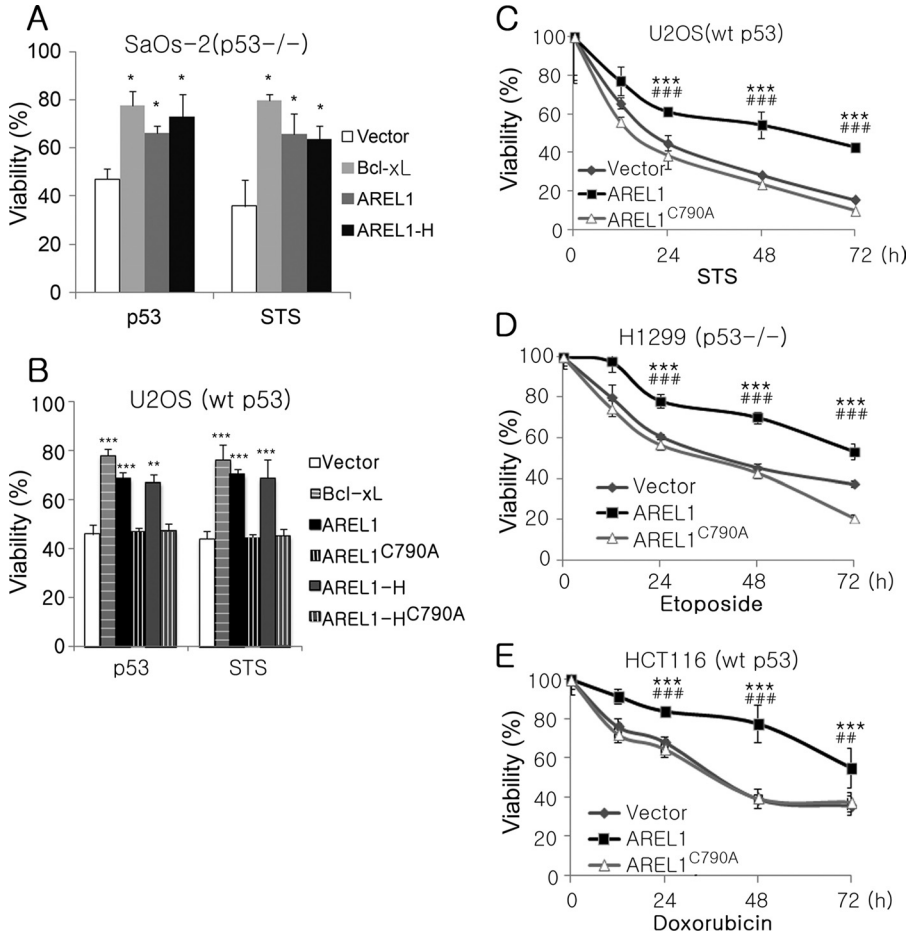

FIGURE 2. **AREL1 inhibits both p53-dependent and -independent apoptosis.** *A*, AREL1 suppresses p53- and staurosporine-induced apoptosis in p53-deficient SaOs-2 cells. SaOs-2 cells were transfected with the indicated plasmids and then either infected with 50 MOI (multiplicity of infection) of adenovirus encoding p53 or staurosporine (*STS*, 30 nM) for 24 h. Cell viability was assessed by colorimetric assay kit (Cell Counting Kit-8) based on MTT assay. The reported values represent the means ± S.E. of three independent experiments performed in duplicate. *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$ *versus* vector control in each case. *B*, AREL1 suppresses p53- and staurosporine-induced apoptosis in p53-positive U2OS cells. U2OS cells were transfected with the indicated plasmids and were then infected with either the p53-encoding adenovirus or staurosporine as in *A*. Cell viability was assessed as in *A*. *C–E*, AREL1 inhibits apoptosis of U2OS ($p53^{+/+}$) cells treated with staurosporine (30 nM) (*C*), H1299 ($p53^{-/-}$) cells treated with etoposide (50 μM) (*D*), and HCT116 ($p53^{+/+}$) cells treated with doxorubicin (1 μM) (*E*). Cells were transfected with an empty control vector (pcDNA6), wild type AREL1, or $AREL1^{C790A}$ and then treated with the indicated apoptosis stimuli for the indicated times. Cell viability was assessed as described in *A*.

