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The mitochondrial ARTS protein promotes apoptosis through targeting XIAP

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ARTS is an unusual septin-like mitochondrial protein that was originally shown to mediate TGF-beta-induced apoptosis. Recently, we found that ARTS is also important for cell killing by other pro-apoptotic factors, such as arabinoside, etoposide, staurosporine and Fas. In *Drosophila*, the IAP antagonists Reaper, Hid and Grim are essential for the induction of virtually all apoptotic cell death. We found that mutations in *peanut*, which encodes a *Drosophila* homologue of ARTS, can dominantly suppress cell killing by Reaper, Hid and Grim, indicating that *peanut* acts downstream or in parallel to these. In mammalian cells, ARTS is released from mitochondria upon pro-apoptotic stimuli and then binds to XIAP. Binding of ARTS to XIAP is direct, as recombinant ARTS and XIAP proteins can bind to each other *in vitro*. ARTS binding to XIAP is specific and related to its pro-apoptotic function, as mutant forms of ARTS (or related septins) that fail to bind XIAP failed to induce apoptosis. ARTS leads to decreased XIAP protein levels and caspase activation. Our data suggest that ARTS induces apoptosis by antagonizing IAPs.

Introduction

Programmed cell death by apoptosis is a major mechanism for regulating cell number and tissue homeostasis (Thompson, 1995; Meier *et al*, 2000). Apoptosis is tightly controlled through the action of both activators and inhibitors of caspases (Song and Steller, 1999; Shi, 2002). The best studied family of caspase inhibitors are the Inhibitors of Apoptosis Proteins (IAPs) (Salvesen and Duckett, 2002). All IAPs contain between one to three baculoviral IAP repeat (BIR) domains. BIR domains can directly interact with caspases and inhibit their apoptotic activity (Deveraux and Reed, 1999; Shi, 2002). Some of the IAP proteins also contain a RING domain and are thought to function as E3-ubiquitin ligases (Yang *et al*, 2000; Hu and Yang, 2003). Among

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these, XIAP has been the best studied. XIAP contains three BIR domains and can directly inhibit caspases 3, 7 and 9 (Deveraux *et al*, 1997; Sun *et al*, 1999, 2000).

In cells that are doomed to die, inhibition of apoptosis has to be overcome. In *Drosophila*, Reaper, Hid and Grim are essential for the induction of virtually all apoptotic cell death (White *et al*, 1994; Bergmann *et al*, 1998). Reaper, Hid and Grim induce apoptosis by binding to and inhibiting *Drosophila* IAP-1 (Diap1) (Wang *et al*, 1999; Goyal *et al*, 2000). Additional IAP antagonists have been identified both in insects and mammals (Du *et al*, 2000; Verhagen *et al*, 2000, 2002; Martins *et al*, 2002; Salvesen and Duckett, 2002). Like in *Drosophila*, these proteins use a short, conserved N-terminal sequence termed IBM (IAP-Binding Motif) for IAP binding and inhibition (Shi, 2002). Binding of Reaper/Hid/Grim to DIAP1 has two consequences: release of caspases bound to the IAP BIR domains causes de-repression of caspases and stimulation of auto-ubiquitination, leading to IAP protein degradation (Martin, 2002). In addition, Diap1 is also the target for caspase cleavage and degradation by the 'N-end rule' pathway (Ditzel *et al*, 2003). Although auto-ubiquitination and degradation of IAPs is also seen in mammalian apoptosis (Yang *et al*, 2000), a role of mammalian IBM proteins in these processes remains to be established.

ARTS is a pro-apoptotic protein derived by differential splicing from the human septin *H5/PNUTL2/CDCrel-2a* (*Sept4*) gene (Larisch *et al*, 2000a, b). ARTS contains a P-loop GTP-binding motif conserved in this family. Yet, unlike most other family members, it is localized to the mitochondria and promotes apoptosis. Although ARTS was initially found to mediate TGF-beta-induced apoptosis, it also promotes apoptosis induced by many other pro-apoptotic stimuli, such as etoposide, arabinoside (ara-C), staurosporine (STS) and Fas. Here we demonstrate that ARTS promotes apoptosis through direct binding and inhibition of XIAP. Upon induction of apoptosis, ARTS is released from the mitochondria and forms a stable complex with XIAP. Binding of ARTS to XIAP causes a significant reduction in XIAP levels and leads to caspase activation and cell death.

