AIMP2 promotes TNFα-dependent apoptosis via ubiquitin-mediated degradation of TRAF2

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Accepted 28 April 2009
Journal of Cell Science 122, 2710-2715 Published by The Company of Biologists 2009
doi:10.1242/jcs.049767

Summary

AIMP2 (aminoacyl-tRNA synthetase interacting multifunctional protein 2; also known as JTV-1) was first identified as p38 in a macromolecular protein complex that consisted of nine different aminoacyl-tRNA synthetases and two other auxiliary factors. AIMP2 also plays pivotal roles in the regulation of cell proliferation and death. Although AIMP2 was previously shown to augment TNFα-induced cell death, its working mechanism in this signal pathway was not understood. Here, we investigate the functional significance and mode of action of AIMP2 in TNFα signaling. TNFα-induced cell death was compromised in AIMP2-deficient or -suppressed cells and exogenous supplementation of AIMP2 augmented apoptotic sensitivity to TNFα signaling. This activity was confirmed by the AIMP2-dependent increase of IκB and suppression of NFκB. We found binding of AIMP2 to TRAF2, a key player in the TNFα signaling pathway. AIMP2 augmented the association of an E3 ubiquitin ligase, c-IAP1, with TRAF2, causing ubiquitin-dependent degradation of TRAF2. These findings suggest that AIMP2 can mediate the pro-apoptotic activity of TNFα via the downregulation of TRAF2 expression.

Key words: AIMP2 (p38, JTV-1), Aminoacyl-tRNA synthetase, TNFα signaling

Introduction

To form a macromolecular complex, several aminoacyl-tRNA synthetases (ARSs) associate with three auxiliary factors called ARS-interacting multifunctional proteins (AIMPs). Many of the enzyme components of the complex were previously shown to be involved in different signaling pathways with their own unique mechanisms (Lee et al., 2004; Park S. G. et al., 2005a). The three AIMPs seem to facilitate the assembly of the whole complex via interactions with each other as well as with their specific target enzymes (Han et al., 2006). These factors also play diverse regulatory roles. AIMP1 (p43) is secreted as a cytokine that controls angiogenesis (Park S. G. et al., 2002), immune response (Ko et al., 2001; Park H. et al., 2002) and tissue regeneration (Park S. G. et al., 2005b), and also as a hormone for glucose homeostasis (Park S. G. et al., 2006). It is also implicated in the regulation of autoimmune phenotype such as lupus (Han et al., 2007). AIMP3 (p18) was demonstrated to be a tumor suppressor responding to DNA damage (Kim et al., 2008; Park B. J. et al., 2005) or oncogenic stimuli (Park B. J. et al., 2006).

AIMP2 (also known as p38 and JTV-1) plays a pivotal role in the control of cell proliferation. It binds FUSE-binding protein (FBP), which is the upstream transcriptional activator of Myc and stimulates ubiquitylation-dependent degradation of FBP in response to TGFβ signaling (Kim et al., 2003). For this reason, mice lacking AIMP2 maintained a higher level of FBP during embryonic development, resulting in Myc overexpression and consequently eventually leads to overproliferation of lung epithelial cells. AIMP2 also responds to DNA damage via direct interaction with p53 (Han et al., 2008). Although AIMP2 seems to augment TNFα-induced cell death (Ko et al., 2005), how it can guide the apoptotic signal was not understood. In this work, we investigated the functional importance and mechanism of action of AIMP2 in TNFα-induced apoptosis.

Results

AIMP2 enhances the pro-apoptotic response to TNFα

We first assessed the significance of AIMP2 in TNFα-induced apoptosis using *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ mouse embryonic fibroblasts (MEFs). To inhibit the induction of target genes by NFκB, we treated the cells with murine TNFα and cycloheximide, and the induction of cell death was monitored by the percentage of sub-G1 cells using flow cytometry. Treatment with TNFα increased the portion of sub-G1 cells to nearly 30% in wild-type cells, but only to about 13% in AIMP2-deficient cells (Fig. 1A). TNFα-induced cell death was reduced when *AIMP2* transcript was suppressed with its specific siRNA (Fig. 1B). Introduction of exogenous *AIMP2* into *AIMP2*$^{-/-}$ MEFs enhanced the apoptotic sensitivity to TNFα (Fig. 1C). We also tested the effect of AIMP2 on colony formation under the effect of TNFα. First, we introduced AIMP2 or empty vector into MEFs of 12.5 embryonic days and grew the transfected cells under the selection of G418. The same numbers of the chemical-resistant transfectants were then further incubated in the absence or presence of TNFα for 1 week. Although both transfectants generated similar numbers of colonies in the absence of TNFα, *AIMP2*-transfected cells formed less colonies in the presence of TNFα compared with the empty-vector transfectants (Fig. 1D,E). All of these results suggest that AIMP2 can enhance apoptotic sensitivity to the TNFα signal.

