The International Journal of Biochemistry & Cell Biology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Short communication

X-linked Inhibitor of Apoptosis Protein promotes the degradation of its antagonist, the pro-apoptotic ARTS protein

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ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form
24 November 2011
Accepted 6 December 2011
Available online xxx

Keywords:
Apoptosis
XIAP
Ubiquitination
Mitochondria
ARTS

ABSTRACT

ARTS (Sept4_i2) is a mitochondrial pro-apoptotic tumor suppressor protein. In response to apoptotic signals, ARTS translocates to the cytosol where it promotes caspase activation through caspase de-repression and proteasome mediated degradation of X-linked Inhibitor of Apoptosis Protein (XIAP). Here we show that XIAP regulates the levels of ARTS by serving as its ubiquitin ligase, thereby providing a potential feedback mechanism to protect against unwanted apoptosis. Using both *in vitro* and *in vivo* ubiquitination assays we found that ARTS is directly ubiquitinated by XIAP. Moreover, we found that XIAP-induced ubiquitination and degradation is prevented by removal of the first four amino acids in the N-terminus of ARTS, which contains a single lysine residue at position 3. Thus, this lysine at position 3 is a likely target for ubiquitination by XIAP. Importantly, although the stabilized ARTS lacking its first 4 residues binds XIAP as well as the full length ARTS, it is more potent in promoting apoptosis than the full length ARTS. This suggests that increased stability of ARTS has a significant effect on its ability to induce apoptosis. Collectively, our data reveal a mutual regulatory mechanism by which ARTS and XIAP control each other's levels through the ubiquitin proteasome system.

1. Introduction

Apoptosis, or programmed cell death is a critical process for regulating cell numbers and maintaining tissue homeostasis (Meier et al., 2000; Thompson, 1995). Caspases, a family of cysteine proteases, are the central executioners of apoptosis (Boyce et al., 2004; Nicholson, 1999; Thornberry and Lazebnik, 1998; Xue et al., 1996; Yuan et al., 1993). The apoptotic process is tightly controlled through the action of both activators and inhibitors of caspases (Bergmann et al., 1998; Shi, 2002; Steller, 2008). There are two main pathways leading to caspase activation: the "intrinsic pathway", also known as the mitochondrial pathway, and the "extrinsic pathway". In the mitochondrial pathway, caspase activation occurs through the release of pro-apoptotic factors from the mitochondria to the cytosol (Olson and Kornbluth, 2001). These factors include cytochrome C, Smac/Diablo (here forth referred to as SMAC), Omi/HtrA2 and ARTS (Du et al., 2000; Gottfried et al., 2004; Green and Kroemer, 2004; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2007). One way in which caspase inhibition occurs is *via* the family of Inhibitors of Apoptosis Proteins (IAPs) (Crook et al., 1993; Salvesen and Duckett, 2002; Srinivasula and Ashwell, 2008). These proteins were originally found in baculoviruses, and contain at least one Baculoviral IAP Repeat (BIR) domain. BIR domains can directly interact with caspases and inhibit their apoptotic activity (Shi, 2002). Thus far, eight mammalian IAP proteins have been identified: NAIP, cIAP1, cIAP2, X-linked IAP (XIAP), MLIAP, ILP2, survivin, BRUCE/Apollon and XAF1 (Liston et al., 2003; Salvesen and Duckett, 2002). Some of the IAPs, such as XIAP, cIAP1 and cIAP2, contain a unique C-terminal RING domain that functions as E3-ligase (Wilson et al., 2002; Yang et al., 2000). The RING domain has been implicated in regulating both caspases and IAP-protein stability *via* proteasome mediated degradation (Ditzel et al., 2003; Holley et al., 2002; Ryoo et al., 2002; Schile et al., 2008; Yang et al., 2000).

The Ubiquitin Proteasome System (UPS) is the main route for protein degradation in eukaryote cells (Ciechanover et al., 2000; Glickman and Ciechanover, 2002). Protein ubiquitination is a post-translational protein modification that involves the ordered action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme

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doi:10.1016/j.biocel.2011.12.005

(E2) and ubiquitin protein ligase (E3) that recognizes and transfers the activated ubiquitin to the target proteins (Ciechanover et al., 1980; Ciechanover and Schwartz, 1998; Hershko et al., 1979).