Results

We previously reported that ARTS induces caspase-3 activity in response to TGF-β treatment in NRP154 and COS-7 cells (Larisch *et al*, 2000b). To examine the effect of ARTS in a wider range of cells and with several different apoptotic inducers, we used two leukemic cells lines (HL-60 and K562) and a lung carcinoma cell line (A549) in addition to NRP154 and COS-7 cells in the current study. All cell lines were transiently transfected with ARTS and a control vector. HL-60 and K562 cells were treated with ara-C, A549 with TGF-β and COS-7 with etoposide. In all these cases, we observed that overexpression of ARTS led to increased caspase-3 activity in response to pro-apoptotic stimuli (*P*-values (ANOVA) of K562 and HL-60 cells were 0.02 and 0.001,
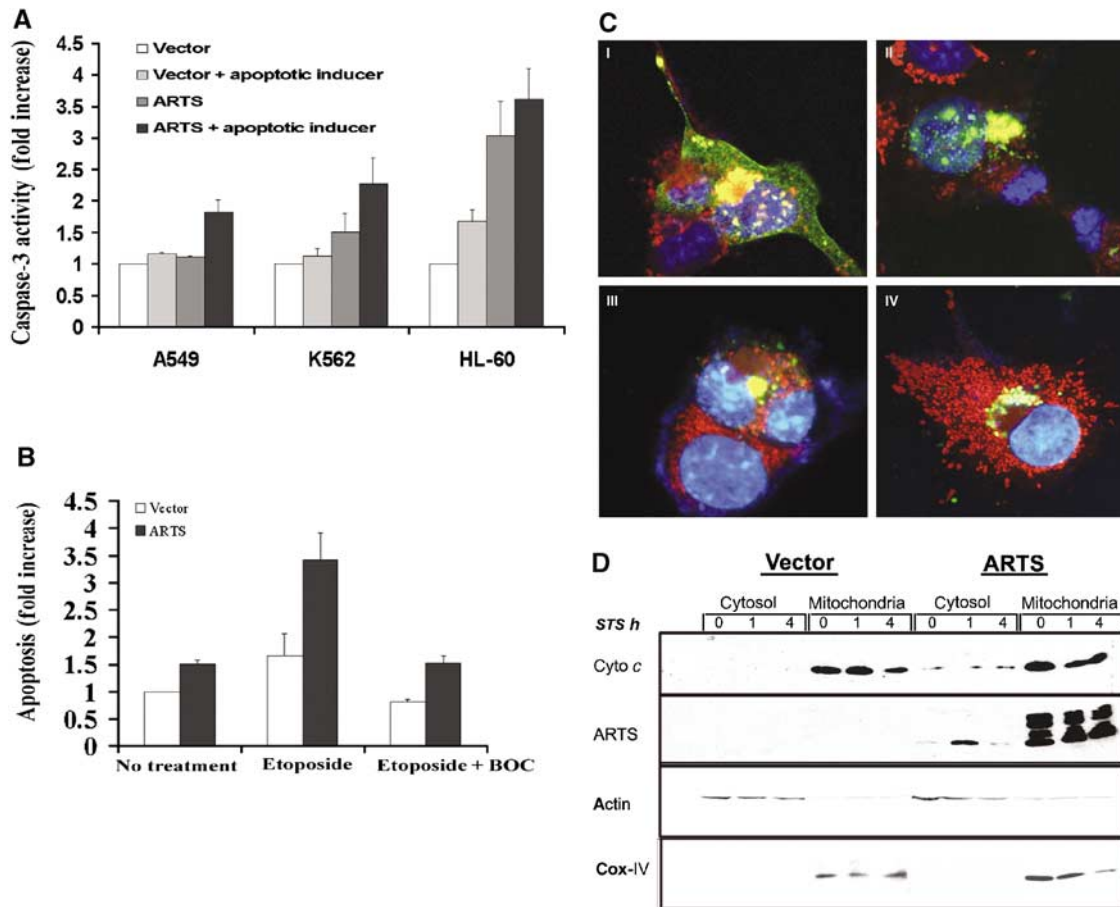
Figure 1 ARTS induces caspase-3 activity in response to a variety of apoptotic inducers, and caspase activity affects ARTS localization. Cells were transiently transfected with either control vector or the AU5-ARTS construct and treated with various apoptotic inducers. (**A**) ARTS induces caspase-3 activity in response to different apoptotic inducers. A549 cells were treated with TGF-β, K562 and HL-60 with ara-C. Caspase activity is presented as fold increase compared to cells transfected with control vector without apoptotic treatment. (**B**) COS-7 cells were treated with 50 μM etoposide for 16 h with and without BOC. (**C**) Immunofluorescence assay of COS-7 cells with MitoTracker® Red CMXRos and anti-ARTS antibodies. The cells were treated with 50 μM of etoposide for 16 h without (C I, II) and with BOC (III, IV). Cells treated with etoposide show typical morphological changes associated with apoptosis and accumulated ARTS in the nucleus (C I, II). Upon addition of caspase inhibitors nuclear translocation of ARTS is blocked, although perinuclear clustering of ARTS-positive mitochondria is still observed (C III, IV). (**D**) COS-7 cells transfected with AU5-ARTS or AU5-empty vector were treated with 1 μM STS for 0, 1 and 4 h prior to cell fractionation. Western blot analysis using anti-cytochrome *c* antibodies (upper panel) show cytochrome *c* restricted to the heavy membrane/mitochondrial fraction. Reduced cytochrome *c* levels were seen after 4 h of treatment with STS. Upon overexpression of ARTS, some cytochrome *c* was found in the cytosol. This is consistent with the increased levels of apoptosis seen under these conditions. The middle panel shows the distribution of ARTS in different cell fractions, using an anti-ARTS antibody (Sigma). In nonapoptotic cells, the vast majority of ARTS was found in the mitochondria. However, low amounts of ARTS protein were also detected in the cytosol of cells overexpressing ARTS. Upon apoptotic induction (1 h with STS), ARTS was released from the mitochondria and elevated levels were found in the cytosol. An antibody against the OxPhosComplex IV subunit IV (COX IV) was used as a mitochondrial marker.

respectively; A549 *P*-value (ANOVA) was 0.09, Figure 1A). The expression of ARTS alone had little or no effect on caspase-3 activity in A549, K562 and COS-7 cells, but it increased caspase-3 activity more than three-fold in HL-60 cells. Although the basis for this differential response is not clear, it should be noted that HL-60 cells are completely devoid of endogenous ARTS, whereas all the other cell lines express various levels of endogenous ARTS protein. We found that variations in expression levels of transfected ARTS are probably responsible for the observed differential response of cells. Higher expression levels of ARTS lead to increased apoptosis (Rotem, Lotan and Larisch, unpublished results).

Next, we wanted to examine the role of caspase activity on the subcellular distribution of ARTS protein. ARTS translocates from mitochondria to the nucleus during apoptosis

(Larisch *et al*, 2000b), but nothing is known about the underlying molecular mechanism. Caspase activity promotes the release of several other mitochondrial proteins, including cytochrome *c* and Smac/Diablo, from mitochondria into the cytosol (Li *et al*, 1998; Bossy-Wetzel and Green, 1999; Adrain *et al*, 2001; Guo *et al*, 2002). Therefore, we tested whether caspase inhibitors affected the mitochondrial release of ARTS. Inhibition of caspases in COS-7 cells transfected with ARTS blocked etoposide-induced apoptosis (*P*-value (ANOVA) 0.0026, Figure 1B). Using immunofluorescence staining, we examined the effect of caspase inhibitors on ARTS protein localization in COS-7 cells (Figure 1C). Transiently transfected cells were treated with etoposide and incubated with or without caspase inhibitors. In the absence of caspase inhibitors, etoposide-treated cells underwent the typical morphological changes associated

with apoptosis and accumulated ARTS in the nucleus (Figure 1C I and II). Interestingly, we saw clusters of ARTS-positive mitochondria in the immediate proximity of the nucleus preceding the nuclear entry of ARTS (Figure 1C I, II and data not shown). Similar clusters of mitochondria were described in Bid-induced apoptosis (Li *et al*, 1998). The addition of caspase inhibitors blocked the nuclear transloca-tion of ARTS, although we still observed the peri-nuclear clustering of ARTS-positive mitochondria (Figure 1C III and IV). Therefore, although ARTS promotes caspase activation, its distribution is also affected by caspase activity. To further confirm the release of ARTS from mitochondria upon pro-apoptotic stimuli, ARTS levels were measured in subcellular fractions from both ARTS-transfected and nontransfected cells. Under nonapoptotic conditions, ARTS was mainly detected in the mitochondria, although low levels of ARTS were also found in the cytosol (Figure 1D). This suggests that some ARTS protein constantly leaks from mitochondria into the cytoplasm. However, because ARTS is a very short-lived protein (Lotan, Rotem and Larisch, unpublished data), it apparently cannot accumulate to levels sufficient for the induction of apoptosis under these conditions. When treated with STS for 1 h, ARTS levels in the cytosol were strongly increased (Figure 1D), and this occurred prior to any detect-able release of cytochrome *c* (Figure 1D, upper panel). ARTS levels decrease following longer exposure to STS, presumably due to the degradation of ARTS in this compartment.