We also examined the effect of AIMP2 on TNFα-induced cell death by monitoring NFκB-dependent luciferase activity, because the proapoptotic activity of TNFα is reflected by the reduced nuclear

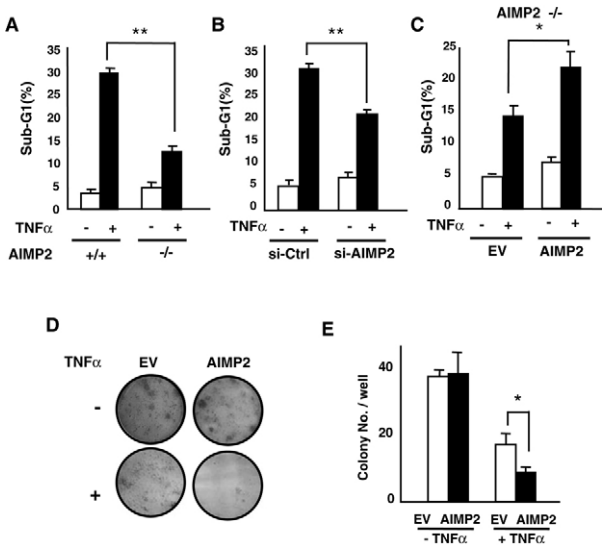

Fig. 1. AIMP2 enhances TNFα-induced cell death. (A) *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs were treated with murine TNFα (30 ng/ml) and cycloheximide (CHX; 10 μg/ml) for 12 hours, and apoptotic response of these cells was determined by counting sub-G1 cells using flow cytometry. All the experiments were conducted in triplicates. ***P*<0.01. (B) *AIMP2*$^{+/+}$ MEFs were transfected with non-specific siRNA (si-Ctrl) or siRNA targeting the *AIMP2* transcript (si-AIMP2), treated with TNFα and CHX, and the effect on cell death compared. ***P*<0.01. (C) *AIMP2*$^{-/-}$ MEFs were transfected with empty vector (EV) or AIMP2, treated with TNFα and CHX, and the cell death compared as above. **P*<0.05. (D) The same numbers of MEFs stably transfected with AIMP2 or EV were incubated in the absence or presence of TNFα for 1 week and the surviving colonies were monitored by Coomassie Blue staining. (E) The number of the colonies represented as a bar graph.

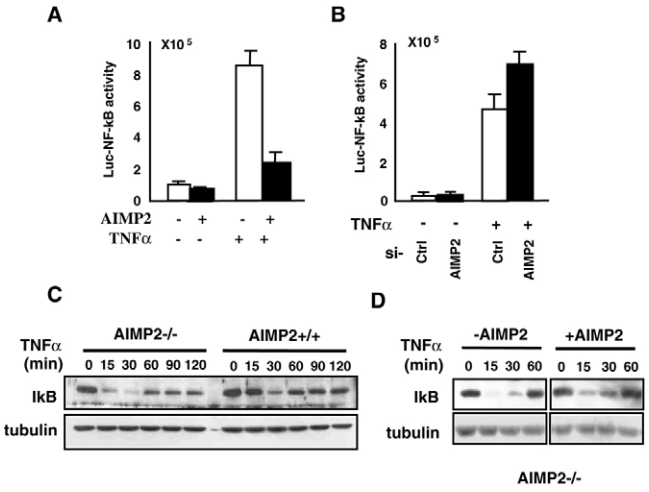

Fig. 2. AIMP2 downregulates NFκB activity. Empty vector (EV) or *AIMP2* (A) or control si-RNA (si-Ctrl) or –AIMP2 (B) were transfected into A549 cells stably expressing NFκB-luciferase, and the effect on luciferase activity was determined by using a luminometer. (C) The effect of AIMP2 on TNFα-dependent NFκB activation was also determined by monitoring the IκB level by western blotting. The TNFα-induced reduction of IκB was compared between *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs. (D) AIMP2 was introduced into *AIMP2*$^{-/-}$ MEFs and the change of IκB level determined.

localization of NFκB. TNFα-dependent NFκB activity was significantly reduced by the exogenous introduction of AIMP2 (Fig. 2A). Conversely, TNFα-dependent NFκB activity was further enhanced by the suppression of *AIMP2* with its specific siRNA (Fig. 2B). The effect of AIMP2 on NFκB activity was further monitored by the TNFα-dependent changes of IκB levels in *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs. IκB levels were significantly reduced within 30 minutes after TNFα treatment in AIMP2-deficient cells and then restored by 60 minutes (Fig. 2C). By contrast, these levels were only slightly affected by TNFα in wild-type cells. The TNFα-dependent reduction of IκB levels in *AIMP2*$^{-/-}$ cells was weakened by the exogenous supplementation of *AIMP2* (Fig. 2D).