Statistical Analysis—The data were represented as mean ± S.E. of the indicated number of measurements. Statistical significance was calculated by two-tailed unpaired *t* test for two data sets and analysis of variance followed by Bonferroni post hoc test for multiple data sets using SPSS18, with $p < 0.05$ considered statistically significant.

RESULTS

Functional cDNA Cloning of a Novel Anti-apoptotic E3 Ubiquitin Ligase, AREL1—A cDNA expression cloning strategy was developed to isolate new anti-apoptotic genes (Fig. 1*A*). Approximately 300,000 independent transfectants were obtained from the human osteosarcoma cell line SaOs-2 after transfection with a cDNA expression library as described previously (15). A recombinant adenovirus encoding p53 was infected into G418-resistant colonies to induce apoptosis (16). Surviving transfectants were rare and appeared at a frequency of $\sim 10^{-3}$. cDNAs were isolated from the transfectants, and their ability to inhibit apoptosis was examined. Of the cDNAs isolated from surviving cells, a 2.4-kb cDNA (originally named 9–31) comprising 342 C-terminal amino acids and encoded by a mouse full-length cDNA (RIKEN cDNA 1110018G07) was identified, which was almost identical (97%) to the human homolog (KIAA0317). This gene was designated AREL1 (apoptosis-resistant, E3 ubiquitin ligase). The location of AREL1 was identified on chromosome 14q24.3, spanning 51,853 bp and 20 exons. Northern blotting showed that 5.4 kb of AREL1 mRNA was detected in various mouse tissues such as brain, testes, heart, liver, lung, and kidney, but barely in skeletal muscle and spleen (data not shown).

AREL1 contains a C-terminal HECT (homologous to E6AP C terminus)-like domain that is similar to the C termini of E3 ubiquitin ligases such as E6-AP (20), Nedd4 (21), SMURF2 (22), and PUB1 (Fig. 1*B*) (23). Therefore, we next examined the ability of the HECT-like domain of human AREL1 to accept ubiquitin from an E2 ubiquitin-conjugating enzyme through a thioester bond. Similar to previous findings showing that the HECT domain of E3 ubiquitin ligases is sufficient for enzymatic activity (20), a fusion protein of GST with the HECT domain of

AREL1 (AREL1-H, comprising amino acids 454 to 823) showed auto-ubiquitination activity in the presence of E1, E2 (UbcH5a), ubiquitin, and ATP (Fig. 1*C*). The formation of AREL1-H ubiquitin adducts was inhibited by DTT, which disrupts thioester complex formation (Fig. 1*C*). HECT E3 ubiquitin ligases contain a highly conserved cysteine residue that is essential for the acceptance of ubiquitin from E2 enzymes (20, 24). AREL1-H^{C790A}, in which cysteine 790 was replaced by alanine, failed to form an ubiquitin thioester complex (Fig. 1*C*). These results indicate that AREL1 encodes a HECT family E3 ubiquitin ligase.

To investigate the anti-apoptotic function of AREL1, apoptotic sensitivity was examined in SaOs-2 cells transiently transfected with human full-length AREL1, the HECT domain of AREL1 (AREL1-H), or Bcl-x_L as a positive control (Fig. 2*A*). Cells transfected with AREL1, AREL1-H, or Bcl-x_L exhibited a similar level of resistance to p53-induced apoptotic death (Fig. 2*A*). To further investigate whether E3 ubiquitin ligase activity is important for the anti-apoptotic functions of AREL1, U2OS cells, which express wild-type p53, were transiently transfected with AREL1^{C790A} or AREL1-H^{C790A}, or with their wild-type counterparts, AREL1 or AREL1-H. Although U2OS cells transfected with AREL1 or AREL1-HECT were resistant to p53-induced apoptosis, those transfected with AREL1^{C790A} or AREL1-H^{C790A} were as sensitive as control cells (Fig. 2*B*), suggesting that the E3 ubiquitin ligase activity of AREL1 is essential for the inhibition of apoptosis.