In order to gain an insight into where in the apoptotic pathway ARTS may act in the apoptotic pathway, we took advantage of the existence of a *Drosophila* homologue of the Sept4 locus. The *peanut* gene of *Drosophila* is highly homo-logous to the mammalian Sept4 locus, which encodes both ARTS and H5 (Neufeld and Rubin, 1994; Larisch *et al*, 2000b). Although it has been previously shown that *peanut* is re-quired for cytokinesis, a role of this locus in apoptosis has not yet been reported (Neufeld and Rubin, 1994). Interestingly, in addition to the previously described cell-surface distribution characteristic for septins, some Peanut protein was also detected in the mitochondria (Neufeld and Rubin, 1994; data not shown), suggesting that this gene may also have an ARTS-like pro-apoptotic function. In order to examine this possibility, we tested the effect of loss-of-function *peanut* mutations on cell killing induced by *Drosophila* Reaper, Hid and Grim. Expression of Reaper, Hid and Grim in the devel-oping *Drosophila* eye induces apoptosis that is sensitive to the dosage of other genes affecting cell death (McCall and Steller, 1997). In animals heterozygous for *peanut* (*pnut1/+* and *pnutXP/+*), the eye ablation phenotypes induced by Reaper, Hid and Grim were significantly reduced (Figure 2). The extent of suppression by *peanut* was greater than the con-sequence of reducing the dosage of the *Drosophila* Apaf1 homologue (Figure 2, rightmost panel), suggesting that *pea-nut* works downstream of Reaper, Hid and Grim, but prox-imal to the conversion with the Apaf-1 activated caspase pathway (Song and Steller, 1999; Zhou *et al*, 1999). One possible target for the pro-apoptotic action of Peanut/ARTS are the IAPs, which act immediately downstream of Reaper, Hid and Grim to inhibit caspases (Goyal, 2001).

In order to explore this possibility, we tested whether ARTS can physically interact with IAPs. For this purpose, we performed GST pull-down assays with GST–ARTS from COS-7 cells that were transiently transfected with pcDNA3
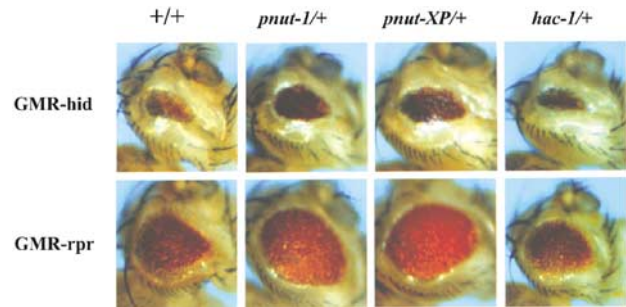

Figure 2 Mutations in *Drosophila peanut* dominantly suppress cell killing induced by Reaper and Hid. Ectopic expression of *reaper*, *hid* and *grim* under the control of the eye-specific GMR promoter induces apoptosis in cells of the developing retina, resulting in rough and reduced compound eyes. This provides a sensitive assay for mutations in other cell death genes (Bergmann *et al*, 1998). Reducing the amount of *peanut* by 50% (in *pnut1/+* or *pnutXP/+* heterozygous animals) significantly increased the eye size of both GMR-*hid* and GMR-*reaper* flies compared to a control background (*+/+*). Similar results were obtained for GMR-*grim* (data not shown). Under the same conditions, reducing the dosage of the *Drosophila* Apaf-1 homolog (*hac-1/+*) had very little effect.

mycXIAP. Western blot analysis with anti-XIAP and anti-Myc shows that XIAP can indeed bind to ARTS *in vitro* (Figure 3A, B). To confirm these results, we also performed the opposite type of pull-down experiment by using GST-XIAP to precipitate ARTS from COS-7 cells transfected with AU5-ARTS. As a control, we used COS-7 cells transfected with AU5-ARTSΔC or AU5-H5. The AU5-ARTSΔC construct lacks 68aa at the ARTS C′-terminus unique sequence. The H5 septin protein is derived by differential splicing from the same locus as ARTS and both proteins share most exons, but H5 is a considerably larger protein that lacks pro-apoptotic activity (Larisch *et al*, 2000b). Significantly, only ARTS, but not ARTSΔC or H5, were able to bind to XIAP (Figure 3C).

In order to test whether ARTS can bind directly to XIAP, we performed GST pull-down assays with GST-XIAP and purified recombinant ARTS protein. As a control, we incubated GST protein with the purified recombinant ARTS protein. Western blot analyses showed that ARTS can indeed bind to XIAP directly (Figure 3D).

Next, we tested whether endogenous ARTS and XIAP interact *in vivo*. For this purpose, we induced apoptosis in COS-7 cells with STS and used an anti-ARTS antibody coupled to protein G/A sepharose beads to recover ARTS–protein complexes. Western blot analysis of these complexes showed a band of the predicted size for XIAP at 57 kDa (Figure 4A). This suggests that in apoptotic cells at least some of the endogenous ARTS and XIAP proteins bind to each other.

Previously, we have shown that ARTS but not H5 or ARTSmGTP induce apoptosis in response to TGF-β (Larisch *et al*, 2000b). We found that mutant ARTS (ARTSmGTP) and H5ΔC did not bind to XIAP in a co-IP assay (Figure 4B). In addition, unlike ARTS, mutant derivatives (ARTSmGTP, ARTSΔC) and related but another nonapoptotic septin (PNUTL2) failed to induce apoptosis (Figure 4C). Therefore, binding of ARTS to XIAP is specific and highly correlated with its apoptotic function.

If significant levels of ARTS protein bind to XIAP in response to pro-apoptotic stimuli, we would expect to see co-localization of these proteins in apoptotic cells. To test
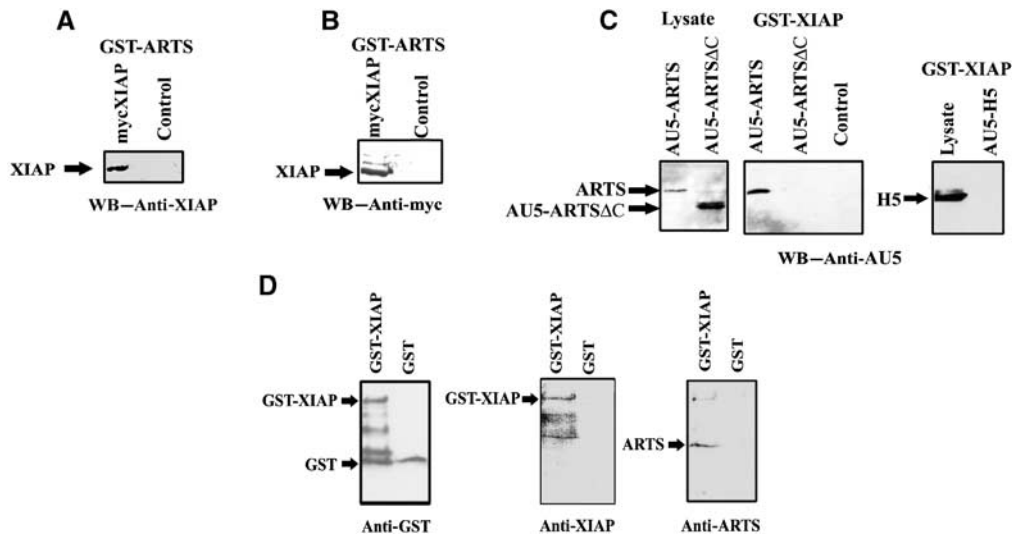
Figure 3 ARTS specifically binds XIAP *in vitro*. *In vitro* binding was tested using the GST pull-down assay. (**A**) COS-7 cells were transient transfected with pcDNA myc XIAP construct. GST pull-down was performed using GST–ARTS and Western blot was caried out with anti-XIAP and (**B**) with anti-myc. (**C**) COS-7 cells were transient transfected with AU5-ARTS, AU5-ARTSΔC or AU5-H5. GST pull-down was performed using GST–XIAP and Western blot was carried out with anti-AU5. Binding to glutathione beads alone served as a negative control. (**D**) Direct binding of ARTS to XIAP is shown in an *in vitro* assay using GST–XIAP and purified recombinant ARTS protein. GST protein served as a negative control.

