

## Structural separation of different extracellular activities in aminoacyl-tRNA synthetase-interacting multi-functional protein, p43/AIMP1

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Received 16 January 2006

Available online 2 February 2006

### Abstract

AIMP1 (previously known as p43) is first found as a factor associated with a macromolecular tRNA synthetase complex. However, it is also secreted and acts on diverse target cells such as endothelial cells, macrophages, and fibroblasts to control angiogenesis, inflammation, and dermal regeneration, respectively. We previously showed that AIMP1 induces the death of endothelial cell but proliferation of fibroblasts and activates macrophages. In this work, we found that elastase 2-cleaved AIMP1 retained its pro-apoptotic activity to endothelial cells but lost the growth-stimulatory activity to fibroblasts. To determine the functional domains responsible for each activity, we generated several deletion fragments of AIMP1 and compared the activities to the target cells. AIMP1 promoted endothelial cell death and caspase-3 activation through its 101–114 amino acid region, fibroblast proliferation through its 6–46 amino acid region, and endothelial migration through its 114–192 amino acid region as revealed by deletion mapping. Thus, this work revealed that AIMP1 uses different regions for its diverse extracellular activities.

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**Keywords:** AIMP1; Domain mapping; Angiogenesis; Inflammation; Fibroblast proliferation

AIMP1 (ARS-interacting multi-functional protein 1) is a protein associated with mammalian macromolecular aminoacyl-tRNA synthetase complex [1,2]. Interestingly, the C-terminal domain of AIMP1 was shown to be equivalent to endothelial monocyte-activating polypeptide II (EMAPII) [1]. EMAP II was initially identified from the culture medium of murine methylcholanthrene A-induced fibrosarcoma cells based on its capacity to induce tissue factor in human umbilical vein endothelial cells (HUVECs) [3]. Murine methylcholanthrene A fibrosarcomas exhibit spontaneous vascular insufficiency manifested by a heterogeneous pattern of thrombohemorrhage and central necrosis, providing an ideal starting point for isolation of tumor-derived mediators that perturb the vasculature

[4,5]. The purified EMAP II possesses a wide range of activities toward endothelial cells (ECs), neutrophils, and monocyte/macrophages *in vitro* [6,7].

Since EMAP II was a potent proinflammatory cytokine and generated *in vitro* by proteolytic cleavage of AIMP1 with caspase-7 [8], AIMP1 was thought to be a precursor of EMAP II. However, AIMP1 itself is secreted from different types of cells, including prostate cancer [9], immune and transfected cells [10]. The secreted AIMP1 works on diverse target cells such as monocyte/macrophages [10–12], endothelial cells [13,14], and fibroblasts [15] and activates monocytes/macrophages to induce various proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin-8 (IL-8), macrophage chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), and IL-1 $\beta$  [10], controls angiogenesis by a dual mechanism involving the migration and death of endothelial cells [14], and promotes fibroblast proliferation [15]. However,

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it is not determined yet how AIMP1 exerts its complex extracellular activities. To address this question, we conducted an extensive series of deletion mapping experiments to determine the activities of the different domains.

**Materials and methods**

**Cell culture.** Bovine aorta endothelial cells (BAECs) were isolated from descending thoracic aortas and grown in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> atmosphere. The primary cultured cells used in this study were between passages 5 and 10. Foreskin fibroblast and U2OS were cultured in DMEM with 10% FBS and 1% antibiotics.

**Cell death assay.** BAECs were treated with deletion fragments of AIMP1 (50 nM) for 24 h and the apoptotic cells were counted as described previously [14]. Briefly, enhanced green fluorescent protein (EGFP) were transfected into BAECs and expressed for 24 h. The transfected cells were treated with AIMP1s for 24 h, and then cell death was determined by counting the apoptotic cells using fluorescence microscopy. The percentage of apoptotic cells was determined by dividing the number of green cells with apoptotic morphology with the total number of green cells. For the caspase assay, BAECs (2 × 10<sup>6</sup>) were harvest and lysed with 300 μl of chilled cell lysis buffer (20 mM Hepes, pH 7.5, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% NP-40, and 0.1 mM PMSF). The cell lysates were

centrifuged at 15,000g for 5 min at 4 °C and the supernatant fractions were used to measure caspase activity. Aliquots of 40 μg of cell lysate protein were incubated for 2 h at 30 °C in an assay buffer (20 mM Hepes at pH 7.5, 2 mM DTT, and 10% glycerol) containing 100 μM Ac-DEVD-*p*-nitroanilide for caspase-3 substrate or Ac-YVAD-*p*-nitroanilide for caspase-1 substrate. The amount of *p*-nitroaniline released by caspase activity was quantitated by measuring the optical density at 405 nm.