However, AREL1 also inhibited apoptosis of SaOs-2 and U2OS cells treated with staurosporine (Fig. 2, *A*–*C*), an agent that induces p53-independent apoptosis (25). Furthermore, AREL1 inhibited apoptosis in p53-deficient H1299 and p53-positive HCT116 cells treated with DNA-damaging agents doxorubicin and etoposide, respectively (Fig. 2, *D* and *E*), suggesting that AREL1 inhibits both p53-dependent and -independent apoptosis. In addition, AREL1 did not affect the cytosolic release of cytochrome *c* from mitochondria upon apoptotic stimulation (data not shown) (see Fig. 4*B*). These results led us to examine downstream of the mitochondrial apoptotic pathway that are activated following cytosolic release of mitochondrial proapoptotic proteins.

AREL1 Binds to and Ubiquitinates the Cytosolic Forms of SMAC, HtrA2, and ARTS—The hypothesis that AREL1 is able to inhibit downstream regulators of the mitochondrial pathway was supported by a yeast two-hybrid screen, in which the HECT domain of AREL1 was used as a bait protein. Among the positive clones that interact with AREL1-HECT in yeast two-hybrid screening, a cDNA encoding HtrA2 was of particular interest because of its proapoptotic functions in the cytosol (10, 11). A GST pulldown assay confirmed the interaction of AREL1-HECT with HtrA2 (Fig. 3*A*). The interactions of AREL1-HECT with IAPs and their inhibitors, including SMAC, ARTS, and XAF-1, were further examined. Interestingly, SMAC and ARTS was also found to interact with AREL1-HECT, but XAF-1 and IAPs (XIAP, cIAP1/2, and survivin) were not (Fig. 3*A*).

Immunofluorescence analysis showed that AREL1 localized to the cytoplasm not to mitochondria or nuclei (Fig. 3, *B* and *C*). Because three IAP antagonists such as SMAC, HtrA2, and ARTS reside in the mitochondria of healthy cells but are

