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AIMP1/p43 downregulates TGF-β signaling via stabilization of smurf2

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ABSTRACT

AIMP1 (also known as p43) is a factor associated with a macromolecular aminoacyl-tRNA synthetase (ARS) complex but also plays diverse regulatory roles in various physiological processes. Here, we report that AIMP1 negatively regulates TGF-β signaling via stabilization of Smurf2. TGF-β-dependent phosphorylation and nuclear localization of R-Smads, induction of target genes, and growth arrest were increased in AIMP1-deficient or -suppressed cells. In AIMP1-deficient or suppressed cells, the Smurf2 level was decreased. Various binding assays demonstrated the direction interaction of the C-terminal region of AIMP1 directly with the Smad7-binding region of Smurf2. The association of Smurf2 with Smad7 and its ubiquitination were inhibited by AIMP1, thereby protecting its autocatalytic degradation stimulated by Smad7. Thus, this work suggests the novel activity of AIMP1 as a component of negative feedback loop of TGF-β signaling.

AIMP1/p43 was first identified as one of the three factors associated with aminoacyl-tRNA synthetase (ARS) complex consisting of nine different enzymes and three non-enzymatic factors [1,2]. Within the complex, AIMP1 makes specific interaction with arginyl-tRNA synthetase, being critical for the activity and stability of the bound enzyme [3,4]. As many of the components for the complex are associated with various signal pathways [5,6], AIMP1 is located in different extra- and intracellular locations for the regulation of diverse processes. For instance, AIMP1 is secreted to control inflammation [7,8], angiogenesis [9] and wound healing process [10]. It is also highly enriched in pancreatic cells and works as glucagon-like hormone for glucose homeostasis [11]. Within the cells, AIMP1 was also found to be critical for the suppression of lupus-type autoimmune disease through the interaction with ER-resident chaperone, gp96 [12]. For these diverse activities of AIMP1, AIMP1-deficient mice suffer from multiple symptoms at various tissues and organs, and show high pre- and post-natal lethality.

Since AIMP1 is ubiquitously found in almost all the cellular compartments and different tissues, more functions are expected to be discovered. Within the multi-ARS complex, AIMP1 is in close proximity with another factor, AIMP2/p38 [13,14] and both are structurally interdependent and critical for the stability of other components [4]. AIMP2/p38 was previously found to mediate TGF-β signaling that is required for lung cell differentiation during development. Thus, AIMP2-deficiency cripples TGF-β signaling and AIMP2$^{-/-}$ mice were neo-natal lethal due to overproliferation of epithelial alveolar cells in lung [15]. Considering the close proximity of AIMP1 and AIMP2 within the multi-ARS complex [16], it is conceivable that AIMP1 may somehow play a role in TGF-β signal pathway as well. Here we investigated the functional significance and working mechanism of AIMP1 in the regulation of TGF-β signal pathway.

Materials and methods

Antibodies and immunoblotting. Anti-c-Myc (9E10), anti-HA, anti-TGF-beta receptor II, anti-p27, anti-p15, anti-PAI-1, anti-ERK1/2, and anti-YY1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Smad2/3, anti-phospho-Smad2, and anti-phospho-Smad3 antibodies were from Cell Signaling Technology (Cell Signaling, MA). Anti-Smurf2 antibody was from Upstate. Anti-AIMP1 antibodies was from Imagene (Seoul, Korea). Proteins were extracted from the indicated cells with RIPA solution containing protease inhibitor cocktail, separated by SDS–PAGE and blotted on PDVF membrane.

Abbreviations: ARS, aminoacyl-tRNA synthetase; AIMP, ARS-interacting multifunctional protein; siRNA, small interfering RNA.

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Measurement of cell proliferation. We treated TGF-β for the indicated times with 2 ng/ml or indicating conc. for 12 h to MEF cell, which is isolated from 12.5d embryo, under the serum free condition. Before 4 h of harvest, we added 1 μCi/ml of [^{3}H]thymidine and measured the incorporated isotope through liquid scintillation counter (Wallac).

Co-immunoprecipitation. For immunoprecipitation, cells were solubilized with RIPA lysis buffer. Extracted proteins were mixed with anti-AIMP1, anti-FLAG, or anti-Myc antibody-precoupled protein A/G and subjected to immunoblot analysis.