this, we performed immunofluorescence double-labeling of COS-7 cells transfected with AU5-ARTS and pcDNA3-Myc-XIAP (Figure 5). Apoptosis was induced with etoposide and cells were stained cells after 2 and 16 h. In nonapoptotic cells, ARTS and XIAP had overall distinct, nonoverlapping distributions (Figure 5, top panel). ARTS was primarily localized to the mitochondria as previously described (Larisch *et al*, 2000b), whereas XIAP staining was cytoplasmic, with some perinuclear concentration. In contrast, 2 h after the induction of apoptosis, there was an extensive overlap between ARTS and XIAP staining (Figure 5, mid panel). Both proteins co-localized as aggregates that were most prominent in the vicinity of the nucleus. After 16 h of induction, most of the staining for both proteins was confined to the nucleus, consistent with previous reports on the localization of these proteins in apoptotic cells (Larisch *et al*, 2000b; Liston *et al*, 2001). However, our data reveal a striking level of co-localization of ARTS and XIAP in cells that are doomed to die, which was previously not recognized. These observations suggest that once ARTS is released from mitochondria in response to apoptotic stimuli, it binds rapidly and efficiently to XIAP, and both proteins appear to remain in a complex throughout much of the apoptosis.

We also used co-IP experiments to examine interactions between endogenous ARTS and XIAP in response to apoptotic stimuli. For this purpose, we initially used cells that express high levels of endogenous ARTS. We induced apoptosis with etoposide for 3 and 6 h and compared binding to non-induced controls (Figure 6A). In living cells, we detected only a small amount of XIAP bound to ARTS, presumably due to a small amount of cells undergoing apoptosis without induction. In contrast, after 3 h of etoposide treatment, a large amount of XIAP was immunoprecipitated together with ARTS, and this amount even increased further after 6 h of apoptotic induction.

As many of our previous studies were performed in COS-7 cells, we repeated co-IP experiments with ARTS and XIAP in

this cell type, even though COS-7 cells express very low levels of endogenous ARTS. COS-7 cells were treated with STS for 1 and 3 h to induce apoptosis. Thereafter, lysates were prepared for co-IP/Western blot analysis using a monoclonal antibody to detect XIAP (Figure 6B). As in cells, we observed very little interaction between ARTS and XIAP without apoptotic induction. After 3 h of incubation with STS, there was a significant increase in ARTS–XIAP binding. As ARTS and XIAP reside in different cellular compartments in living cells, we considered the possibility that caspase activity and execution of apoptosis may be required to release ARTS from mitochondria and allow interaction with XIAP. To test this possibility, we repeated co-IP experiments in the presence of the broad-spectrum caspase inhibitor BOC (Figure 6B). Treatment of BOC did not inhibit the binding of ARTS to XIAP in response to apoptotic stimuli. If anything, the amount of XIAP immunoprecipitated by ARTS under these conditions was even greater. Therefore, ARTS–XIAP interactions appear not to depend on caspase activity and do not occur as a consequence of apoptosis. We also noted a decrease in XIAP protein levels (compare 'IP–XIAP' 0 h with 1 and 3 h induction) that was readily detectable after 1 h. As this reduction of XIAP protein was not sensitive to the caspase inhibitor BOC, it cannot be simply the consequence of caspase activation and apoptosis. Taken together, these results demonstrate that the induction of apoptosis promotes interactions between ARTS and XIAP, presumably due to the release of ARTS from mitochondria. Moreover, cytochrome *c* and Smac/Diablo release from mitochondria is reported to occur in a caspase-dependent manner (Adrain *et al*, 2001). Our data suggest that the release of ARTS from mitochondria is a caspase-independent event. Thus, it seems that the release of ARTS may precede the release of both cytochrome *c* and Smac/Diablo from mitochondria, and ARTS may therefore act upstream of both Smac/Diablo and cytochrome *c*.

It was previously shown that XIAP is degraded upon the induction of apoptosis (Yang *et al*, 2000). As overexpression
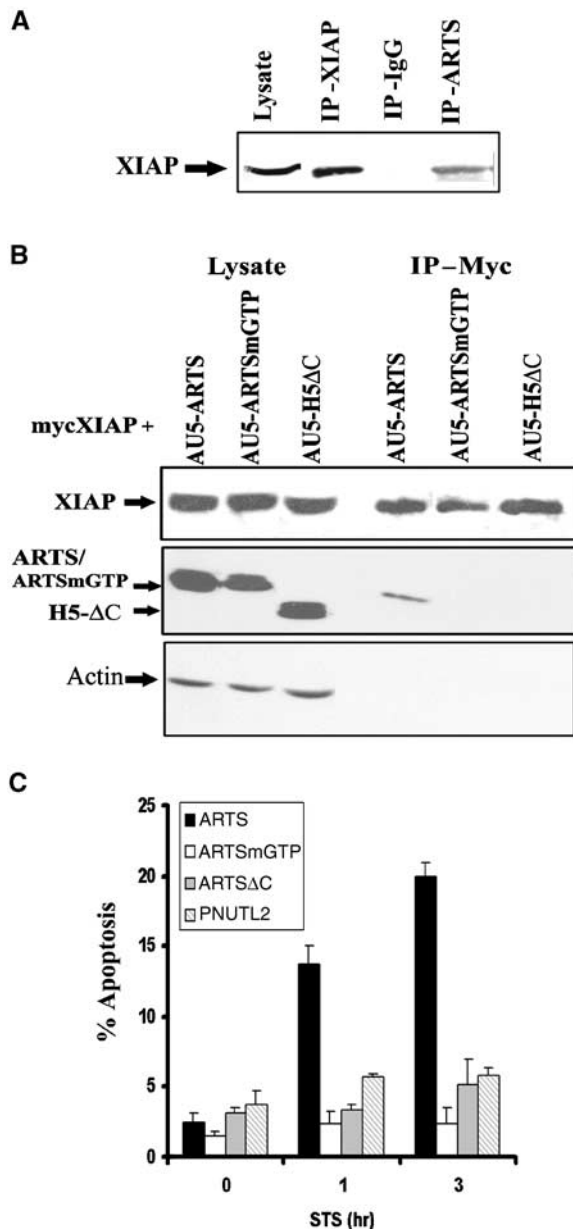
A