**Cell migration assay.** Endothelial cell migration assays were performed by using a Transwell chamber (24-well chamber) with polycarbonate membranes (8.0 μm pore size, Costar). The wells were coated with 0.5 mg/ml gelatin (Sigma) in PBS and allowed to air-dry. BAECs were suspended in serum-free DMEM and added to the upper chamber at 2–5 × 10<sup>4</sup> cells per well. A chemotactic stimulus, VEGF (0.7 nM), or one of the indicated concentrations of AIMP1 was placed in the lower chamber, and the cells were allowed to migrate for 6 h at 37 °C in a 5% CO<sub>2</sub> incubator. After incubation, non-migrant cells were removed from the upper face of the membrane with a cotton swab. The migrant cells (those attached to lower face) were fixed in 100% methanol and visualized by the hematoxylin (Sigma) staining. The migrant cells were counted in high power fields.

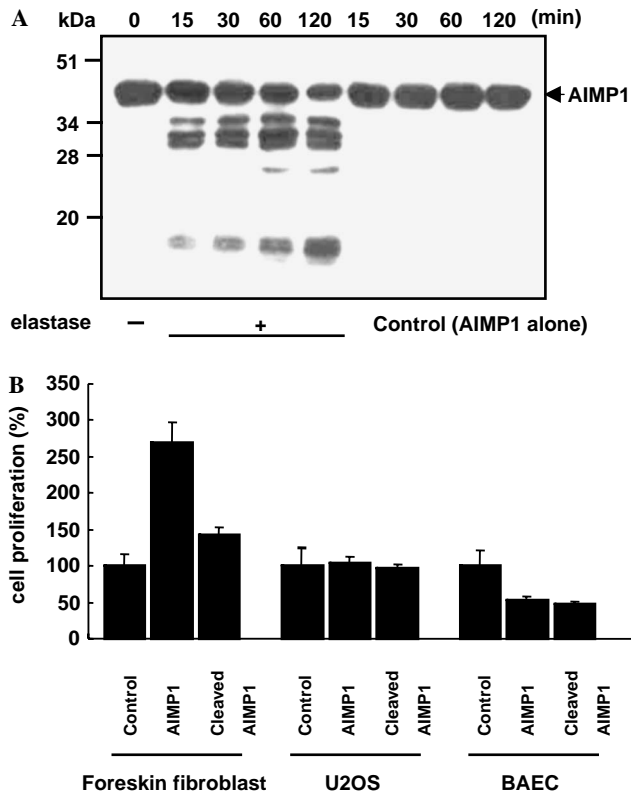


Fig. 1. AIMP1 cleaved by elastase 2 has endothelial cell proliferation inhibitory activity, but loose fibroblast proliferation activity. (A) AIMP1 protein (4 μg) was incubated with elastase 2 (1 U/ml) for the indicated times at 37 °C and subjected to SDS–PAGE. Peptides were visualized with Coomassie blue. (B) Foreskin fibroblast, U2OS, and BAECs grown in 24-well plates were treated with 100 nM AIMP1 or cleaved AIMP1 for 12 h. At the end of incubation, [<sup>3</sup>H]thymidine was added to each well and the cells were incubated further for 4 h at 37 °C. [<sup>3</sup>H]Thymidine incorporation was measured as described in Materials and methods. Data represent means ± SD.