RT-PCR. Total RNAs were isolated using Trizol following the protocol of the manufacturer (Invitrogen). One microgram of the isolated RNA was used in reverse transcription reaction using M-MLV reverse transcriptase. After the reaction, the mixture was diluted threefold with DW used in PCR with gene specific primers: PAI-1-R, 5′-AGATGTCTCCAGCCCTCACCT-3′; PAI-1-F, 5′-TTGCTTGAC CGTGCTCCGGAA-3′; GAPDH-R, 5′-CCATGA CGAACATGGGGGCAT-3′; GAPDH-F, 5′-TTTGG TCGTATTGGGCGCCTG-3′.

In vitro binding assay. For GST-pull down, we incubated with A549 cell lysates with bead-conjugated GST or GST-AIMP1 proteins for 1 h. After collection of bead-associated proteins, we performed immunoblot analysis.

Luciferase reporter assays. Mv1Lu cells were transfected with p3TPlux (0.1 μg) and the indicated amounts of plasmid encoding AIMP1. The total amount of DNA per well was kept constant by adding empty vector. To suppress the expression of AIMP1, AIMP1 siRNA (0–60 pmol, Invitrogen), or a control siRNA (invitrogen) was introduced to Mv1Lu cells with p3TPlux (0.2 μg). TGF-β1 was treated at 80 pM for 6 h. Luciferase activity was measured using Luciferase Assay System (Promega).

Immunofluorescence microscopy. A549 cells transfected with control siRNA or AIMP1 siRNA were fixed using methanol, blocked and incubated with anti-Smurf2 antibody. Cells were stained with Alexa fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nucleus was stained with DAPI.