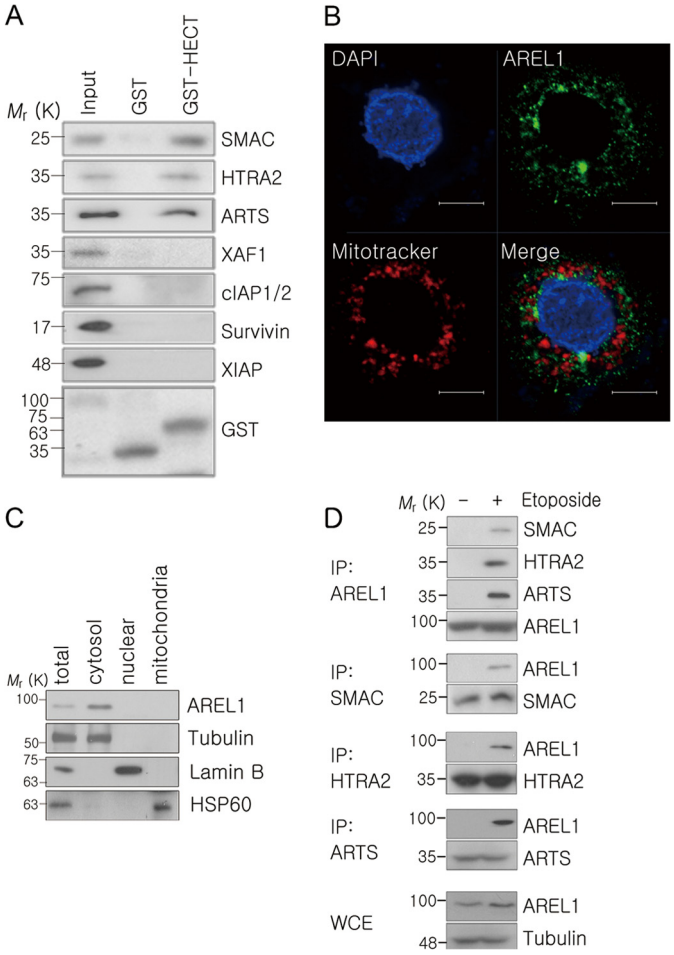

FIGURE 3. **AREL1 interacts with IAP antagonists in the cytosol.** *A*, GST pulldown assay. Pulldowns were performed using extracts of H1299 cells and were followed by Western blotting with the indicated antibodies. The *input* loaded represents 5% of the total protein. *B*, subcellular localization of AREL1 (*green*) was assessed in H1299 cells by immunofluorescence staining with polyclonal anti-AREL1 serum (1:100). DAPI (*blue*) and MitoTracker (*red*) were used to stain nuclei and mitochondria, respectively. *Scale bar* is 100 μm. *C*, AREL1 in the cytosolic fractions. H1299 cells were fractionated into soluble cytosolic, nuclear, and mitochondrial fractions. Western blot analysis was performed with the indicated antibodies. Tubulin, lamin B, and HSP60 were used to confirm the purity of each subcellular fraction. *D*, immunoprecipitation (*IP*) and Western blot analysis. Cell extracts were prepared 6 h after treatment with etoposide and subjected to either Western blotting or immunoprecipitation/Western blot analysis. *WCE*, whole cell extract.

released into the cytosol upon apoptotic stimulation, AREL1 may interact with cytosolic forms of SMAC, HtrA2, and ARTS but not with the mitochondria-associated forms of these proteins. To examine this idea, cells were treated with etoposide to release the IAP antagonists into the cytosol, and the *in vivo* interactions were examined by Western blot analysis of immunoprecipitated fractions. Endogenous AREL1 interacted with SMAC, HtrA2, and ARTS in etoposide-treated cells but not in healthy cells (Fig. 3*D*). Therefore, these results suggest that AREL1 interacts with the cytosolic forms of SMAC, HtrA2, and ARTS upon apoptotic stimulation but does not associate with mitochondria-localized proteins in healthy cells.

Based on the apoptosis-specific interaction between AREL1 and cytosolic forms of the IAP antagonists, we next examined whether AREL1 directly ubiquitinated those proteins. An *in vitro* ubiquitination assay indicated that AREL1-HECT directly