Proapoptotic interaction of AIMP2 with TRAF2

We then investigated the mode of action of AIMP2 in the TNFα signaling pathway. Although AIMP2 could potentially function at various points in the pathway, we focused on TNF-receptor-associated factor 2 (TRAF2) as a potential target protein of AIMP2 because it plays a pivotal role in the apoptotic response to TNFα (Lee et al., 1997; Tada et al., 2001; Xia and Chen, 2005). Besides, TNFα induces the ubiquitylation of TRAF2 (Li et al., 2002) and AIMP2 mediates the ubiquitylation of FBP, the previously identified target protein in the TGFβ signaling pathway (Kim et al., 2003). Thus, we tested whether the ubiquitylation of TRAF2 could be influenced by AIMP2 in a TNFα-dependent manner. We treated HeLa cells with TNFα, and the interaction of AIMP2 and TRAF2 was examined by co-immunoprecipitation. When the cells were treated with TNFα, binding of endogenous AIMP2 to TRAF2 increased with time (Fig. 3A). A yeast two-hybrid assay showed that AIMP2 interacted with both TRAF2 and KRS (lysyl-tRNA synthetase), which was used as the positive control (Kim et al., 2002; Quevillon et al., 1999) (Fig. 3B). To assess whether AIMP2 could directly bind TRAF2, we prepared GST-AIMP2 and mixed it with TRAF2 that had been synthesized in vitro. TRAF2 was precipitated with GST-AIMP2, but not with GST (Fig. 3C). Deletion mapping determined that the peptide region of AIMP2 spanning aa 84-225 was responsible for the interaction with TRAF2 (Fig. 3D).

To evaluate the effect of the interaction between AIMP2 and TRAF2 on the TNFα-dependent proapoptotic activity of AIMP2, we examined the significance of TRAF2 in the proapoptotic activity of AIMP2 by using TRAF2-positive and -negative cells. Exogenous introduction of AIMP2 augmented TNFα-induced cell death in TRAF2-positive, but not in TRAF2-negative, cells (Fig. 3E). When we introduced TRAF2 into TRAF2-deficient cells, the apoptotic sensitivity to TNFα was partially reduced and the additional introduction of AIMP2 restored the sensitivity to TNFα (Fig. 3E, right). These results suggest the importance of TRAF2 in the TNFα-dependent proapoptotic activity of AIMP2.

AIMP2 mediates the ubiquitylation of TRAF2

We examined the effect of AIMP2 on the delivery of ubiquitin to TRAF2. After the introduction of AIMP2 along with HA-tagged ubiquitin, we inhibited proteasome activity with MG132. We then immunoprecipitated TRAF2 with its antibody and its ubiquitylation was monitored by immunoblotting with anti-HA antibody. The amount of ubiquitylated TRAF2 was increased in a dose-dependent manner by the introduction of AIMP2 (Fig. 4A, left). Ubiquitylation of the precipitated TRAF2 was also monitored by immunoblotting with anti-TRAF2 antibody. The TRAF2 bands of higher molecular weight were increased by the introduction of AIMP2 (Fig. 4A, right). We repeated the same experiment as above but without exogenously