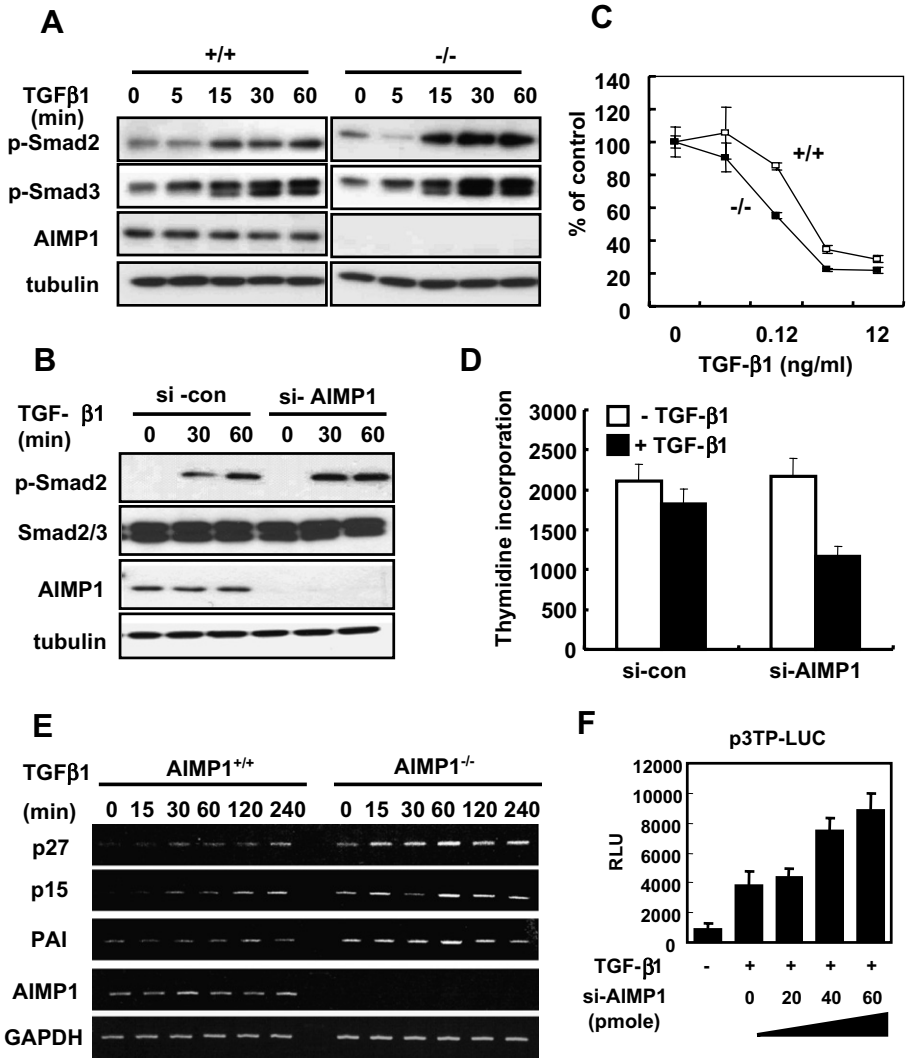

Fig. 1. Suppression of AIMP1 enhances TGF-β signaling. (A) 12.5d AIMP1$^{+/+}$ and AIMP1$^{-/-}$ MEF cells were treated with TGF-β1 (2 ng/ml) and phosphorylation of Smad2 and Smad3 was determined by their specific antibodies at time interval. Tubulin was used for loading control. (B) Suppression of AIMP1 using specific si-AIMP1 promotes phosphorylation of Smad2 in response to TGF-β in A549 cells, without alternation of total Smad expression. Tubulin was used as loading control. (C) AIMP1$^{+/+}$ and AIMP1$^{-/-}$ MEFs were treated with indicted concentration of TGF-β1 for 24 h. [^{3}H]Thymidine was then added and incubated for 4 h before of harvest and incorporated thymidine was quantified using liquid scintillation counter. The radioactivity of the control cells without TGF-b treatment was taken as 100%. The experiments were repeated three times. (D) A549 cells were transfected with si-control or si-AIMP1, and then treated with TGF-β1 (2 ng/ml) for 24 h, and cell proliferation was measured by thymidine incorporation as above. (E) Expression of p27, p15, and PAI was compared between AIMP1$^{+/+}$ and AIMP1$^{-/-}$ MEFs by RT-PCR at the indicated time interval. (F) To monitor the effect of AIMP1 knockdown on target gene expression, 3TP-lux plasmid is transfected with AIMP1 siRNA in Mv1Lu cells for 36 h and measured the luciferase activity of using luminometer. Data shown is the representative of three independent experiments.

Results

Suppression of AIMP1 enhances TGF-β signaling

AIMP1-deficient mice were generated by gene trap method in C57BL/6 strain as previously described [10]. To determine the possible role of AIMP1 in TGF-β signal pathway, we prepared mouse embryonic fibroblasts (MEFs) from AIMP1$^{+/+}$ and AIMP1$^{-/-}$ mice, and compared the response of TGF-β signal mediator, R-Smads to TGF-β treatment by Western blotting of phosphorylated Smad2 and Smad3. Phosphorylation of Smad2 and 3 was enhanced by the depletion of AIMP1 (Fig. 1A). To confirm the role of AIMP1 in TGF-β signaling, we reduced AIMP1 level using its specific siRNA in human lung adenocarcinoma A549 cells. Knockdown of AIMP1 promoted phosphorylation of Smad2 (Fig. 1B). The similar effects were also observed in other cell lines, U2OS and Mv1Lu (data not shown).

We then compared the sensitivity of AIMP1$^{+/+}$ and AIMP1$^{-/-}$ MEFs to TGF-β-dependent growth inhibition. AIMP1$^{-/-}$ cells became more sensitive to the growth inhibition effect of TGF-β (Fig. 1C). We also compared the TGF-β-induced cell cycle arrest in the control or si-AIMP1 transfected Mv1Lu cells. TGF-β-induced growth inhibition was more evident in si-AIMP1 transfected cells than in si-control transfected cells (Fig. 1D).

We also monitored the expression of R-Smad targets in AIMP1$^{+/+}$ and AIMP1$^{-/-}$ MEFs. The expression of the Smad target genes p27, p15, and PAI-1 was enhanced in AIMP1$^{-/-}$ MEFs than that in the normal cells as determined by RT-PCR analysis with their specific primers (Fig. 1E). To determine the dosage effect of AIMP1 on TGF-β signaling, we performed the reporter gene assay using a mink lung epithelial cell line, Mv1Lu. TGF- β-mediated transcriptional activities were measured by p3TP-lux, which is one of the standard reporters for assessing TGF-β signaling activity [17]. When Mv1Lu cells were transfected with p3TP-lux together with increasing amount of AIMP1 siRNA, TGF-β-induced luciferase activity was increased according to the amount of transfected AIMP1 siRNA (Fig. 1F).