B

C

Figure 4 ARTS binding to XIAP is specific and related to its apoptotic function. (**A**) *In vivo* binding of ARTS to XIAP was tested using co-immunoprecipitation using anti-ARTS, anti-XIAP or mouse IgG as a negative control. Western blot analysis was carried out using anti-XIAP antibodies. (**B**) Co-transfected COS-7 cells with pcDNA-mycXIAP and AU5-ARTS, AU5-ARTSmGTP or H5 ΔC were co-immunoprecipitated using agarose-anti-myc beads. Western blot analysis was carried out using rabbit anti-myc and anti-ARTS (NT) antibodies directed against the common N′ terminus of ARTS and H5. Mutant forms of ARTS as well as H5 failed to bind to XIAP. (**C**) COS-7 cells were transient transfected with AU5-ARTS, AU5-ARTSmGTP, AU5-ARTSΔC or AU5-PNUTL2. Percent apoptosis was determined with the TMR kit. Unlike ARTS, mutant forms of ARTS as well as related septin do not promote apoptosis in STS-treated cells. The *P*-values (ANOVA) were 0.03 for non-treated cells, compared to 0.000004 for 1 h and 0.00001 for 3 h STS treatment.

of ARTS promotes apoptosis and was associated with downregulation of XIAP, we further investigated the consequence of increased levels of ARTS on XIAP protein. COS-7 cells were transiently transfected with pcDNA3-myc-XIAP and AU5-ARTS, and XIAP levels were assessed both with

anti-Myc and anti-XIAP antibodies. The amount of XIAP was significantly decreased in cells overexpressing ARTS compared to cells transfected with XIAP alone (Figure 7, see also Figure 6B for endogenous levels of ARTS). Similar results were obtained in COS-7 cells that had been stably transfected with ARTS expression construct (Figure 7B). These cells, which overexpress high levels of ARTS, show high levels of the apoptotic marker H2A.X that is detected only in apoptotic cells (Figure 7B; Paull *et al*, 2000). In addition, we found that COS-7 cells transiently transfected with ARTS and treated with STS had reduced XIAP protein levels, while cells transfected with a nonapoptotic-related septin (PNUTL2) showed no effect (Figure 7C). Furthermore, treatment of cells with the proteasome inhibitor MG132 blocked ARTS-induced reduction of XIAP levels, indicating that proteasome-mediated protein degradation is involved in ARTS-mediated downregulation of XIAP (Supplementary Figure 1). We therefore suggest that ARTS promotes apoptosis through binding to and inhibiting the antiapoptotic function of XIAP. At least part of this inhibition of XIAP appears to involve a reduction of XIAP protein levels via proteasome-mediated protein degradation (Figure 8, see Discussion).

Discussion

IAPs are a conserved family of proteins that can inhibit caspases (Salvesen and Duckett, 2002). In cells that are doomed to die, this inhibition of death has to be overcome (Song and Steller, 1999; Shi, 2002). Recent studies in both *Drosophila* and mammalian systems showed that decreasing IAP levels by ubiquitination increases caspase activity and leads to apoptosis (Martin, 2002). In the absence of the *Drosophila* IAP antagonists Reaper, Hid and Grim, virtually no apoptosis can occur (White *et al*, 1994; McCall and Steller, 1997), demonstrating the importance of IAP inactivation for the induction or cell death. In this study, we show that ARTS also induces apoptosis, at least in part, by targeting IAPs.

Several lines of evidence show that ARTS interacts with XIAP upon induction of apoptosis. In living cells, the localization of ARTS and XIAP is distinct, but both proteins co-localize extensively in apoptotic cells. Likewise, GST pull-down and co-IP assays demonstrate that ARTS and XIAP are in a complex under apoptotic conditions. As recombinant ARTS protein can bind XIAP *in vitro*, this interaction appears to be direct. This interaction appears to be highly specific, since it was not seen with nonapoptotic proteins closely related to ARTS.

The mitochondrial localization of ARTS and its ability to bind XIAP are reminiscent of Smac/Diablo and Omi/HtrA2, but unlike these proteins ARTS does not contain a recognizable IBM (Shi, 2002; Vaux and Silke, 2003). Possibly, binding of ARTS to XIAP requires the unique C-terminus of ARTS, a stretch of 27 amino acids not found in H5 (Larisch *et al*, 2000b). Consistent with this idea, we show that a C-terminal deletion of ARTS lacking this region did not bind to XIAP *in vitro* and did not induce apoptosis (Figures 3C and 4C, respectively). These results suggest that the unique C′ terminus of ARTS is important for its binding to XIAP. Interestingly, a GTP-binding domain mutant of ARTS also did not bind to XIAP and did not induce apoptosis. Therefore, this domain seems to be required for XIAP binding in combination with the unique C′ terminus of ARTS. One consequence of ARTS
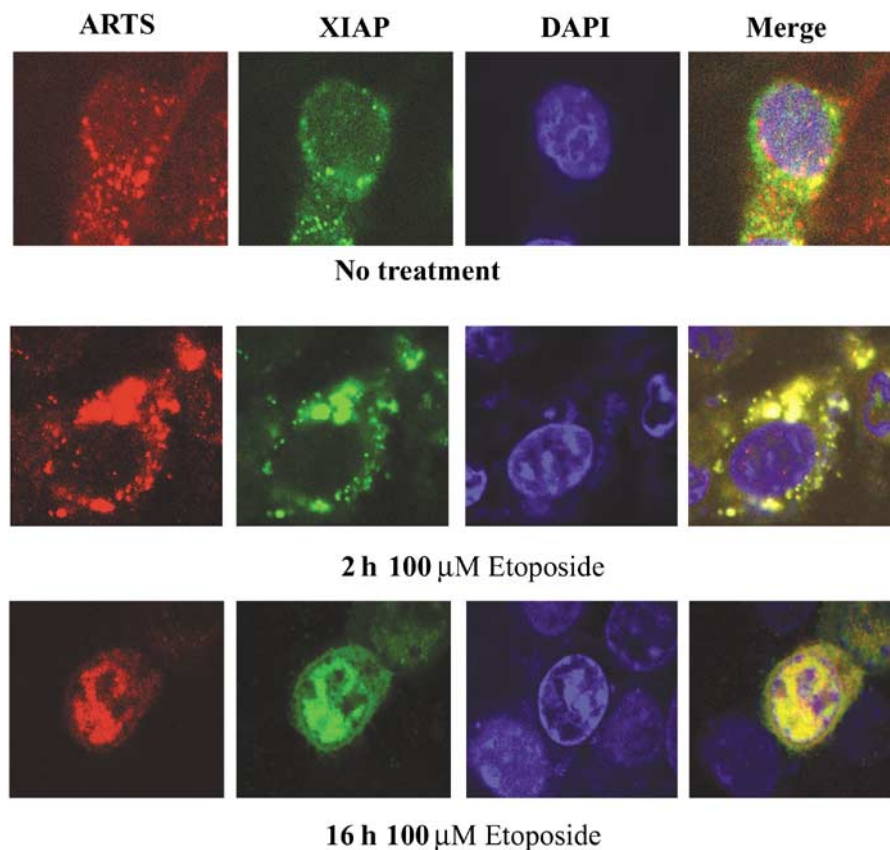
Figure 5 ARTS and XIAP co-localize during apoptosis. Immunofluorescence assay of COS-7 cells with anti-ARTS antibodies (Red), and anti-XIAP antibodies (green). The nucleus is stained with Dapi (blue). Cells were treated with 100 μM etoposide for 2 h (mid panel) and for 16 h (lower panel), as compared to non-treated cells (upper panel). The cells were analyzed using confocal microscopy (× 60). The merged images (right panel) are shown in yellow. Nonapoptotic cells had very little overlap between ARTS and XIAP staining (top panel). In nonapoptotic cells, ARTS was primarily localized to mitochondria, whereas XIAP staining was cytoplasmic, with some perinuclear concentration. At 2 h after the induction of apoptosis, co-localization of ARTS and XIAP occurred mainly in the vicinity of the nucleus (mid panel). After 16 h of apoptotic induction, both proteins perfectly co-localized in the nucleus.