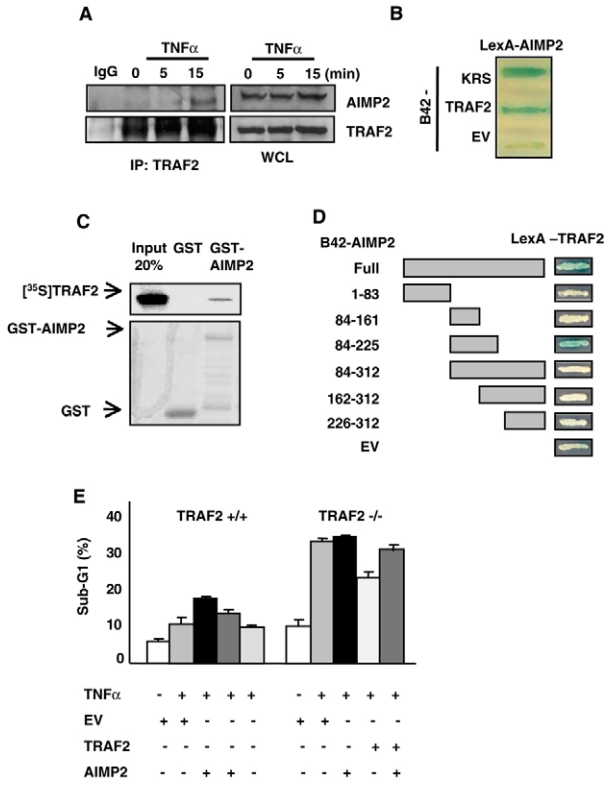

Fig. 3. Functional interaction between AIMP2 and TRAF2. (A) HeLa cells were treated with TNFα, and the interaction of AIMP2 with TRAF2 was determined by co-immunoprecipitation at the indicated time points. The extracted proteins were immunoprecipitated with anti-TRAF2 antibody and co-precipitated AIMP2 was determined by western blotting with anti-AIMP2 antibody. (B) The interaction of LexA-AIMP2 with B42-KRS or B42-TRAF2 was tested by the formation of a blue colony on the X-gal plate using yeast two-hybrid assay. (C) AIMP2 was expressed as GST fusion proteins. TRAF2 was synthesized by in vitro translation, mixed with GST or GST-AIMP2 and precipitated with glutathione-Sepharose. TRAF2 that was co-precipitated with GST proteins was determined by autoradiograph. 20% of radioactive input TRAF2 was loaded for comparison. (D) Different deletion fragments of AIMP2 were expressed as B42 fusion proteins, and their interactions with the LexA-TRAF2 fusion protein were tested by the yeast two-hybrid method. The positive interaction was shown by blue-colony formation on yeast medium containing X-gal. (E) AIMP2 was transfected into *TRAF2*$^{+/+}$ and *TRAF2*$^{-/-}$ MEFs, and cell death was measured in the presence or absence of TNFα by flow cytometry as described above. Transfection efficiency was monitored by the introduction of GFP.

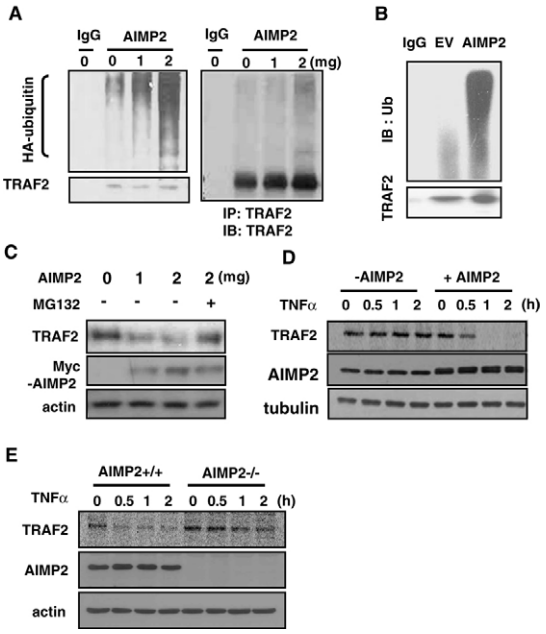

Fig. 4. AIMP2 controls TRAF2 stability via ubiquitylation. (A) Different amounts of AIMP2 and HA-tagged ubiquitin were expressed in HEK 293 cells. Cells were treated with MG-132 for 4 hours and then TNFα was added for 1 hour. After lysis of the cells, TRAF2 was immunoprecipitated with anti-TRAF2 antibody and its ubiquitylation was monitored by western blotting with anti-HA antibody (left). The immunoprecipitated TRAF2 was subjected to electrophoresis and immunoblotted with its specific antibody (right). (B) HEK 293 cells were transfected with empty vector (EV) or *AIMP2*, treated with MG-132 for 4 hours and then with TNFα for 1 hour. Proteins extracted from the cells were subjected to immunoprecipitation with anti-TRAF2 antibody and immunoblotted with anti-ubiquitin (Ub) antibody. (C) *Myc-AIMP2* was introduced into HEK 293 cells at the indicated amounts and its effect on TRAF2 level was determined after incubation with TNFα (30 ng/ml) by immunoblotting with anti-TRAF2 antibody. (D) HEK 293 cells were transfected with EV (–AIMP2) or *AIMP2* (+AIMP2) and the turnover rate of TRAF2 was determined by pulse-chase experiment after TNFα treatment. The cellular level of TRAF2 was monitored by autoradiography at the indicated times. (E) The same experiment as above was conducted using *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs.

introduced HA-ubiquitin. TRAF2 was immunoprecipitated and the effect of AIMP2 on the ubiquitylation of TRAF2 with endogenous ubiquitin was determined by immunoblotting with anti-ubiquitin antibody. As the data above shows, transfection of AIMP2 significantly increased the amount of ubiquitylated TRAF2 (Fig. 4B).

AIMP2 mediates the TNFα-dependent reduction of TRAF2

We examined whether AIMP2-mediated ubiquitylation of TRAF2 would result in the reduction in TRAF2 level. TRAF2 was decreased as Myc-AIMP2 was introduced (Fig. 4C). To see whether the reduction of TRAF2 by AIMP2 involves proteasomes, we treated the cells with MG132 and checked the TRAF2 level. The AIMP2-dependent reduction of TRAF2 was abolished when the cells were treated with MG132 (Fig. 4C). We also checked the effect of AIMP2 on the turnover rate of TRAF2 in A549 cells by pulse-chase experiment. Exogenous introduction of AIMP2 rapidly reduced the TRAF2 level (Fig. 4D) compared with the empty vector transfection. When we compared the turnover of TRAF2 between *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs by the same method, the TRAF2 level was more stably maintained in the AIMP2-deficient cells (Fig. 4E).