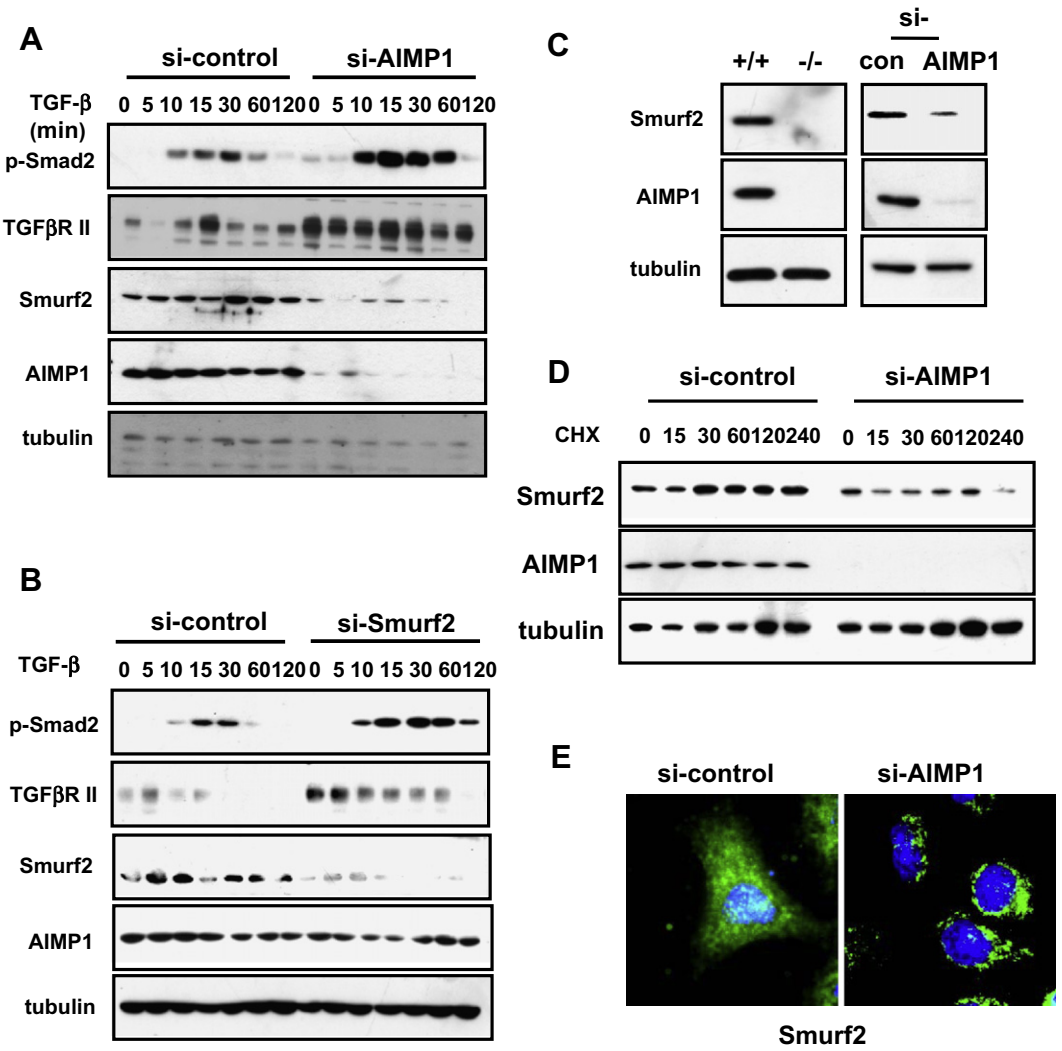

Fig. 2. Deficiency of AIMP1 destabilizes Smurf2. (A) A549 cells were transfected with si-control or si-AIMP1 and treated with TGF-β1 for the indicated times. The cell lysates were immunoblotted with anti-p-Smad2, TGF-β receptor II, Smurf2, AIMP1, and tubulin (loading control) antibodies, respectively. (B) A549 cells were transfected with si-control or si-Smurf2, and treated with TGF-β1 for the indicated times. The cell lysates were immunoblotted with anti-p-Smad2, TGF-β receptor II, Smurf2, AIMP1, and tubulin antibodies, respectively. (C) The cell lysates from AIMP1$^{+/+}$ or AIMP1$^{-/-}$ MEFs, and si-control or si-AIMP1-transfected A549 cells were immunoblotted with anti-Smurf2, AIMP1, and tubulin antibodies, respectively. (D) Si-control or si-AIMP1-transfected A549 cells were treated with ALLN (50 μM) for 3 h. The culture medium was replaced with fresh medium and cycloheximide (CHX) was added to the final concentration of 20 μg/ml. At the indicated times after the addition of CHX, the cell lysates were prepared and examined the Smurf2 level by Western blot analysis. (E) The effect of AIMP1 suppression on Smurf2 was also monitored by immunofluorescence staining of A549 with anti-Smurf2 antibody (green). The cells were counterstained with DAPI. Data shown is the representative of three independent experiments.