XIAP, the best studied IAP, contains three BIR domains that can directly bind and inhibit caspases 3, 7 and 9 (Deveraux et al., 1997; Sun et al., 2000). In addition, it contains a RING domain that bestows E3-ligase activity (Schile et al., 2008; Yang et al., 2000) and an ubiquitin-associated (UBA) domain, which enables the binding of ubiquitin conjugates *via* lysine 63 (Gyrd-Hansen et al., 2008; Ikeda and Dikic, 2008; Rajalingam and Dikic, 2009). It has been shown that XIAP plays a role as an E3-ligase for several pro-apoptotic proteins such as caspases, SMAC and AIF (Galban and Duckett, 2010; Morizane et al., 2005; Schile et al., 2008; Suzuki et al., 2001). In dying cells, caspases inhibition by XIAP has to be overcome to enable the initiation of apoptosis (Albeck et al., 2008). This is achieved, at least in part, by IAP-antagonist proteins (Galban and Duckett, 2010; Verhagen et al., 2007). The best characterized mammalian IAP-antagonist is SMAC, which resides in the mitochondrial inter-membrane space. Following apoptotic induction, SMAC is released from mitochondria to the cytosol where it binds XIAP (Du et al., 2000; Verhagen et al., 2000). Another mitochondrial protein that promotes apoptosis through binding and antagonizing XIAP is ARTS (Gottfried et al., 2004; Larisch-Bloch et al., 2000; Larisch et al., 2000).

ARTS (Sept4_i2) is derived by alternative splicing from the *Sept4* gene (Larisch, 2004; Larisch et al., 2000). Unlike other septins which are localized to actin-rich regions and function during cytokinesis and cellular morphogenesis, ARTS is localized at mitochondrial outer membrane (MOM) (Edison et al., 2011). Upon induction of apoptosis, ARTS translocates from the mitochondria to the cytosol, directly binds and antagonizes XIAP, causing activation of caspases and cell death (Bornstein et al., 2011; Edison et al., 2011; Gottfried et al., 2004; Reingewertz et al., 2011). In particular, ARTS stimulates the ubiquitination and degradation of XIAP (Bornstein et al., 2011; Garrison et al., 2010; Gottfried et al., 2004). ARTS expression is frequently lost in Acute Lymphoblastic Leukemia (ALL) and lymphoma patients, indicating that it is a tumor suppressor protein (Elhasid et al., 2004). Moreover, *Sept4*/ARTS deficient mice exhibit increased tumor incidence, increased numbers of hematopoietic stem and progenitor cells, elevated XIAP protein levels, and increased resistance to cell death (Garcia-Fernandez et al., 2010). Importantly, the apoptosis, stem cell and tumor phenotypes of *Sept4*/ARTS-null mice are all suppressed by inactivation of XIAP. These findings confirm that XIAP is a major target for ARTS-induced caspase activation and tumor suppression (Garcia-Fernandez et al., 2010; Kissel et al., 2005).

In this study, we show that XIAP promotes the degradation of its antagonist, ARTS by functioning as its E3-ligase. Moreover, removal of the first four amino acids in ARTS N-terminus, containing a lysine residue at position 3, protects ARTS from degradation by XIAP. Furthermore, this mutant form of ARTS accumulates in the cytosol and is a much more potent inducer of apoptosis than full-length ARTS. Collectively, these data indicate that ARTS is targeted for degradation by XIAP, and that this degradation is important for regulation of the pro-apoptotic function of ARTS.

2. Materials and methods

2.1. Antibodies

Antibodies to the various proteins were purchased from the indicated companies, and used as instructed. The following antibodies were used: anti-ARTS antibody (Sigma, St. Louis) which is the only currently commercially available antibody directed against the unique C-terminus of ARTS; anti-XIAP (#610716, BD); anti-caspase 9 (22-2-96, Lab); anti-caspase 3 (#9661, Cell Signaling); anti-ubiquitin (SC-8017, Santa Cruz); anti-GST (B-14, SC-138, Santa Cruz); anti-Myc (SC-40, Santa Cruz); anti-HA (#2367, Cell Signaling) and anti-actin (#69100, MP biomedicals).

2.2. Cell cultures and treatments (transfection, induction of apoptosis and proteasome inhibition)

COS-7 and HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l D-glucose. BT and K562 cells were grown in RPMI medium. Media were supplemented with 10% fetal calf serum (FCS), penicillin 100 U/ml, streptomycin 100 mg/ml, Sodium pyruvate 1 mM and glutamine 2 mM (Biological Industries, Israel). XIAPdelRING deficient MEFs were prepared from XIAPdelRING deficient mice described in Schile et al. (2008).

jetPEI™ (Polyplus Transfection), Effectene (Qiagen) and Transfectol (GeneChoice) were used according to the manufacturer's protocol.