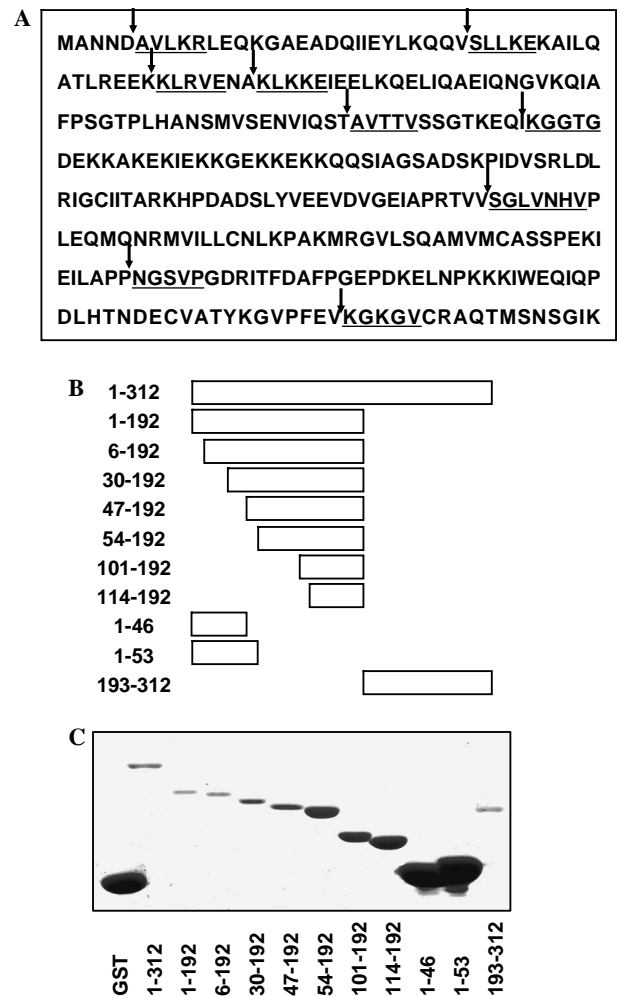


Fig. 2. Identification of elastase 2 cleavage sites of AIMP1 and generation of deletion mutants of AIMP1. (A) Schematic representation of elastase 2 cleavage sites of AIMP1. Elastase 2-cleaved AIMP1 peptides were separated into SDS–PAGE and stained with Coomassie blue. Each band was analyzed by N-terminal amino acid sequencing. Arrows indicate cleavage sites and underlines indicate the results of N-terminal amino acid sequencing. (B) A schematic drawing of the AIMP1 deletion mutants used in this study. (C) SDS–PAGE of each purified proteins.

**Cell proliferation assay.** We monitored the cell proliferation by measuring the incorporation of [<sup>3</sup>H]thymidine to the replicating DNA as well as by directly counting cell numbers. We plated the indicated cells ( $5 \times 10^3$  cells) on 24-well dish, cultivated them for 12 h, and serum-starved them for 3 h. We then added the indicated amount of AIMP1 deletion fragments to each well, and cultivated for 12 h. After 4 h of incubation with tritium-labeled thymidine (1  $\mu$ Ci/well), the cultured cells were washed with PBS three times, fixed with 5% TCA for 10 min, and washed again with PBS three times. We lysed the cells with 0.5 N NaOH and quantified the incorporated thymidine with a liquid scintillation counter.

**Construction and purification of AIMP1 deletions.** The DNA fragments coding for AIMP1-(1–192), AIMP1-(6–192), AIMP1-(30–192), AIMP1-(47–192), AIMP1-(54–192), AIMP1-(101–192), AIMP1-(114–192), AIMP1-(1–46), AIMP1-(1–53), and AIMP1-(193–312) regions were synthesized by PCR with specific primer sets. The specific PCR products were digested with *Eco*RI and *Xho*I and ligated into pGEX4T3 cut with the same enzymes. Each of the full-length AIMP1 and AIMP1-deleted constructs was expressed as GST-tag fusion protein in *Escherichia coli* BL21 (DE3) and purified by GSH agarose. To remove lipopolysaccharide, the protein solution was dialyzed in pyrogen-free buffer (10 mM potassium phosphate buffer, pH 6.0, 100 mM NaCl). After dialysis, the protein was

loaded to polymyxin resin (Bio-Rad) pre-equilibrated with the same buffer, incubated for 20 min, and eluted. The concentration of the residual lipopolysaccharide (LPS) was below 20 pg/ml when determined using the Limulus Amebocyte Lysate QCL-1000 kit (BioWhittacker).

**Results and discussion**

*Elastase 2-cleaved AIMP1 still inhibits endothelial cell proliferation*

Since AIMP1 shows complex signaling activities on different target cells, we hypothesized that AIMP1 may use different structural motifs or domains for its diverse activities. To prove this possibility, we first tried to cleave AIMP1 using several proteases and examined whether the cleaved fragments of AIMP1 still have ligand activity. When AIMP1 was cleaved with neutrophil elastase 2, it was separated into small fragments as shown in Fig. 1A. Interestingly, elastase 2-cleaved AIMP1 inhibited