AIMP1 deficiency destabilizes smurf2

To address how AIMP1 suppresses TGF-β signaling, we checked the status of upstream components regulating R-Smad phosphorylation in TGF-β signaling pathway. In si-AIMP1-transfected cells, the level of TGF-β receptor II was increased (Fig. 2A second row) while E3 ubiquitin ligase, Smurf2, which mediates TGF-β receptor turnover, was significantly reduced (Fig. 2A, third row). Based on this observation, we hypothesized that the reduction of Smurf2 level in the absence of AIMP1 may result in the increase of TGF-β receptor and R-Smad phosphorylation. To see if this is the case, we suppressed Smurf2 with its specific siRNA and checked its effect on the level of TGF-β receptor II and phospho-Smad2. As expected, the level of TGF-β receptor II and phospho-Smad2 were increased by the suppression of Smurf2 (Fig. 2B). The reduction of Smurf2 level was also observed in AIMP1$^{-/-}$ MEFs as well as si-AIMP1 transfected cells (Fig. 2C). To compare the turnover rate of Smurf2 between si-control and si-AIMP1-transfected cells, we

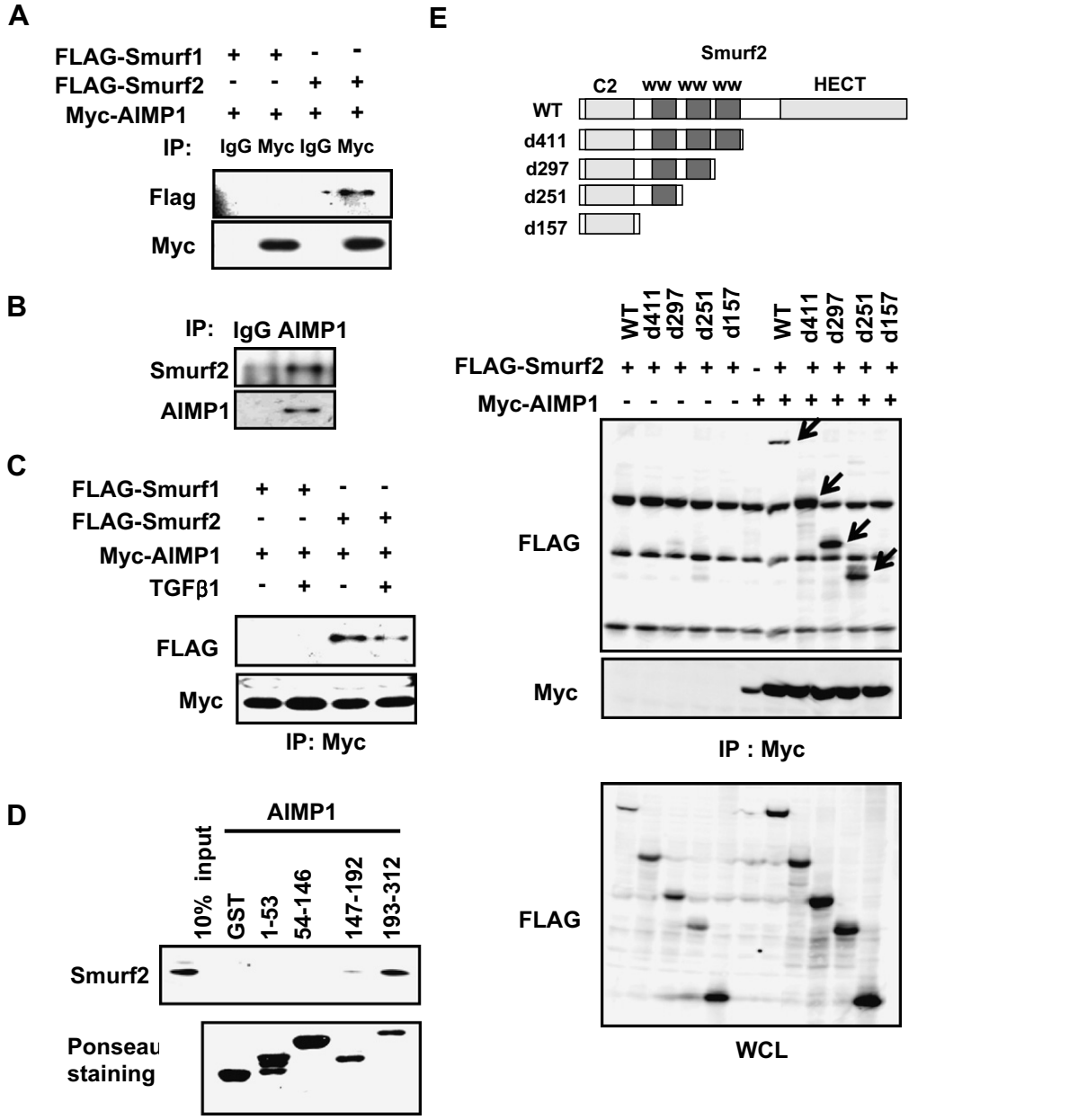

Fig. 3. Specific and direct binding of AIMP1 with Smurf2. (A) AIMP1-transfected A549 cells were also co-transfected with either FLAG-Smurf1 or -Smurf2. Protein extracts were immunoprecipitated with anti-Myc antibody and the co-precipitated Smurf2 was determined by immunoblotting with anti-FLAG antibody. (B) Protein extracts from the control or TGF-β-treated A549 cells were immunoprecipitated with anti-AIMP1 antibody and co-precipitated Smurf2 was determined by immunoblotting with anti-Smurf2 antibody. (C) TGF-β-dependent dissociation of AIMP1 from Smurf2. A549 cells, co-transfected