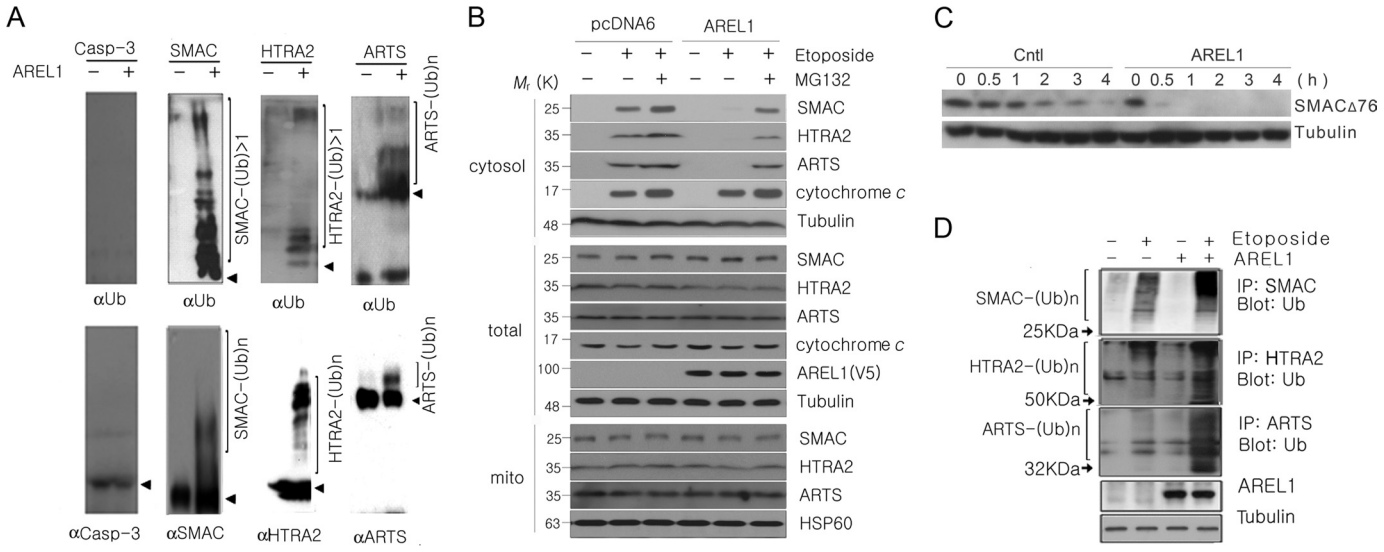

FIGURE 4. **AREL1 targets the cytosolic forms of SMAC, HtrA2, and ARTS.** *A*, *in vitro* ubiquitination of ARTS, HtrA2, and SMAC by AREL1-HECT. GST-HECT proteins were incubated with the indicated proteins in the presence of E1, E2, ubiquitin (*Ub*), and ATP. *Arrowheads* indicate non-ubiquitinated proteins. Caspase-3 (*Casp-3*) was used as a negative control. *B*, AREL1 targets the cytosolic forms of the IAP antagonists. Total cell extracts and subcellular fractions (mitochondrial (*mito*) and cytosolic) were prepared from H1299 cells transfected with empty vector (pcDNA6) or AREL1 treated with etoposide (20 μM) for 6 h in the presence or absence of MG132 (10 μM) and used for Western blotting with the indicated antibodies. *C*, AREL1 shortened the half-life of cytosolic SMAC proteins. A FLAG-tagged SMAC mutant (SMACΔ76) was transfected with AREL1 or an empty vector. The SMACΔ76 proteins were detected with anti-FLAG antibody after treatment with cycloheximide (10 μg/ml). The half-life of the SMAC proteins was calculated to be ~2 h in HCT116 cells, but shorter than 0.5 h in AREL1-expressing cells. *D*, ubiquitination of endogenous XIAP in AREL1-expressing cells. H1299 cells transfected with AREL1 or an empty vector were treated with etoposide (20 μM) for 6 h in the presence of a proteasome inhibitor, MG132 (10 μM), and then lysed with denaturation buffer containing 6 M Urea followed by immunoprecipitation and Western blotting with the indicated antibodies. *Cntl*, control.

ubiquitinated all three proteins in the presence of E1, E2, and ATP (Fig. 4*A*). To further investigate whether AREL1 specifically promotes the degradation of the cytosolic forms of the IAP antagonists, cytosolic fractions were prepared from H1299 cells transfected with AREL1 or an empty vector to examine the protein levels of SMAC, HtrA2, and ARTS.

Consistent with previous reports showing that SMAC, HtrA2, and ARTS are released into the cytosol along with cytochrome *c* (26), the cytosolic forms of the mitochondrial pro-apoptotic proteins increased upon treatment with etoposide (Fig. 4*B*). However, this increase in cytosolic forms of SMAC, HtrA2, and ARTS was not observed in AREL1-expressing cells (Fig. 4*B*). Furthermore, a proteasome inhibitor, MG132 inhibited the degradation of cytosolic forms of the IAP antagonists in AREL1-expressing cells (Fig. 4*B*), implying that AREL1 promotes proteasomal degradation of the cytosolic SMAC, HtrA2, and ARTS. In addition, the half-life of a SMAC mutant (SMACΔ76), which lacks the IAP-binding motif and mitochondrial import signals and localized at the cytosol (27), was clearly shortened by AREL1 (Fig. 4*C*).