binding to XIAP appears to be a reduction in XIAP protein levels. As this reduction of XIAP protein was also seen in cells treated with the caspase inhibitor BOC, it cannot simply be the consequence of caspase activation and apoptosis. Collectively, these results suggest that ARTS promotes apoptosis by binding to IAPs and decreasing their protein levels, which in turn de-represses caspases. Presumably, ARTS can interact with multiple IAPs, since inactivation of XIAP alone cannot account for the induction of apoptosis (Harlin *et al*, 2001).

ARTS and XIAP co-localize during most, if not all, of apoptosis and eventually both accumulate in the nucleus. The role of this nuclear complex is not clear at this time. As both proteins are subject to ubiquitin-mediated degradation (Yang *et al*, 2000; Lotan, Roten and Larisch, unpublished results), accumulation in the nucleus may simply be the result of differential protein stability in different cellular compartments. Alternatively, both proteins may play a role in the nucleus. Importantly, in apoptotic cells, which do not overexpress ARTS, only low levels of diffuse XIAP staining were seen (Supplementary Figure 2). In contrast, in cells overexpressing ARTS, XIAP was found at significantly higher levels in the nucleus. Thus, it appears that ARTS may be responsible for XIAP translocation to the nucleus. Interestingly, it has been reported that another XIAP-asso-

ciated protein, XAF1, triggers the redistribution of XIAP from the cytosol to the nucleus when overexpressed (Liston *et al*, 2001). Therefore, it is possible that nuclear translocation of XIAP–antagonist complexes is a common feature in apoptosis.

Several studies have shown feedback and feed-forward loops in the control of caspases (Slee *et al*, 1999). For example, IAPs are both inhibitors of and targets for caspases: mammalian XIAP and *Drosophila* Diap1 are cleaved by caspases during apoptosis, and this cleavage appears to be functionally significant (Deveraux *et al*, 1999; Ditzel *et al*, 2003). In addition, the release of cytochrome *c* promotes caspase activation, and caspase activity can promote the release of cytochrome *c* via cleavage of Bid (Li *et al*, 1998; Bossy-Wetzel and Green, 1999). Interestingly, binding of ARTS to XIAP does not require caspase activity and the execution of cell death. On the other hand, caspase inhibitors blocked the nuclear translocation of ARTS. Taken together, our results suggest the following role of ARTS for the induction of apoptosis (Figure 8). Upon apoptotic stimuli, ARTS is released from mitochondria by a caspase-independent mechanism that remains to be determined. The exit of ARTS from mitochondria enables ARTS to bind XIAP and reduce its protein levels, presumably by proteosome-mediated degradation. As a result, caspases become de-repressed and apoptosis is facilitated. ARTS shares its mitochondrial localization and
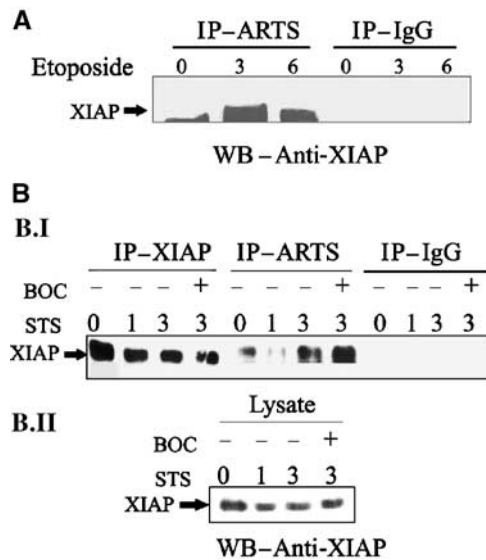
Figure 6 Upon apoptotic induction, ARTS binds higher levels of XIAP in a caspase-independent manner. (**A**) NRP-154 cells without apoptotic treatment, or treated with $100\,\mu M$ etoposide for 3 and 6 h were used for co-immunoprecipitation using anti-ARTS antibodies. Western blot analysis was carried out with anti-XIAP antibodies. (**B.I**) COS-7 cells without apoptotic treatment, or treated with $1\,\mu M$ STS for 1 and 3 h were used for co-immunoprecipitation using anti-ARTS antibodies. COS-7 cells were also treated with BOC (caspase inhibitor). (**B.II**) XIAP levels in the same lysates used for IP (B. I) are shown. During apoptotic induction, both NRP-154 cells and COS-7 cells show a significant increase of ARTS binding to XIAP. ARTS–XIAP interactions do not depend on caspase activity and do not occur as a consequence of apoptosis.

IAP-binding properties with Smac/Diablo, although there is no resemblance in the sequence of both proteins. Also, a reduction of IAP protein levels by Smac/Diablo has not yet been reported. In this respect, the function of ARTS is similar to that of the *Drosophila* IAP antagonists Reaper and Grim, even though again there is no detectable amino-acid similarity between ARTS and these proteins. As septins are thought to serve as scaffold proteins (Field and Kellogg, 1999; Kartmann and Roth, 2001; Kinoshita *et al*, 2002), it is possible that ARTS promotes the assembly of multi-protein complexes between IAPs and other cell death regulators, including IAP antagonists and ubiquitin pathway proteins. Alternatively, ARTS may employ novel protein motifs to carry out its IAP-inhibiting activities. In either case, our results demonstrate that ARTS is an important mitochondrial protein that promotes apoptosis through regulation of IAPs.