with Myc-AIMP1/FLAG-Smurf1 and Myc-AIMP1/FLAG-Smurf2 were treated with TGF-β for 30 min. Protein extracts were immunoprecipitated with anti-Myc antibody and the co-precipitated Smurf2 was determined by immunoblotting with anti-FLAG antibody. (D) The different deletion fragments of AIMP1 were expressed as GST fusion proteins in *E. coli*, and their expressions were confirmed by Ponceau staining. Protein extracts from FLAG-Smurf2-transfected 293 cells were mixed with each of the GST-AIMP1 fragments and affinity precipitated with glutathione–Sepharose beads. The co-precipitation of Smurf2 was determined by immunoblotting with anti-FLAG antibody. (E, upper panel) The C2, WW, and HECT domains of Smurf2 were schematically demonstrated. A549 cells were co-transfected with Myc-AIMP1 and each of the different fragments of Smurf2 fused to FLAG. Protein extracts were immunoprecipitated with anti-Myc antibody and co-precipitation of the Smurf2 fragments was determined by immunoblotting with anti-FLAG antibody (indicated by arrows). WCL stands for whole cell lysate. WCL stands for whole cell lysate. Data shown are representative of three independent experiments.

first treated the cells with ALLN to inhibit proteasome activity. The cells were then incubated in fresh medium, and compared cellular turnover of Smurf2 between si-control or si-AIMP1-transfected cells while blocking *de novo* protein synthesis with cycloheximide. The Smurf2 level decreased more rapidly under the suppression of AIMP1 (Fig. 2D), suggesting that AIMP1 stabilizes Smurf2 in TGF-β-dependent manner. Decrease of Smurf2 by the suppression of AIMP1 was also observed by immunofluorescence staining (Fig. 2E).