Apoptotic induction: HeLa and BT cells were incubated with Staurosporine (STS) (1.75 μM for HeLa cells and 0.75 μM for BT cells) for different time periods. HeLa and K562 cells were incubated with different concentration of etoposide for 16 h.

Proteasome inhibition: COS-7 cells were incubated with MG132 (20 μM) for 6 h, and HeLa cells were incubated with MG132 (10 μM) for 6 h.

2.3. Plasmid constructs

ARTS: pEF1-AU5 and pEF1-AU5-ARTS constructs contain an AU5 tag that is attached to the N-terminus of ARTS (Larisch et al., 2000). pEF1-AU5-ARTS N-terminus deletions, -4aa, -21aa were generated using PCR with the following forwards primers: dell-4aa GAAGATCTTTTCCTGGAGGACACCACGG; dell-21aa GAAGATCTTTCTCAGGAAATGCGAGCTG; and reverse primer: ARTS-R TACCGCTCGAGCTAGTGGCAGCCCTGCCC. The pCS2-6Myc ARTS construct contain 6Myc tag that is attached to the N-terminus of ARTS (Edison et al., 2011).

XIAP: the mammalian expression vector pcDNA3 encoding Myc tagged wild-type XIAP and the mammalian expression vector pEBG encoding N-terminus GST fusion XIAP or XIAPΔRING were a kind gift from Colin Duckett (Burstein et al., 2004). pEGZ-flag XIAP construct contain flag tag attached to XIAP (Dogan et al., 2008). The pEGZ-flag XIAP-H467A was generated using QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies (Catalog #200522) using the following primers: forward 5′-GCTATCGTTTTTGTTCCTTGTGGAGCTCTAGTCACTTGTAAACAATG-3′; reverse 5′-CATTGTTTACAAGTGACTAGAGCTCCACAAGGAACAA-AAACGATAGC-3′.

2.4. Western blot analysis

Western blot analysis was performed as described in Lotan et al. (2005). Visualization was performed using LAS4000 luminescent image analyzer (Fujifilm). Densitometry analyses of Western blot results were done using TotalLab TL100 graphic software.

2.5. GST pull-down binding assay

COS-7 cells were co-transfected with GST-XIAP together with different constructs of ARTS. The GST pull down binding assay was performed as described in Gottfried et al. (2004).

2.6. In vivo ubiquitination – immunoprecipitation assay

COS-7 cells were transiently transfected with different constructs of AU5-ARTS, GST-XIAP and HA-ubiquitin. *In vivo*

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ubiquitination assays were performed as previously described in Lotan et al. (2005). HeLa cells were transiently transfected with 6Myc-ARTS, Flag-XIAP or XIAP-H467A and HA-ubiquitin constructs. *In vivo* ubiquitination assays were performed as previously described in Dogan et al. (2008).

2.7. In vitro ubiquitination assay

The assay was performed in 24 μl reaction mixture (40 mM Tris–HCl, pH 7.6, 2.5 mM MgCl2, 0.5 mM DTT and 2 mM ATP). The following purified components were added: 0.2 μg UbcH5c serving as E2, 4 μg ubiquitin and 1 μg of ARTS labeled with 35S that was generated using the TNT-Quick Coupled Transcription/Translation System (Promega) and 0.5 μg or 1 μg of purified GST-XIAP. The reactions were incubated for 30 min at 37 °C.

2.8. Cell fractionation

Cell fractionations were carried out in two different ways; One by using Dounce homogenizer as described in Chandra et al. (2002), Gottfried et al. (2004) and the other by digitonin fractionation as described in Adrain et al. (2001), Edison et al. (2011). The exact method used in each experiment is specifically described in the appropriate figure legend.

2.9. Apoptotic assays (caspase 9 activity and TUNEL assays)

COS-7 cells were transiently transfected with different constructs of AU5-ARTS and empty vector. Caspase 9 fluorimetric assay was performed according to the manufacturer's instructions (R&D Systems). TUNEL assay (*in situ* cell death detection kit, Roche, #12-156-792910) was performed on as described in Edison et al. (2011).