AIMP2 facilitates the association of c-IAP1 with TRAF2

We then investigated how AIMP2 can mediate the ubiquitylation of TRAF2. AIMP2 itself did not show any of the E1, E2 or E3 enzyme activities that are the key components of ubiquitin coupling systems (data not shown). We then checked the possibility that AIMP2 facilitates the association of E3 ubiquitin ligase to its target proteins using c-IAP1, which is the known ubiquitin ligase responsible for the ubiquitylation of TRAF2 (Li et al., 2002; Wu et al., 2005). To determine the importance of c-IAP1 for AIMP2-dependent downregulation of TRAF2, we suppressed the expression of c-IAP1 and checked whether AIMP2 could still reduce the TRAF2 level. In control-siRNA-transfected cells, transfection of AIMP2 decreased TRAF2 levels (Fig. 5A, left). However, when

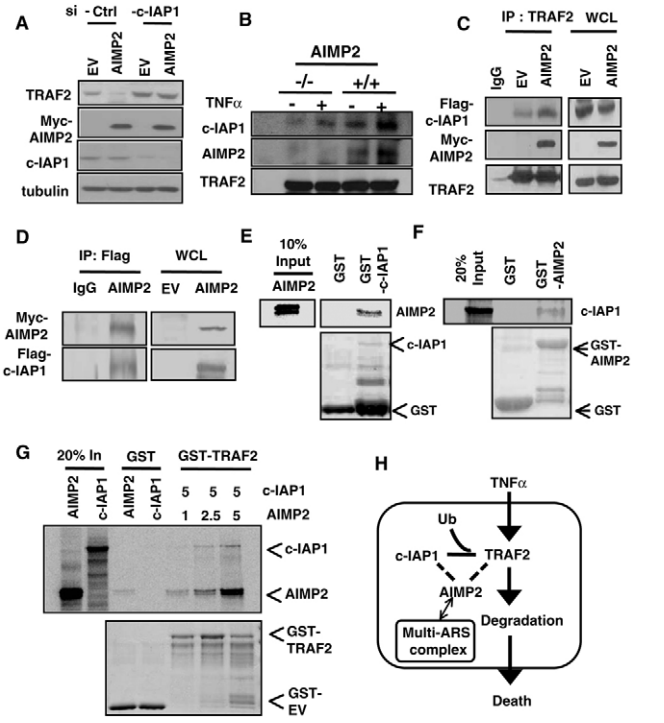

Fig. 5. AIMP2 mediates the association of c-IAP1 with TRAF2. (A) HEK 293 cells were transfected with control siRNA (si-Ctrl) or siRNA against *c-IAP1* (si-c-IAP1), and either AIMP2 or EV (empty vector), treated with TNFα, and the cellular level of TRAF2 was determined by western blotting. (B) *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs were treated for 4 hours with MG-132 (20 μM) to inhibit proteasomal degradation. TNFα (10 ng/ml) was then added and the cells were incubated for 1 hour. The cells were treated with the PBS buffer containing 1% NP-40 and protease inhibitor cocktail. The endogenous TRAF2 was immunoprecipitated and co-precipitation of endogenous c-IAP1 and AIMP2 was determined by western blotting. (C) Myc-AIMP2 was transfected into HEK 293 cells with FLAG–c-IAP1 and Myc-TRAF2. After the addition of TNFα to MG132-pretreated cells, the cellular TRAF2 was immunoprecipitated with its specific antibody from the protein extracts, and co-precipitation of AIMP2 and c-IAP1 with TRAF2 was determined by western blotting with anti-Myc and -FLAG antibodies, respectively. (D) FLAG–c-IAP1 was transfected into HEK 293 cells with either *Myc-AIMP2* or empty vector. FLAG–c-IAP1 was immunoprecipitated with anti-FLAG antibody and co-immunoprecipitation of Myc-AIMP2 was determined by western blotting with anti-Myc antibody. (E) AIMP2 was prepared by in vitro translation in the presence of [^{35}S]methionine, mixed with either GST or GST–c-IAP1 and precipitated with glutathione-Sepharose. AIMP2 co-precipitated with GST or GST–c-IAP1 was detected by autoradiography. (F) AIMP2 was expressed as GST fusion proteins, mixed with [^{35}S]methionine-labeled c-IAP1 and precipitated with glutathione-Sepharose. c-IAP1 co-precipitated with GST-AIMP2 was detected by autoradiography. (G) The effect of AIMP2 on the interaction of c-IAP1 with TRAF2 was determined by in vitro pull-down assay. GST-TRAF2 was mixed with the fixed amount of c-IAP1 (5 μl of the in vitro translation reaction) and the increasing amounts of AIMP2 (the indicated volume of the reaction), precipitated with glutathione-Sepharose, and the co-precipitated proteins were detected by autoradiography. (H) Schematic representation of AIMP2 function in the TNFα signaling pathway. AIMP2 mediates the association of c-IAP1 with TRAF2 for ubiquitylation. The ubiquitylated TRAF2 undergoes proteasome-mediated degradation, leading to TNFα-induced cell death.