AIMP1 specifically interacts with Smurf2

To understand how AIMP1 would stabilize Smurf2, we first examined the interaction of AIMP1 with Smurf2. AIMP1 was specifically co-immunoprecipitated with Smurf2, but not Smurf1 (Fig. 3A). The interaction was also confirmed by co-immunoprecipitation of endogenous AIMP1 and Smurf2 (Fig. 3B). The association of the two proteins was decreased by TGF-β treatment (Fig. 3C). The direct interaction of the two proteins was tested by *in vitro* pull-down assay. We prepared GST-fused AIMP1 fragments, mixed them with Smurf2, and precipitated with glutathione–Sepharose bead. The C-terminal region spanning aa 193-312 of AIMP1 showed the capability of interacting with Smurf2 (Fig. 3D). We also determined the region of Smurf2 that is involved in the interaction with AIMP1. Smurf2 consists of the N-terminal C2 domain involved in membrane localization, three WW domains that interact with proline-rich sequences of the substrates or adaptor proteins and the C-terminal HECT domain [18–20]. Among different peptide fragments of Smurf2, those containing different numbers of the WW domains of Smurf2 showed the interaction with AIMP1 (Fig. 3E arrows), but the C2 domain alone did not bind AIMP1, suggesting that the WW domain is required for the association with AIMP1.

AIMPI inhibits smad7-mediated smurf2 degradation

Since both of the PY motif of Smad7 [21–23] and the C-terminal region of AIMP1 bind to the WW domains of Smurf2, we checked whether AIMP1 can compete with Smad7 for Smurf2 binding. Increase of AIMP1 reduced the binding of Smad7 to Smurf2 in dose-dependent manner (Fig. 4A). Smad7 was shown to stimulate the autocatalytic E3 ligase activity of Smurf2 by recruiting the E2, UbcH7, to the HECT domain of Smurf2 [24]. Thus, we monitored the effect of AIMP1 on Smad7-mediated Smurf2 ubiquitination. Overexpression of Smad7 stimulated Smurf2 ubiquitination, but this activity was inhibited by the increase of AIMP1 (Fig. 4B), further demonstrating the inhibitory activity of AIMP1 against Smad7. Since downregulation of Smurf2 increased the level of TGF-β receptor II, we checked whether the stabilization of Smurf2 by AIMP1 would result in ubiquitination of TGF-β receptor II induced by TGF-β. Overexpression of AIMP1 enhanced TGF-β receptor II ubiquitination in TGF-β-dependent manner (Fig. 4C), suggesting that the stabilization of Smurf2 by AIMP1 results in the negative regulation of TGF-β signaling.