We next examined the ubiquitination of endogenous SMAC, HtrA2, and ARTS by AREL1. Ubiquitination of the IAP antagonists was detected in H1299 cells treated with etoposide and was significantly increased in AREL1-expressing cells compared with vector-transfected cells (Fig. 4*D*). Therefore, these results suggest that AREL1 binds to and ubiquitinates SMAC, HtrA2, and ARTS, resulting in the degradation of their cytosolic forms after their release from mitochondria following apoptosis stimulation.

Anti-apoptotic Functions of AREL1-mediated Ubiquitination of the IAP Antagonists—To evaluate the functional significance of AREL1-mediated ubiquitination of the three IAP antagonists, we next examined the effects of simultaneous knockdown of the three IAP antagonists on apoptosis induction, XIAP degradation, and caspase-3 cleavage. Knockdown of any one of the IAP antagonists in H1299 cells did not confer apoptotic resistance; however, knockdown of all three IAP antagonists conferred a level of apoptotic resistance that was similar to that observed after AREL1 overexpression (Fig. 5*A*). The knockdown of any two of the IAP antagonists was less effective than the knockdown of all of the IAP antagonists (Fig. 5*A*). The triple knockdown also conferred apoptosis resistance to U2OS and HCT116 cells treated with staurosporine and doxorubicin, respectively (Fig. 5, *B* and *C*). Because XIAP is the most potent caspase inhibitor among IAP family proteins and a common cytosolic target for SMAC, HtrA2, and ARTS (26, 28), we examined the influence of the triple knockdown on XIAP and caspase-3. XIAP degradation and caspase-3 cleavage were detected in etoposide-treated H1299 cells, staurosporine-treated U2OS cells, and doxorubicin-treated HCT116 cells, but these were significantly suppressed by the knockdown of the three IAP antagonists (Fig. 5, *D–F*). Furthermore, the inhibition of XIAP degradation was also seen in AREL1-expressing cells (Fig. 5*G*). In addition, the effects of AREL1 knockdown were examined. In contrast to AREL1 overexpression, AREL1 knockdown increased apoptotic sensitivity in etoposide-treated H1299 cells, staurosporine-treated U2OS cells, and doxorubicin-treated HCT116 cells (Fig. 6, *A–C*). Furthermore, the AREL1 knockdown enhanced XIAP degradation and caspase-3 cleavage (Fig. 6, *D–F*), further supporting anti-apoptosis functions of AREL1. Therefore, these results suggest that AREL1-mediated ubiquitination and degradation of the cytosolic forms of the three IAP antagonists contributes to the inhi-