c-IAP1 expression was suppressed with its specific siRNA, this effect of AIMP2 on TRAF2 levels was not observed (Fig. 5A, right), implying the importance of c-IAP1 for the effect of AIMP2 on TRAF2. To see whether AIMP2 would facilitate the binding of c-IAP1 to TRAF2 via trimeric-complex formation, we immunoprecipitated TRAF2 in *AIMP2*$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs, and the amount of co-precipitated c-IAP1 was compared. Although the association of c-IAP1 with TRAF2 was observed even in AIMP2-deficient cells, the two proteins formed a trimeric complex with AIMP2 and their association was enhanced by the presence of AIMP2 and by TNFα treatment (Fig. 5B). To further confirm this, FLAG–c-IAP1 was introduced into human embryonic kidney (HEK) 293 cells along with Myc-AIMP2. Then, we immunoprecipitated TRAF2 with its specific antibody, and co-precipitation of AIMP2 and c-IAP1 was determined with their specific antibodies. The amount of c-IAP1 that co-immunoprecipitated with TRAF2 was increased by the introduction of AIMP2 (Fig. 5C), further suggesting the stimulatory role of AIMP2 in the association of c-IAP1 and TRAF2. Although the results above suggest that there is the possibility of the formation of a trimeric complex that includes AIMP2, TRAF2 and c-IAP1, the direct interaction between AIMP2 and c-IAP1 is to be determined. We thus tested the cellular interaction of AIMP2 with TRAF2 by co-immunoprecipitation. FLAG–c-IAP1 was transfected into HEK 293 cells with either Myc-encoding empty vector or Myc-AIMP2, immunoprecipitated with anti-FLAG antibody and co-precipitation of AIMP2 was determined by western blotting with anti-Myc antibody. AIMP2 was co-precipitated with c-IAP1 (Fig. 5D). Direct interaction of AIMP2 with c-IAP1 was further examined by an in vitro pull-down assay using GST–c-IAP1 and radioactively labeled AIMP2. AIMP2 was co-precipitated with GST–c-IAP1, but not with GST (Fig. 5E). Conversely, when GST-AIMP2 was mixed with radioactive c-IAP1 and precipitated, c-IAP1 was pulled down with GST-AIMP2, but not with GST (Fig. 5F). After knowing the direct pair-wise interactions of AIMP2 with c-IAP1 as well as with TRAF2, the effect of AIMP2 on the association of TRAF2 with c-IAP1 was further tested by in vitro pull-down assay. Different amounts of AIMP2 were added to the binding mixture of GST-TRAF2 and c-IAP1. GST-TRAF2 in the mixture was precipitated with glutathione-Sepharose, and the amount of co-precipitated c-IAP1 was determined. The precipitated c-IAP1 with GST-TRAF2 was increased by the addition of AIMP2 in a dose-dependent manner (Fig. 5G). Taken together, AIMP2 might facilitate the recruitment of the E3 ligase c-IAP1 to the substrate TRAF2 so that TRAF2 can enter proteasome-mediated degradation. Via this activity, AIMP2 can sensitize the cells to TNFα-induced apoptosis (Fig. 5H).

Discussion

AIMP2 was previously suggested to be a substrate of Parkin, a RING-type E3 ubiquitin ligase (Ko et al., 2005). In our current work, we demonstrated that AIMP2 mediates the proapoptotic activity of TNFα via ubiquitin-mediated degradation of TRAF2. Thus, AIMP2 is not only the target for ubiquitylation but also seems to serve as an adaptor to mediate ubiquitin delivery to its target proteins such as TRAF2 (Fig. 4A,B) as well as FBP, which we have previously identified (Kim et al., 2003). Although it is not yet completely clear how AIMP2 can help the delivery of ubiquitin to different target proteins, it can be differentially modified depending on its upstream stimuli and guided for its cellular localization and/or to its target proteins. In this regard, it is worth noting that phosphorylation of AIMP2 takes place upon DNA damage and is required for dissociation from the multi-ARS complex and nuclear localization (Han et al., 2008). c-IAP1 appears to give a complex effect on TNFα-dependent NFκB activity and cell death (Varfolomeev and Vucic, 2008; Wu et al., 2007). In this work, we used c-IAP1 as one representative E3 ubiquitin ligase that can be recruited to TRAF2.

c-IAP1 appears to directly bind TRAF2 even without AIMP2 and TNFα (Fig. 5B) (Shu et al., 1996). However, the assembly of the two proteins seems to be further augmented by AIMP2 and TNFα in the presence of the proteasome inhibitor MG132 (Fig. 5B), which would result in an apoptotic response of the cells. In addition to c-IAP1, AIMP2 might also mediate the delivery of other E3 ubiquitin ligases, such as Siah2, to TRAF2 (Habelhah et al., 2002). It is yet to be seen whether AIMP2 would work as a general adaptor to link diverse pairs of E3 ubiquitin ligases and target proteins.