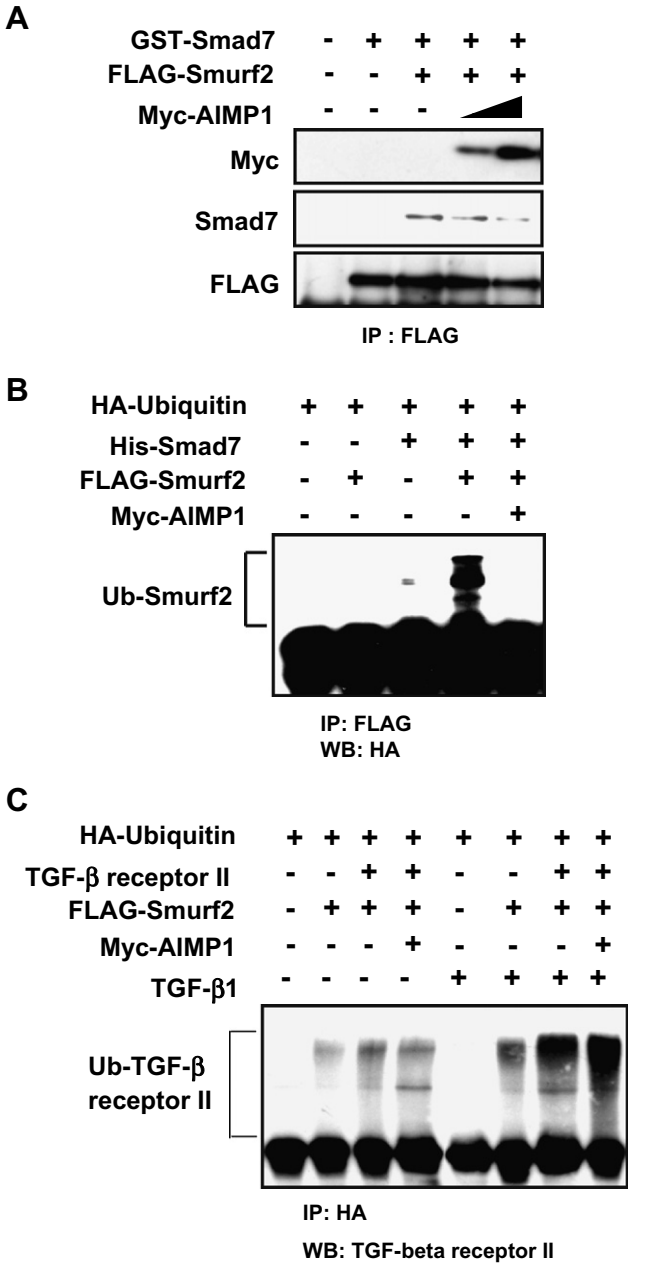

Fig. 4. AIMP1 prevents Smad7-mediated ubiquitination of Smurf2. (A) A549 cells were transfected with GST-Smad7, FLAG-Smurf2, and the indicated amounts of Myc-AIMP1. Protein extracts were immunoprecipitated with anti-FLAG antibody, and co-precipitation of AIMP1 and Smad7 was determined by immunoblotting with anti-Myc and -Smad7 antibodies. (B) The effect of AIMP1 on Smad7-mediated Smurf2 ubiquitination was determined. A549 cells transfected with HA-Ubiquitin, His-Smad7, FLAG-Smurf2, and Myc-AIMP1, were treated with ALLN (50 μM) for 3 h to block the proteasome activity. Smurf2 was precipitated from the cell lysates with anti-FLAG antibody and ubiquitination of Smurf2 was evaluated by immunoblotted with anti-HA antibody. (C) The effect of AIMP1 on Smurf2-mediated ubiquitination of TGF-β receptor II was determined. A549 cells transfected with HA-ubiquitin, HA-TGF-β receptor II, FLAG-Smurf2, and Myc-AIMP1 were treated with ALLN (50 μM) and treated with TGF-β1 for 2 h. TGF-β receptor II was precipitated from the cell lysates with anti-HA antibody and ubiquitination was evaluated by immunoblotted with anti-HA antibody. Data is the representative of three independent experiments.

Discussion

AIMP1 exerts its biological activities via its interactions with diverse target molecules. It binds arginyl-tRNA synthetase via its 72 aa N-terminal extension within the multi-ARS complex, helping the catalytic activity and cellular stability of the bound enzyme [3,4]. The central part of AIMP1 holds the C-terminal domain of heat shock protein gp96 in endoplasmic reticulum, thereby preventing false activation of autoimmune reaction [12]. AIMP1 also shows diverse cytokine activities via its different peptide regions [25], each of which would be involved in the interaction with their cognate receptors. In this work, we found that the OB-fold containing C-terminal region of AIMP1 [26] is also used for the association with the negative regulator of TGF-β signal, Smurf2 (Fig. 3D). Thus, AIMP1 appears to use the entire polypeptide region for its diverse biological activities.

Several molecular mechanisms have been proposed for the negative regulation of TGF-β-induced Smad2/3 phosphorylation. FKBP12 blocks TGF-β receptor I phosphorylation, causing inhibition of receptor-mediated R-Smads phosphorylation [27]. Smad7 competitively inhibits the interaction of R-Smads with receptors [21,28]. Smurf2 E3 ligase targets R-Smads for destruction [23,29,30], and Smad7-Smurf2 cooperatively brings the activated receptor to degradation route [23]. At this moment, we do not know yet the molecular details on the AIMP1-mediated stabilization of Smurf2. Smurf2 is kept inactive by the intramolecular association of the N-terminal C2 and the C-terminal HECT domains. The binding of Smad7 to the WW domain of Smurf2 releases the intramolecular inhibitory interaction of the C2 and HECT domains [31], and the resulting Smurf2-Smad7 complex is exported to cytoplasm and targets TGFβ receptor for degradation [23]. Since AIMP1 is bound to Smurf2 without TGF-β treatment and their dissociation was enhanced by TGF-β (Fig. 3C), it may provide additional mechanism for the protection of Smurf2 from self destruction or be required for the sequestration of Smurf2.

Since AIMP1 binds to WW domains of Smurf2 (Fig. 3E), AIMP1 is expected to have the WW domain-binding PY motif similar to Smad7. In this regard, it is noteworthy that the 193–312 aa Smurf2-binding region of AIMP1 has two proline-rich sequences, PPNG and PGEP although further investigation is needed to determine whether these motifs are actually required for the interaction with Smurf2. Although further investigation is needed to understand how diverse activities of AIMP1 is partitioned and regulated, this work reports a component of translational machinery, AIMP1, as a novel negative component for TGF-β signal pathway.

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