3. Results

3.1. XIAP regulates the protein levels of ARTS by serving as its E3-ligase

We have previously shown that under non-apoptotic conditions the levels of ARTS are kept low through constant ubiquitin-mediated degradation (Lotan et al., 2005). Yet, in response to pro-apoptotic stimuli a significant increase in the protein levels of ARTS was detected (Fig. 1A and Suppl. Fig. 1A). We therefore hypothesized that the observed increase in ARTS may result from inhibition of its ubiquitination and degradation. XIAP is an E3-ligase and its E3-ligase activity is important for both self-conjugation and caspase regulation (Galban and Duckett, 2010; Morizane et al., 2005; Schile et al., 2008; Yang et al., 2000). Since ARTS was shown to bind directly to XIAP and promote apoptosis through antagonizing XIAP activity (Bornstein et al., 2011; Garrison et al., 2010; Gottfried et al., 2004; Reingewertz et al., 2011), we tested whether ARTS is a substrate of XIAP. An *in vitro* ubiquitination assay using recombinant ARTS in the presence or absence of GST-XIAP revealed a significant increase in the ubiquitination of ARTS upon addition of XIAP (Fig. 1B). To verify that XIAP E3-ligase activity is required for the regulation of the protein levels of ARTS, we used an XIAP construct lacking its RING domain (XIAPdelRING) (Fig. 1C.I) and an XIAP-H467A mutant construct compromised in its E3 ligase activity (Fig. 1C.II). These *in vivo* ubiquitination assays done with both COS-7 and HeLa cells demonstrated a significant reduction in the ubiquitination of ARTS when co-transfected with mutant XIAP as compared to its ubiquitination when co-transfected with full length XIAP (Fig. 1C). This suggests that the E3-ligase activity of XIAP is required for the ubiquitination of ARTS. Next, we showed that the E3-ligase activity of XIAP is also required for down-regulating the protein levels of ARTS. Fig. 1D revealed that co-transfection of ARTS and full length XIAP strongly reduces the levels of ARTS, while co-transfection of ARTS and XIAPdelRING does not change ARTS protein levels (Fig. 1D). Moreover, MEFs derived from XIAPdelRING mice expressed elevated levels of ARTS when compared with WT MEFs (Fig. 1E). Thus we conclude that ARTS and XIAP interact in living cells, and that XIAP serves as the E3-ligase of ARTS and is responsible for the ubiquitination and degradation of ARTS.

3.2. Deletion of the first four amino acids in ARTS containing Lysine3 results in its stabilization in the cytosol

Proteins are targeted for UPS-mediated degradation by the covalent modification of ubiquitin to a specific lysine residue (Glickman and Ciechanover, 2002). We observed that the N-terminal sequence of ARTS contains a lysine residue at position 3 (Fig. 2A, top panel). To find out if this particular lysine plays a role in the ubiquitination and degradation of ARTS by XIAP, we prepared a deletion mutant of ARTS lacking its first four amino acids (ARTSdel-4aa) including the lysine at position 3 (Fig. 2A, bottom panel). Cellular fractionation of COS-7 cells transfected with either full length or mutant ARTS revealed that deletion of the first four amino acids in ARTS is sufficient to increase the stability of this mutant, allowing it to accumulate both at the mitochondria and cytosol (Fig. 2B). Furthermore, we show that ARTSdel-4aa binds to XIAP equally well as full length ARTS (Fig. 2C). This indicates that deletion of these particular amino acids does not prevent the binding of ARTS to XIAP.

To specifically investigate whether deletion of the first four amino acids in ARTS protects it from ubiquitination and degradation by XIAP, we performed an *in vivo* ubiquitination assay comparing the ubiquitination of full length ARTS to that of ARTSdel-4aa. To allow accumulation of potential polyubiquitinated forms of ARTS, we pretreated COS-7 cells over-expressing ARTS, ARTSdel-4aa and XIAP with the proteasome inhibitor MG132 for 6 h. Consistent with previous results, these experiments showed that full-length ARTS undergoes constant ubiquitination in the presence of exogenous XIAP, as revealed by the appearance of high levels of poly-ubiquitinated-ARTS (Fig. 2D). Importantly, although ARTSdel-4aa could strongly bind to XIAP (Fig. 2C), a significant decrease in its ubiquitination was demonstrated in the presence of XIAP (Fig. 2D). These results suggest that the first four amino acids in ARTS are essential for the ubiquitination of ARTS by XIAP.

Finally, we hypothesized that if ARTSdel-4aa is more resistant to XIAP-induced degradation, then it should accumulate in the cytosol and become more potent in promoting caspase activation and apoptosis as compared to full length ARTS. Indeed, transfection of ARTSdel-4aa into COS-7 cells resulted in stronger activation of caspase 9 (Fig. 2E.I) and higher percentage of TUNEL positive cells (Suppl. Fig. 2) as compared to full length ARTS. Similarly, Western blot analysis of ARTSdel-4aa transfected cells, revealed a strong increase in three apoptotic markers: active caspase 9, active caspase 3 and H2AX (Fig. 2E.II). These results indicate that ARTSdel-4aa is a more potent inducer of apoptosis than full-length ARTS.