Materials and methods

Mammalian cell culture and plasmids
K562 and HL-60 cells were grown in RPMI 1640 medium. A549, COS-7 cells were grown in Dulbecco's modified Eagle medium (DMED) with 4.5 g/l D-glucose. All cells were grown at $37^{\circ}C$ in 5% CO_2 atmosphere. All media were supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin ($100\,\mu g/ml$) and glutamine (2 mM), medium (Biological Industries, Israel). Treatment of cells with proteosome inhibitor (MG132, Calbiochem) at $20\,\mu M$ for 2 h was carried out prior to the addition of the apoptotic inducer. COS-7, K562 and HL-60 were transient transfected using electroporation (Easyject plus—Equibio). A549 was transient transfected using lipofectamine^{TM} (Invitrogen), according to the manufacturer's protocol.
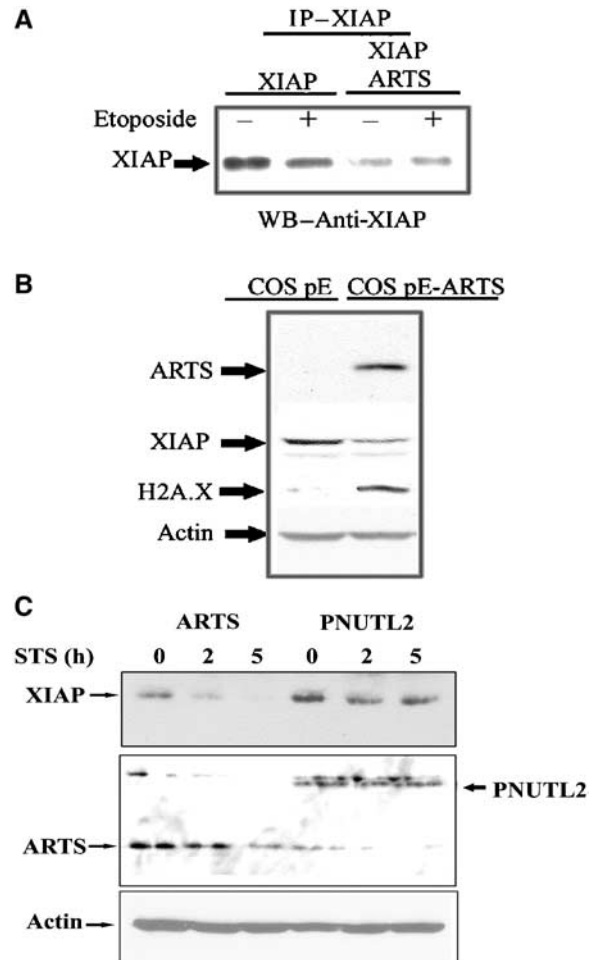

Figure 7 ARTS causes downregulation of XIAP levels during apoptosis. (**A**) COS-7 cells were transient transfected with either pcDNA3 myc XIAP alone, or co-transfected with pcDNA3 myc XIAP and AU5-ARTS. Cells with or without treatment with $100\,\mu M$ etoposide are shown. Immunoprecipitation with anti-myc antibodies was carried out, followed by Western blot analysis with anti-XIAP antibodies. (**B**) COS pE cells and COS pE stably transfected with AU5-ARTS. Analysis was carried out with anti-XIAP, anti-AU5, anti-H2A.X and anti-actin antibodies. COS pE-ARTS cells expressing high levels of ARTS exhibited significantly reduced levels of XIAP, which probably leads to the observed increase in apoptosis. The phospho-histone H2A.X, detected only under apoptotic conditions, was used to identify cells undergoing apoptosis. (**C**) COS-7 cells were transient transfected with AU5-ARTS or AU5-PNUTL2. Cells were treated with $1\,\mu M$ STS for 0, 2 and 5 h. Western blot analysis was carried out using anti-XIAP and anti-ARTS (NT) antibodies, which recognize the common N′ terminus of ARTS and H5. XIAP levels show a significant reduction only in lysates from ARTS-transfected cells. ARTS, but not the nonapoptotic-related septin (PNUTL2), causes downregulation of XIAP levels during apoptosis.

pEF1-AU5 and pEF1-AU5-ARTS constructs were used for all ARTS transient transfection experiments. AU5 tag was attached to the N′ terminus of ARTS (Larisch *et al*, 2000b). The AU5-ARTSmGTP construct was generated by site-directed mutagenesis (QuickChange^{TM}, Stratagene) with the primer ggagagtctggcctgga gaatcccacacttgtcaatagcc replacing three amino acids (G K S with E N P) at the GTP-binding site.

The AU5-ARTS ΔC construct lacks 68aa at the ARTS C′-terminus unique sequence and was generated using PCR with the primers: L1: *Bam*HI—atcaagcgtttcctggaggacaccacgg and S-207: *Eco*RI—ctatgccacaggcttccagcactc.

pEF1-AU5-H5, pEF1-AU5-PNTUL2 isoform 3, AU5-ARTSmGTP, AU5-H5 ΔC and AU5-ARTS ΔC were obtained from Dr Seong-Jin Kim, NCI/NIH.
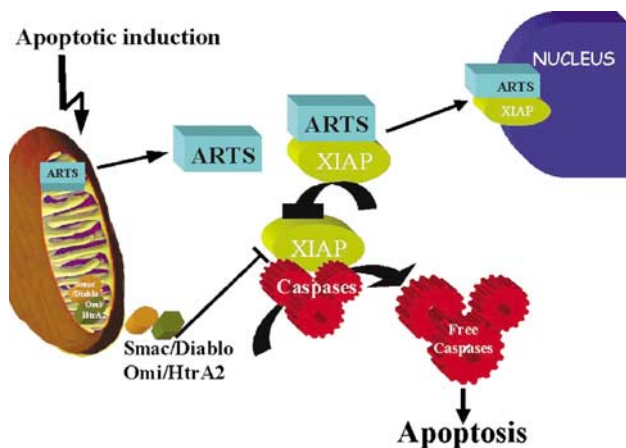
Figure 8 Schematic model of ARTS mechanism of action in apoptosis. In living cells, ARTS is localized to the mitochondria. Upon pro-apoptotic stimuli, ARTS is released from mitochondria in a caspase-independent manner and binds to XIAP. Eventually, the ARTS–XIAP complex translocates to the nucleus. As the release of Smac/Diablo from mitochondria is thought to require caspase activity, it appears that ARTS acts at an earlier stage during apoptosis. ARTS binding to XIAP downregulates XIAP levels, which releases caspase activation, leading to apoptotic cell death.