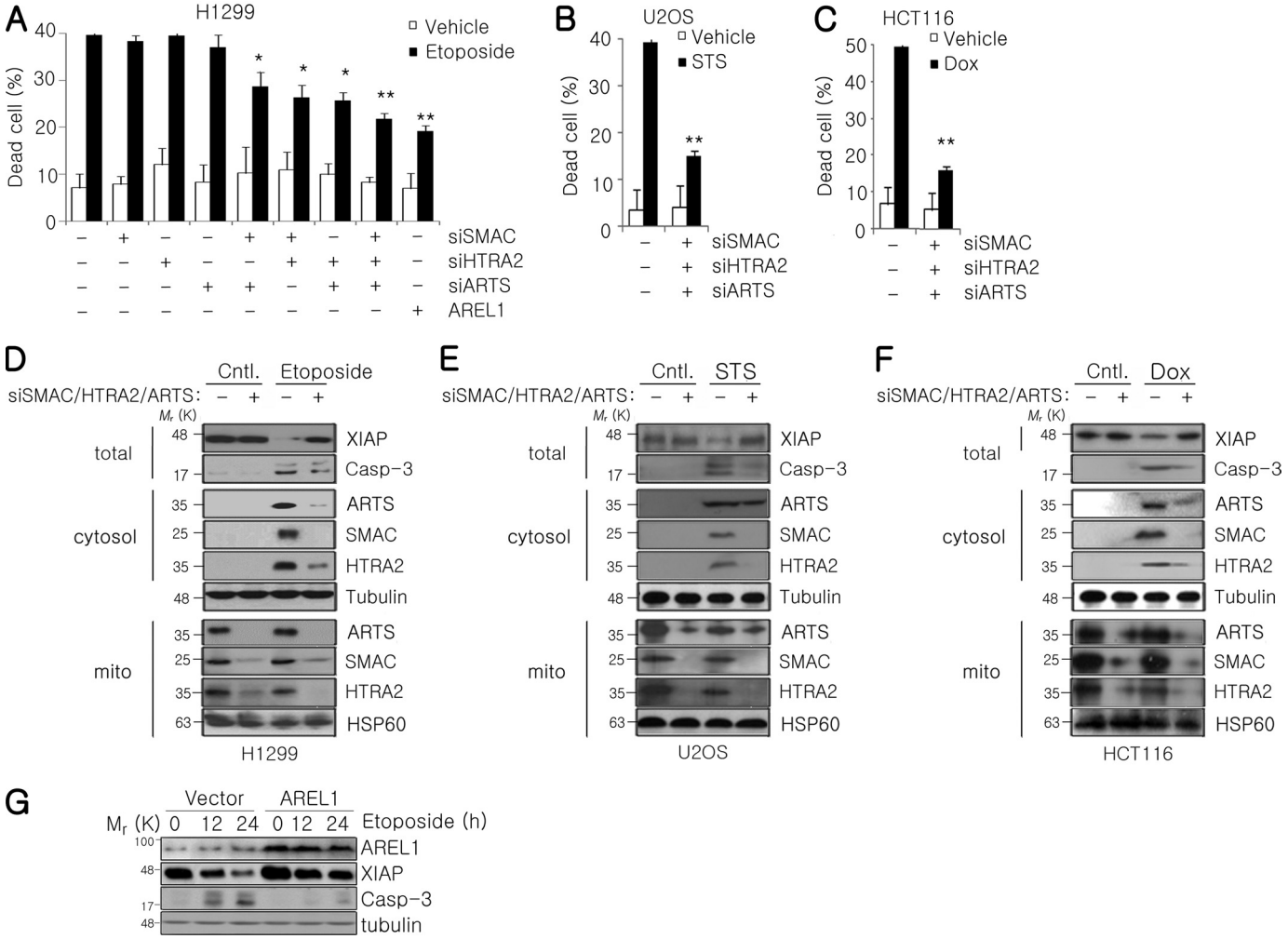

FIGURE 5. **Anti-apoptotic effects of simultaneous knockdown of three IAP antagonists.** *A*, apoptotic sensitivity of H1299 cells transfected with siRNAs directed against one, two, or three IAP antagonists following treatment with etoposide for 24 h. Viability was assessed using the Cell Counting Kit-8 assay. Reported values represent the means ± S.E. of three independent experiments performed in duplicate. *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$ *versus* siRNA control (*siCntl*). *B* and *C*, apoptosis of U2OS and HCT116 cells that were transfected with siRNAs against three IAP antagonists following treated with staurosporine and doxorubicin (*Dox*), respectively, for 24 h. Viability was assessed as described in *A*. *D–F*, the effects of triple knockdown on XIAP degradation and caspase-3 cleavage. H1299, U2OS, and HCT116 cells were transfected with a mixture of siRNAs against SMAC, HtrA2, and ARTS for 12 h and then treated with etoposide, staurosporine, and doxorubicin, respectively. Cytosolic and mitochondrial (*mito*) fractions were prepared 6 h after treatment with apoptosis stimuli and analyzed by Western blotting with the indicated antibodies. *F*, inhibition of XIAP degradation and caspase-3 (*Casp-3*) cleavage by AREL1. H1299 cells transfected with AREL1 or an empty vector were treated with etoposide (20 μM) for the indicated times, and then analyzed by Western blotting with the indicated antibodies. *Cntl.*, control.

bition of apoptosis induction, XIAP degradation, and caspase-3 activation.

DISCUSSION

In the present study, we cloned and identified a novel anti-apoptotic E3 ubiquitin ligase, AREL1, which binds to and ubiquitinates the IAP antagonists, SMAC, HtrA2, and ARTS released into the cytosol upon apoptotic stimulation (Figs. 1–4). Because simultaneous knockdown for three IAP antagonists inhibited apoptosis induction as well as caspase-3 activation and XIAP degradation (Fig. 5), AREL1-mediated ubiquitination of the IAP antagonists will contribute apoptotic resistance in human tumor cells treated with DNA damaging agents.

Although AREL1 was originally identified as a suppressor of p53-induced apoptosis (Fig. 1*A*), overexpression of AREL1 conferred apoptotic resistance to staurosporine in both p53-positive and -deficient tumor cells (Fig. 2). Furthermore, apoptosis induced by DNA damaging agents such as etoposide and doxorubicin in p53-deficient H1299 and p53-positive HCT116 cells was also suppressed by AREL1 expression (Fig. 2). Because AREL1 did not affect the cytosolic release of mitochondrial proapoptotic proteins such as cytochrome *c* (Fig. 4*B*) but inhibited caspase-3 activation (Fig. 5*F*), we examined whether AREL1 influences a downstream pathway that is activated following mitochondrial outer membrane permeabilization. Furthermore, based on the results of two hybrid screens and GST pulldown assays (Fig. 3*A*), we found that AREL1 binds to and ubiquitinates the cytosolic forms of the IAP antagonists but not mitochondrial-associated proteins (Figs. 3 and 4).