Interestingly, elevated levels of ARTS are detected in lysates of HeLa cells as soon as 30 min following treatment with STS (Fig. 1A). Furthermore, significant levels of ARTS are found at the cytosol of HeLa cells at the same time point following apoptotic induction (Fig. 2F). This early accumulation of ARTS is associated with a strong decrease in levels of XIAP seen at this time point (Fig. 2F). We therefore suggest that under non apoptotic conditions XIAP promotes the ubiquitination and degradation of ARTS. Once apoptosis is triggered, ARTS translocation to the cytosol changes the balance towards ARTS mediated ubiquitination and degradation of XIAP.

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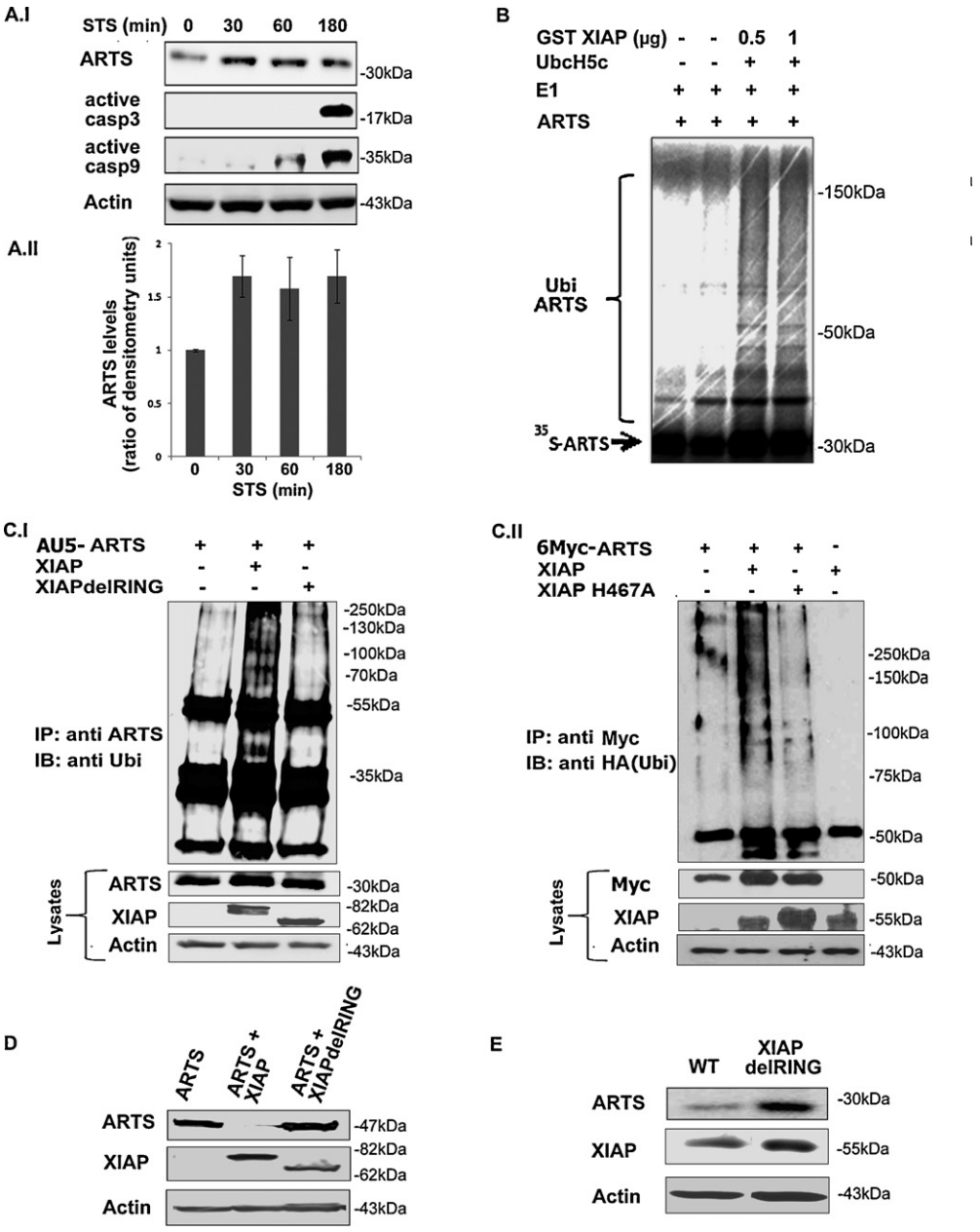