Although the expression level of AIMP2 does not seem to be significantly changed within 30 minutes after TNFα treatment, the interaction of AIMP2 with TRAF2 was increased (Fig. 3A). This led us to investigate the source of AIMP2. One probable candidate is the multi-tRNA synthetase complex that harbors AIMP2. It is possible that AIMP2 is recruited from the complex to TRAF2 upon TNFα signaling. In response to DNA damage, AIMP2 is dissociated from the multi-tRNA synthetase complex and translocated to the nucleus for interaction with p53 (Han et al., 2008). In this case, AIMP2 blocks MDM2 from binding to p53, thereby protecting p53 from ubiquitylation. Likewise, glutamyl-prolyl-tRNA synthetase (EPRS), another component of the multi-tRNA synthetase complex, has also been shown to be phosphorylated, dissociated from the complex and recruited to its new target site in response to IFNγ (Sampath et al., 2004). On the basis of these observations, the primary response of AIMP2 to TNFα signaling might occur at the post-transcriptional level.

AIMP2 controls cell fate via multiple signaling pathways. For instance, it can exert anti-proliferative activity via TGFβ, which is crucial in lung cell differentiation (Kim et al., 2003). AIMP2 also enhances cell death in response to DNA damage via p53. Here we added another function of AIMP2: to augment TNFα-dependent cell death via TRAF2. Because of a broad functional spectrum of AIMP2, any disruption of AIMP2 activity or expression can lead to pathological outcomes. For example, AIMP2 level is abnormally increased in patients with Parkinson's disease (Ko et al., 2005). Because TNFα is broadly implicated in many diseases, such as cancer (Mocellin and Nitti, 2008) and inflammation (Bradley, 2008), it would be interesting to see whether dysfunction of AIMP2 in this signaling pathway is involved in TNFα-associated diseases.

Materials and Methods

Cell culture and reagents

HEK 293 cells were cultivated in Dulbecco's modified Eagle's medium (HyClone, USA) supplemented with 10% fetal bovine serum and 50 μg/ml penicillin and streptomycin in a 5% CO_2 incubator. Myc- and FLAG-tagged TRAF2 were kindly provided by Soo Young Lee (Ewha Womans University, Korea). Geneporter (GTS, USA) and Lipofectamine 2000 (Invitrogen, USA) were used as transfection reagents. Human and mouse TNFα (Sigma, USA), and MG-132 (Calbiochem, USA), were treated at the concentration of 30 ng/ml and 20 μM in serum-free condition, respectively. siRNAs of 5′-AGAGCUUGCAGAGACAGGUUAGACU-3′ and 5′-CAGCUAUAUCCUAGCUGUAGCUUUA-3′ specifically targeting *AIMP2* and *c-IAP1*, respectively, were obtained from Invitrogen. Stealth universal RNAi (Invitrogen) was used as a non-specific control.

Western blot analysis

Monoclonal antibody (#324) that recognizes the peptide region of aa 84-225 was used for immunoblotting of AIMP2. The cultivated cell lines were dissolved in lysis buffer containing 0.5% Triton X-100 and protease inhibitor cocktail. The extracted proteins were separated by 10% SDS-PAGE and blotted using ECL system following the manufacturer's instructions.

Flow cytometry

AIMP2$^{+/+}$ and *AIMP2*$^{-/-}$ MEFs of 12.5 embryonic days or the indicated cells transfected with the plasmid encoding AIMP2 were cultivated in the absence or presence of TNFα (30 ng/ml) for 24 hours, fixed with 70% ethanol for 1 hour at 4°C and washed twice with ice-cold PBS. Then, 1×10^6 cells were stained with propidium iodide (50 μg/ml) containing 0.1% sodium citrate, 0.3% NP-40 (nonyl phenoxylpolyethoxylethanol 40) and 50 μg/ml RNaseA for 40 minutes, and subjected to flow cytometry (FACSCalibur, Becton-Dickinson) for the determination of apoptotic cells by counting sub-G1 cells and Annexin V staining following the manufacturer's instructions (Invitrogen). For each sample, 20,000 cells were analyzed using Cell Quest Pro software. All of the experiments were repeated three times.

Co-immunoprecipitation

Cells were dissolved in the lysis buffer containing 0.5% Triton X-100, 0.1% SDS and protease inhibitor cocktail, and the lysates were centrifuged at 15,000 ***g*** for 30 minutes. We then separated the extracted proteins by SDS-PAGE. To examine the interaction of AIMP2 with TRAF2, the cells were lysed and the protein extracts were incubated with normal IgG and protein G agarose for 2 hours, and then centrifuged to remove nonspecific IgG-binding proteins. We mixed the supernatant with anti-TRAF2 antibody (Santa Cruz), incubated for 2 hours at 4°C with agitation, and added protein G agarose. After washing three times with the ice-cold lysis buffer, the precipitates were dissolved in the SDS sample buffer and separated by SDS-PAGE. After the proteins were transferred to polyvinylidene fluoride (PVDF) membrane, co-immunoprecipitation of AIMP2 was determined with anti-AIMP2 antibody.