Certain cell lines show resistance to apoptosis after knockdown of SMAC, HtrA2, or ARTS (9, 13, 29). However, the pres-

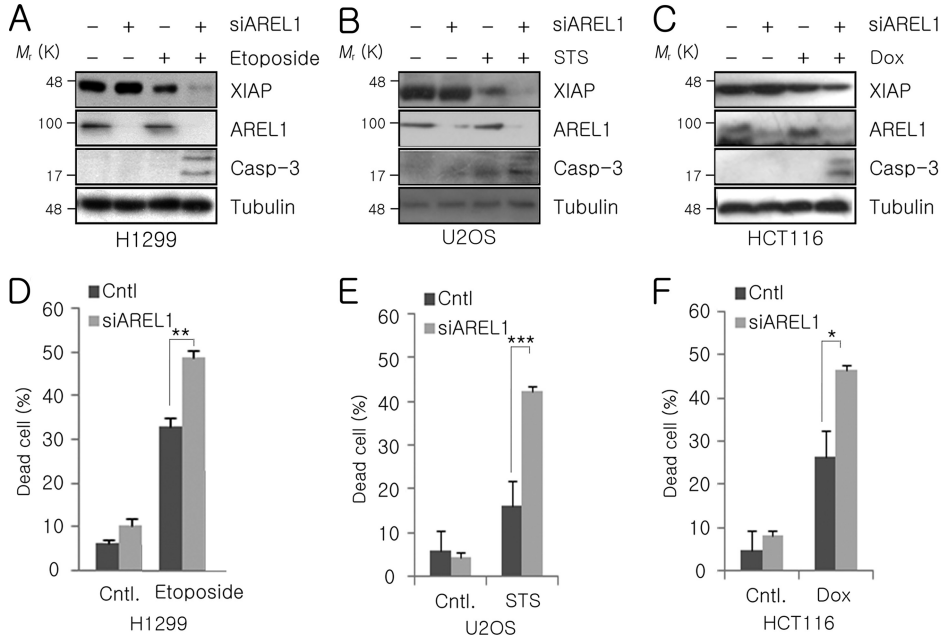

FIGURE 6. **The effects of AREL1 knockdown on apoptosis.** *A–C*, enhanced XIAP degradation and caspase-3 (*Casp-3*) cleavage following AREL1 knockdown. H1299 cells (*A*), U2OS (*B*), and HCT116 (*C*) were transfected with either control siRNA (−) or siRNA specific for AREL1 (+) and then treated with etoposide, staurosporine (*STS*), and doxorubicin (*Dox*), respectively, for 6 h. Western blot analysis were carried out with the indicated antibodies. *D–F*, enhanced apoptotic death by AREL1 knockdown. H1299 cells (*D*), U2OS (*E*), and HCT116 (*F*) harboring either control siRNA (−) or AREL1-specific siRNA were treated with the indicated apoptotic stimuli for 24 h. Viability was assessed using the Cell Counting Kit 8 assay. Reported values represent the means ± S.E. of three independent experiments performed in duplicate. *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$ *versus* control siRNA (*Cntl.*).

ent study showed that knockdown of all three IAP antagonists exerted a stronger anti-apoptosis effect than knockdown of any one or two of three IAP antagonists (Fig. 5), implying that the three IAP antagonists are functionally redundant with respect to each other in tumor cells tested. Therefore, these results suggest that degradation of three IAP antagonists by AREL1 may confer apoptotic resistance upon a wider range of tumor cells that require two or more IAP antagonists for apoptosis induction.

The cytosolic forms of mitochondrial IAP antagonists target many proteins involved in various signaling pathways. SMAC binds to and promotes the degradation of cIAP1/2, which regulates both the classical and alternative NF-κB signaling pathways rather than the catalytic activity of caspases (6). However, cIAP1/2 degradation was not prominent in many types of tumor cells such as H1299, A549, or 293T cells upon treatment with apoptotic stimuli (data not shown). These results coincide with a recent report showing that many human tumor cell lines do not appear to be sensitive to SMAC mimetic chemical compounds, which cause a loss of both cIAP1 and -2 and enhance the sensitivity to TNFα-induced apoptosis in certain tumor cells (30). Therefore, the effects of AREL1 on cIAP1/2 stability need to be studied in other experiment systems, in which endogenous SMAC proteins efficiently target cIAP1/2. Like SMAC, HtrA2 also targets many proteins located in the cytosol and within mitochondria (29). Because AREL1 interacted with HtrA2 released into the cytosol, but not with mitochondria-associated proteins, cytosolic targets of HtrA2 may be influenced by AREL1. Therefore, the E3 ubiquitin ligase activity of AREL1 for the three IAP antagonists may influence many of the regulatory pathways involved in apoptosis, depending on the tumor cell context and the type of apoptotic stimuli.

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