Fig. 1. XIAP regulates the protein levels of ARTS by serving as its E3-ligase. (A) Protein levels of ARTS are elevated upon induction of apoptosis. (I) HeLa cells were treated with 1.75 μM STS for increasing periods of time. Western blot analysis of lysates was done using the indicated antibodies. (II) Densitometry analyses of ARTS protein levels are presented as fold of induction (mean ± S.E., $n = 3$). (B) *In vitro* ubiquitination assay was performed with addition of an E1, E2-UbcH5c, recombinant ARTS protein labeled with ^{35}S and in the presence or absence of purified GST-XIAP. Addition of purified XIAP promoted the ubiquitination of ARTS. (C) *In vivo* ubiquitination assays. (I) COS-7 cells transiently transfected with AU5-ARTS together with GST-XIAP or XIAPdelRING and HA-ubiquitin. Cells were treated with 20 μM MG132 for 6 h. Immunoprecipitation (IP) assay was carried out with anti-ARTS antibody followed by immune-blotting (IB) with the indicated antibodies. (II) HeLa cells were transiently transfected with 6Myc-ARTS together with flag-XIAP or XIAP-H467A and HA-ubiquitin. Cells were treated with 10 μM MG132. Immunoprecipitation (IP) assays were carried out with anti-Myc antibody followed by immune-blotting (IB) with the indicated antibodies. (D) COS-7 cells were transfected with 6myc-ARTS construct alone or co-transfected with 6myc-ARTS construct together with GST-XIAP or GST-XIAPdelRING. Western blot analysis was performed with the indicated antibodies. Levels of ARTS remain unchanged in cells transfected with XIAPdelRING. (E) MEFs from XIAPdelRING and control MEFs were lysed and subjected to Western blot analysis with the indicated antibodies. XIAPdelRING MEFs expressed elevated levels of ARTS when compared to control MEFs.

4. Discussion

XIAP is considered to be the most potent inhibitor of caspases (Deveraux and Reed, 1999). XIAP inhibits apoptosis by binding to active caspase 3, 7 and 9 (Huang et al., 2001; Schile et al., 2008; Yang et al., 2000). Moreover, recent studies have shown that XIAP can act upstream of Mitochondrial Outer Membrane Permeabilization (MOMP) (Albeck et al., 2008; Flanagan et al., 2011; Owens et al., 2010). In this study we show that XIAP also promotes the ubiquitination and degradation of its antagonist ARTS.

Using both *in vitro* and *in vivo* ubiquitination assays we found that ARTS is directly ubiquitinated by XIAP and that XIAP serves as the specific E3-ligase for ARTS. Additionally we found that XIAP-induced ubiquitination and degradation is prevented by removal of the first four amino acids in the N-terminus of ARTS, which contains a lysine residue at position 3. Thus, this lysine at position 3 is a likely target for ubiquitination by XIAP. Importantly, though the