Stably transfected COS pE-ARTS cells were generated using the pEF1-IRES vector (Hobbs *et al*, 1998). Briefly, the construct contains EF-1 promoter followed by a multi-cloning site, EMC (Encephalo-myocarditis virus) internal ribosome entry sites (IRES) and puromycin resistance gene. AU5-tagged ARTS was inserted into the *Xho*I site downstream of the EF-1 promoter. The construct was stably transfected into COS-7 cells. COS pE cells stably transfected with pEF1-AU5 empty vector served as control. The cells were maintained in medium containing 4 µg/ml puromycin.

The mammalian expression construct encoding Myc epitope-tagged wild-type XIAP in pcDNA3 and the pGEX-XIAP were obtained from Dr Colin S Duckett.

For the GST-pull-down assays, we cloned full-length ARTS into the pGEX 4T (Pharmacia Biotech) construct; GST–ARTS fusion protein was generated using the PCR method with the following primers: *Bam*HI—5′-TCGAGGATCCATCAAGCGTTTCCTGGAGGACA CCACGG-3′ and *Eco*RI—5′-CTAGTGGCAGCCCTGCCCCTGGTGC-3′, and cloned into *Bam*HI and *Eco*RI sites in pGEX 4T.

Apoptosis assays

In order to induce apoptosis, cells were treated with different apoptotic agents 40 h after transient transfections. The following apoptotic agents were used: TGF-beta (10 ng/ml) for 24 h in medium containing 1% FCS, 100 µg/ml etoposide for 2 and 16 h (Sigma), STS (1 µM) for 0–3 h (Sigma) and arabinoside-c (ara-C, cytosar) 100 µM for 4 h (Pharmacia). For caspase inhibition, we added 40 µM of BOC-Asp(Ome)CH$_2$F (Enzyme Systems Products) 1 h prior to addition of the apoptotic agents. Apoptotic cells were detected using the anti-H2A.X antibody (Upstate; Paull *et al*, 2000) or TUNEL. For TUNEL assays, COS-7 cells were transiently transfected with AU5-ARTS, AU5-ARTS ΔC, AU5-ARTSmGTP and AU5-H5. At 24 h after transfection, the cells were treated with STS (1 µM) for 1 and 3 h. The cells were fixed and permeabilized. Apoptosis levels were determined using the TUNEL *In situ* cell death detection TMR kit (Roche) according to the manufacturer's protocol. All slides were coded and the experiments were carried out in a blind manner.

Caspase-3 activity assay

Caspase-3 activity was tested in K562, HL-60 and A549 cells using caspase-3 activity assay kit (Roche) according to the manufacturer's protocol. Caspase-3 activity was tested in COS-7 cells by immuno-fluorescence staining with anti-active caspase-3 antibodies 1:4000 (R&D systems). Results are presented as fold increase relative to results in cells transfected with control vector without treatment with apoptotic inducers.

GST pull-down experiments

For *in vitro* binding studies, recombinant GST–ARTS or GST–XIAP fusion proteins were purified from bacteria. After sonication, 0.1% Triton X-100 and protease inhibitors (Mini-Complete^{TM}, Roche) were added to the bacterial extract, followed by 10 000 rpm centrifugation. Supernatants of bacterial extracts were collected and incubated in the presence of glutathione-sepharose 4B beads (Amersham Biosciences) for 30 min at 4°C. The beads were washed three times. COS-7 cells were transient transfected with pcDNA3-mycXIAP construct, pEF1-AU5-ARTS construct or pEF1-AU5-H5 construct as a control. The cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8), 1% NP-40, 0.1% SDS, 0.5% deoxycholate acid containing protease inhibitors (mini Complete, Roche)). Each sample was divided into two tubes; one was rotated for 4 h at 4°C with GST–ARTS fusion protein coupled with the glutathione beads, or GST–XIAP fusion protein. The second tube served as control and was incubated with glutathione beads alone. Samples were centrifuged at 4000 rpm at 4°C for 4 min and washed five times in lysis buffer. Elution of proteins from beads was carried out by 5 min boiling in sample buffer. Proteins were separated on 12.5% SDS–PAGE gel, followed by Western blot analysis using monoclonal anti-ARTS antibodies (Sigma) or monoclonal anti-XIAP antibodies (BD Transduction Laboratories) or monoclonal anti-myc antibodies.

In vitro binding assay

Recombinant ARTS protein was generated with the TNT-Quick Coupled Transcription/Translation System (Promega) and incubated overnight at 4°C with either recombinant GST-XIAP bound to glutathione beads, or with GST alone bound to glutathione beads (beads were washed and treated as described in the above paragraph). SDS–PAGE analysis and Western blot was performed using monoclonal anti-ARTS antibodies (Sigma) and anti-GST antibodies (Gibco).

Co-immunoprecipitation

Cells were grown in 100 mm culture dishes and treated with or without apoptotic agents. Protein extracts were prepared with lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 8), 1% NP-40, 0.5% deoxycholate acid with protease inhibitors (mini complete, Roche). Protein levels were determined and equal amounts were used for each sample. Lysates were pre-cleared with 1 mg mouse IgG (Sigma) coupled with protein A/G sepharose mix (Amersham Biosciences). Complexes were incubated overnight at 4°C, followed by low-speed centrifugation. Supernatants were immunoprecipitated using 5 µl of monoclonal anti-ARTS antibodies (Sigma) for 4 h or monoclonal anti-XIAP antibodies (BD Transduction Laboratories) or monoclonal anti-myc antibodies (Clontech). As a control, we used mouse IgG (Sigma). Protein A/G sepharose beads were added to immunoprecipitate complex for 1 h, collected and washed four times with PBS.

For Western blot analysis, antibodies against XIAP (BD Transduction Laboratories), Myc (Santa Cruz), ARTS/ARTSmGTP/H5 (ProSci Incorporated) were used.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS and permeabilized with 0.5% Triton-X in PBS for 5 min, and incubated with primary antibodies for 2 h at RT (rabbit polyclonal anti-ARTS 1:20 000 (Sigma), monoclonal anti-ARTS (Sigma) and monoclonal anti-XIAP 1:500 (BD Transduction Laboratories). Cells were washed (×3) with PBS/0.1% Triton-X and incubated with FITC conjugated anti-mouse and Rhodamin^{TX} conjugated anti-rabbit secondary antibodies (Jackson). Image analysis was carried out using confocal laser microscopy (Zeiss LSM 510).

Cell fractionation

Cell fractionation was carried out as described (Chandra *et al*, 2002). Briefly, cells were homogenized in 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 in the presence of 250 mM sucrose and protease inhibitors (Mini-Complete^{TM}, Roche). Homogenates were centrifuged at 500 *g* for 5 min at 4°C, and the supernatant was centrifuged at 10 000 *g* for 20 min to obtain mitochondria. The mitochondrial pellet was washed and solubilized in TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 5 mM CaCl$_2$)

containing protease inhibitors. Protein concentration was determined by Micro-BCA kit (Pierce).

Anti-OxPhosComplex IV subunit IV antibodies (COX IV, Molecular Probes) were used to identify specifically the mitochondrial subcellular fraction.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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