Yeast two-hybrid assay

The full-length and different deletion fragments of *AIMP2* were cloned to both pLexA and pB42 vectors. The full-length *TRAF2* and *KRS* were ligated to the pLexA and pB42 vectors at *Eco*RI and *Xho*I sites. The positive interactions were determined by the formation of blue colonies on the X-gal-containing yeast medium as described previously (Rho et al., 1999).

In vitro pull-down assay

We expressed GST-AIMP2 or GST in *Escherichia coli* Rosetta (DE3) strain. We mixed the protein extracts containing GST-AIMP2 or GST with glutathione-Sepharose in the PBS buffer containing 1% Triton X-100 and 0.5% N-laurylsarcosine at 4°C for 2 hours. We synthesized human TRAF2 by in vitro translation in the presence of [^{35}S]methionine using pcDNA3.1/V5-His-TOPO AIMP2 as the template, added it to the GST protein mixtures, incubated at 4°C for 6 hours with agitation in the PBS buffer containing 1% Triton X-100, 0.5% N-laurylsarcosine, 1 mM DTT (dithiothreitol), 2 mM EDTA (ethylenediaminetetraacetic acid) and 300 μM phenylmethylsulfonyl fluoride, and washed six times with the same buffer containing 0.5% Triton X-100. We then eluted the Sepharose-bead-bound proteins with the SDS sample buffer, separated by SDS-PAGE and determined AIMP2 by autoradiography. Similarly, the interaction of c-IAP1 with AIMP2 was determined using GST–c-IAP1 with radioactively synthesized AIMP2 or GST-AIMP2 with radioactive c-IAP-1. The effect of AIMP2 on the interaction between TRAF2 with c-IAP1 was also examined by in vitro pull-down assay. We expressed GST-TRAF2 in *E. coli* Rosetta DE3 and mixed the protein extracts with glutathione-Sepharose in the PBS buffer containing 1% Triton X-100 and 0.5% N-laurylsarcosine at 4°C for 2 hours. Then, in vitro synthesized c-IAP1 and AIMP2 at the indicated amounts were added to the GST protein mixtures, incubated at 4°C for 6 hours with agitation in the PBS buffer containing 1% Triton X-100, 0.5% N-laurylsarcosine, 1 mM DTT, 2 mM EDTA and 300 μM phenylmethylsulfonyl fluoride, and washed six times with the same buffer containing 0.5% Triton X-100. GST-TRAF2 and GST–c-IAP1 were kind gifts from Ze'ev Ronai (Burnham Institute, CA) and Tae Ho Lee (Yonsei University, Korea), respectively.

Reporter-gene assay

To test the NFκB-dependent transcriptional activity, NFκB-luciferase vector (kindly provided by Jonathan Ashwell, NIH) was co-transfected into A549 cells with pcDNA3.1 empty vector and the cells were selected by G418 to establish the stable cell lines. After the selection of a single colony, we subsequently introduced empty vector (as control) or *AIMP2* into the cells, and treated with TNFα for 18 hours. The cell lysates were prepared and reacted by luciferase assay kit following the manufacturer's protocol (Promega) and the luciferase activity was analyzed by using luminometer.

Ubiquitylation assay

To determine AIMP2-dependent ubiquitylation of TRAF2, we transfected HA-tagged ubiquitin into HEK 293 cells together with AIMP2, and incubated for 24 hours. Then, we treated the cells with MG-132 (40 μM) for 4 hours to inhibit proteasome activity and incubated the cells with TNFα for 1 hour. The cells were lysed with 150 mM Tris-HCl (pH 7.5) buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, protease inhibitor and phosphatase inhibitor for 30 minutes. The lysates were centrifuged and the supernatants were mixed with anti-TRAF2 antibody (C-20, Santa Cruz) for 2 hours and precipitated with protein A agarose beads for 6 hours. The precipitated TRAF2 was eluted and separated by SDS-PAGE and immunoblotted for TRAF2 as well as HA-ubiquitin. Ubiquitylation of endogenous TRAF2 was monitored by using anti-ubiquitin antibody.

Pulse-chase experiments

Pulse-chase analysis was performed using [^{35}S]methionine. A549 cells were transfected with pcDNA3 empty vector or vector expressing *Myc*-tagged *AIMP2* using

GenePorter (GTS) and incubated for 24 hours after transfection. Then, the cells were incubated with methionine-free medium for 1 hour, and [^{35}S]methionine (50 μCi/ml) was added and incubated for 1 hour. After washing off the radioactive methionine with fresh medium, we treated the cells with TNFα and incubated further. TRAF2 was immunoprecipitated, separated by 12% SDS-PAGE and detected by autoradiography using BAS (FLA-3000, FujiFilm). The amount of TRAF2 was quantified by Multi-gauge program (V3.0, FujiFilm).

This work was supported by the grants of Acceleration Research of KOSEF (2009-0063498), the 21st Frontier Functional Proteomics Research (FPR0881-250) and R31-2008-000-10103-0 from the WCU project of the MEST and the KOSEF.

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