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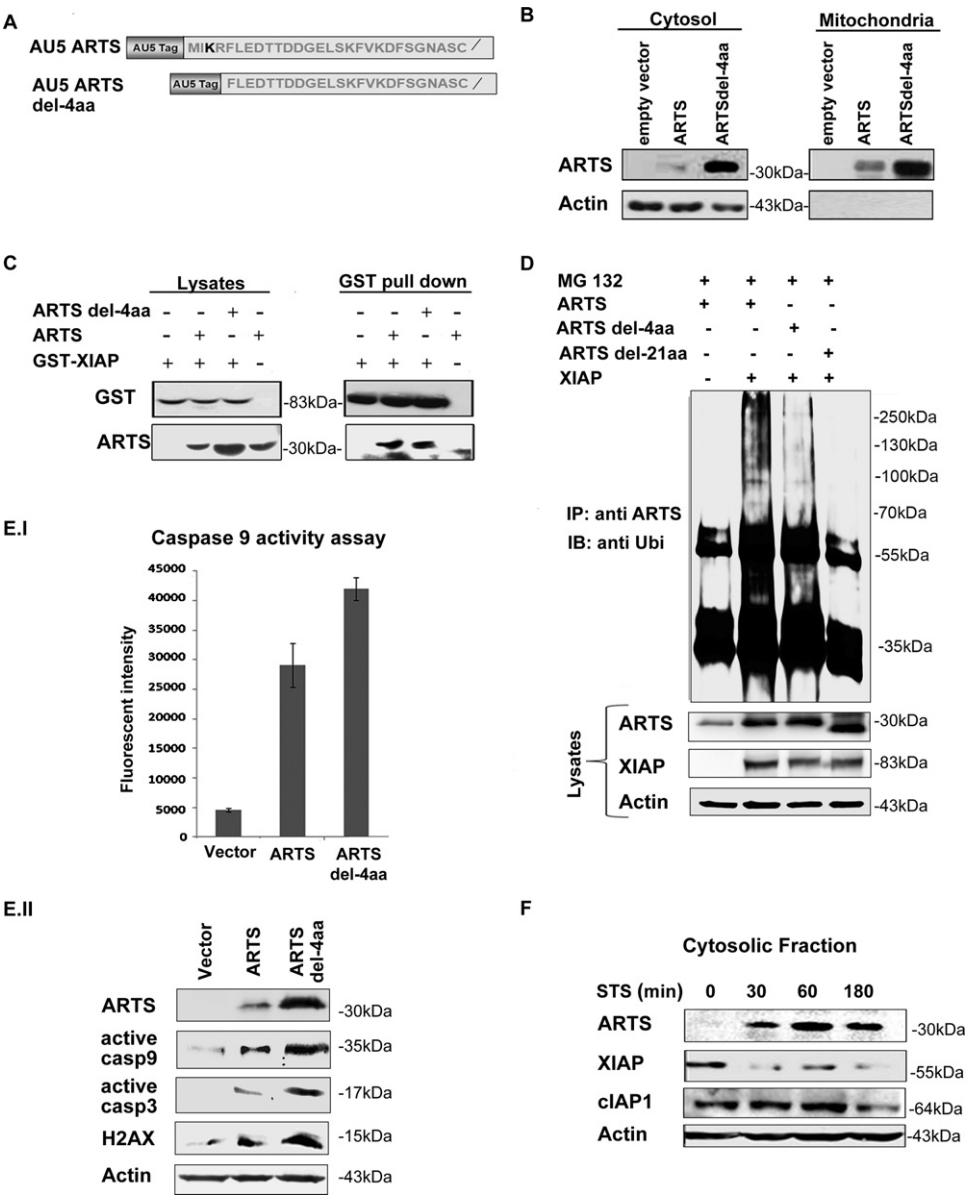

Fig. 2. Deletion of the first four amino acids in ARTS containing Lysine3 results in its stabilization in the cytosol. (A) Schematic diagram exhibiting ARTS constructs used in this study: full length ARTS and N-terminal deletion ARTS which lacks its first four aa (ARTS del-4aa), including lysine at position 3. (B) COS-7 cells were transiently transfected with full length ARTS, mutant ARTS or empty vector. Cellular fractionation assay was performed by Dounce homogenizer fractionation technique. Western blot analysis of mitochondrial and cytosolic fractions was performed using anti-ARTS and anti-actin antibodies. Mutant ARTS accumulated in the cytosol. (C) COS-7 cells were transiently transfected with GST-XIAP together with full length or mutant ARTS. GST Pull down assays were carried out followed by Western blot analysis with anti-ARTS and anti-GST antibodies. Full length and mutant ARTS bind equally well to XIAP. (D) *In vivo* ubiquitination assay was performed on COS-7 cells co-transfected with GST-XIAP and various ARTS constructs (AU5-ARTS, AU5-ARTS del-4aa and AU5-ARTS del-21aa, deleted at their N-terminal part). Cells were treated with 20 μM MG132 for 6 h. Immunoprecipitation (IP) assay was carried out with anti-ARTS antibody, followed by immune-bloting (IB) with the indicated antibodies. Full length ARTS is constantly ubiquitinated in living cells (IP, top panel). Deletion of the first 4aa and 21aa in ARTS results in a significant reduction in ubiquitination of ARTS. (E) Mutant ARTS is a much more potent inducer of apoptosis then full-length ARTS. COS-7 cells were transiently transfected with AU5-ARTS, AU5-ARTSdel-4aa or AU5-empty vector. (I) Caspase 9 activity assays were performed (mean ± S.E., $n = 3$). (II) Western blot analysis was performed using the indicated antibodies. Mutant form of ARTS is more potent in inducing apoptosis than the full length ARTS. (F) ARTS translocates to the cytosol 30 min after STS induction. HeLa cells were treated with 1.75 μM STS for increasing periods of time. Digitonin fractionation assay was performed, followed by Western blot analysis using anti-C-terminus ARTS (CT-ARTS), anti-N-terminus ARTS (NT ARTS), anti-XIAP, anti-cIAP1 and anti-actin antibodies.

stabilized mutant ARTS binds XIAP as well as the full length ARTS, it is more potent in promoting apoptosis than the full length ARTS. This suggests that increased stability of ARTS has a significant effect on its ability to induce apoptosis.

We hypothesize that ARTS and XIAP can interact with each other both in living cells and in cells undergoing apoptosis. We and others have shown that upon apoptotic stimuli ARTS promotes caspase activation by inducing ubiquitin–proteasome-mediated degradation of XIAP (Bornstein et al., 2011; Garrison et al., 2010; Gottfried et al., 2004). Here we show that the interaction between ARTS and XIAP can also occur under non apoptotic conditions. First, we show that XIAPdelRING MEFs exhibit increased levels of ARTS when compared to WT MEFs (Fig. 1E). Similar results were seen using XIAP-null MEFs (data not shown). This suggests that XIAP regulates the steady state levels of ARTS. Second, *in vivo* ubiquitination assays and immunoprecipitation assays performed in non-apoptotic cells indicate that the binding of ARTS and XIAP can also occur in living cells (Figs. 1C, 2C and D).

We propose that the presence or absence of pro-apoptotic signals controls and affects the equilibrium between ARTS and XIAP.

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Under non apoptotic conditions, the levels of ARTS are kept low through constant ubiquitination and degradation by XIAP. Once apoptosis is triggered, ARTS translocates to the cytosole and its levels are elevated (Fig. 1 and Suppl. Fig. 1) which in turn, results in the degradation of XIAP and caspase activation (Bornstein et al., 2011; Edison et al., 2011; Gottfried et al., 2004).

Several studies have shown regulation of IAP-antagonists by IAPs; this mechanism seems to be conserved throughout evolution as the *Drosophila* IAP homolog DIAP-1 regulates the protein levels of its antagonists Reaper, HID, Grim (Olson et al., 2003). In mammalian cells, XIAP promotes the ubiquitination of SMAC and AIF through its E3-ligase activity (Galban and Duckett, 2010; MacFarlane et al., 2002; Morizane et al., 2005). Moreover, Bruce-Apollon, Op-IAP, Livin, cIAP1 and cIAP2 all mediate the ubiquitination of SMAC (Hao et al., 2004; Hu and Yang, 2003; Ma et al., 2006; Wilkinson et al., 2004). SMAC was suggested to function as the mammalian homologue of Reaper, HID, GRIM due to structural homology of their IAP-Binding Motif (IBM) (Du et al., 2000; Shi, 2002). However, SMAC is localized inside mitochondria, in the mitochondrial inter membrane space, and requires MOMP for its release to the cytosol (Burri et al., 2005; Verhagen et al., 2007). We have found that although ARTS does not contain a classical transmembrane domain it is localized at the MOM, presumably through association with an integral MOM-bound protein (Edison et al., 2011). Likewise, the *Drosophila* IAP antagonists Reaper binds to HID at the MOM (Sandu et al., 2010). Taking into consideration the localization of ARTS at the MOM and its mutual regulation with XIAP, we suggest that ARTS mechanism of action highly resembles that of Reaper/HID/Grim leading to caspase activation and cell death.

Collectively, our data reveal a mutual regulatory mechanism by which ARTS and XIAP control each other's levels through the UPS.

Acknowledgments

We thank John Silke, Colin Duckett and Yuri Lazebnick for generously providing us with constructs and antibodies used in this manuscript. We are also grateful to Carrie Anderson and Juliana Kagan for technical assistance. This work was supported by funds from BSF (US Israel Binational Science Foundation) grant #2003085 (to S.L), ISF (Israel Science Foundation), grant #1264/06 (to S.L) and from ENP (Emmy Noether programme) grant #RA1739/1-1 (to K.R. from the DFG). This work was also made possible through a generous contribution by Ms. Helen Steyer and Mr. Tommy Steyer, USA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2011.12.005.

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Please cite this article in press as: Bornstein B, et al. X-linked Inhibitor of Apoptosis Protein promotes the degradation of its antagonist, the pro-apoptotic ARTS protein. Int J Biochem Cell Biol (2011), doi:10.1016/j.biocel.2011.12.005

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