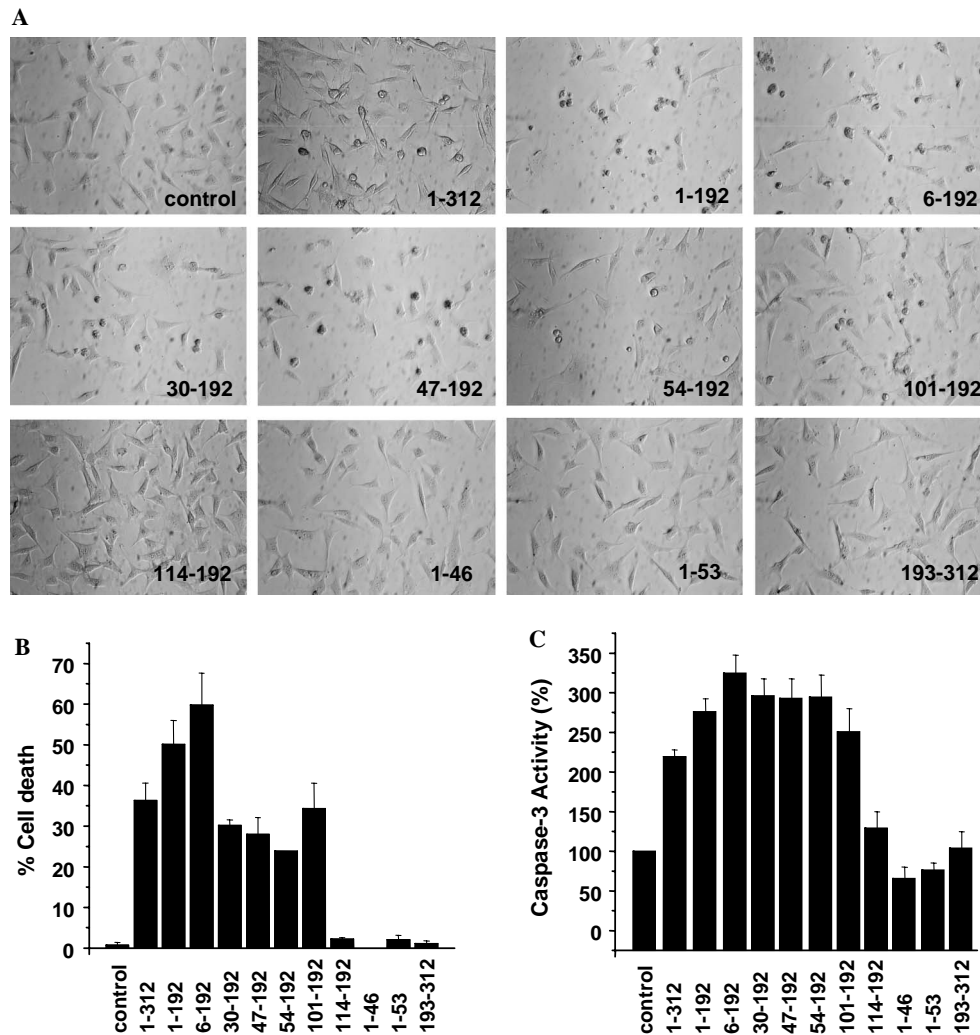


Fig. 3. Identification of endothelial cell death-inducing domain of AIMP1. (A) BAECs were treated with 50 nM AIMP1 mutants for 24 h, and the apoptotic cells were visualized by morphological characteristics. (B) BAECs were treated with 50 nM AIMP1 proteins for 24 h, and the apoptotic cells were counted as described in Materials and methods. (C) The activity of caspase-3 was measured from BAECs treated with 0 or 50 nM AIMP1 proteins for 16 h. Data represent means  $\pm$  SD.

endothelial cell proliferation, but could not induce fibroblast proliferation (Fig. 1B), implicating that AIMP1 may use different domains for two ligand activities and elastase 2 specifically disrupted the region of AIMP1 which is important to fibroblast proliferation.

#### Identification of elastase 2-cleaved sites of AIMP1 and expression of deletion fragments of AIMP1

To identify the region of AIMP1 which is cleaved by elastase 2, elastase 2-cleaved AIMP1 fragments were resolved by 15% SDS-PAGE and N-terminal amino acids of fragments were determined by N-terminal amino acid sequencing (Fig. 2A). According to this sequence information, several deletion derivatives of AIMP1 were constructed. The mutant AIMP1 constructs used in this study are shown schematically in Fig. 2B. The mutant proteins were purified as glutathione-S-transferase (GST) fusion proteins using a bacterial expression system, and the purified proteins were analyzed by SDS-PAGE (Fig. 2C). Endotoxin

from the purified proteins was removed by using a polymyxin affinity column.

#### Identification of cell death-inducing domain of AIMP1

To investigate endothelial cell death by AIMP1 mutants, the purified recombinant proteins were added to BAECs. Twenty four hours after incubation, we determined endothelial cell death by morphological change (Figs. 3A and B). In addition, in our previous report, AIMP1 induced endothelial cell apoptosis through caspase-3 activation [14]. To make these data more convincing, we also monitored cell death by measuring caspase-3 activity after the treatment of the AIMP1 mutant recombinant proteins (50 nM) for 16 h. (Fig. 3C). Since AIMP1-(1–312), AIMP1-(1–192), AIMP1-(6–192), AIMP1-(30–192), AIMP1-(47–192), AIMP1-(54–192), and AIMP1-(101–192), but not AIMP1-(114–192), AIMP1-(1–46), AIMP1-(1–53), and AIMP1-(193–312), showed high cell death activity and caspase-3 activation, the middle region of

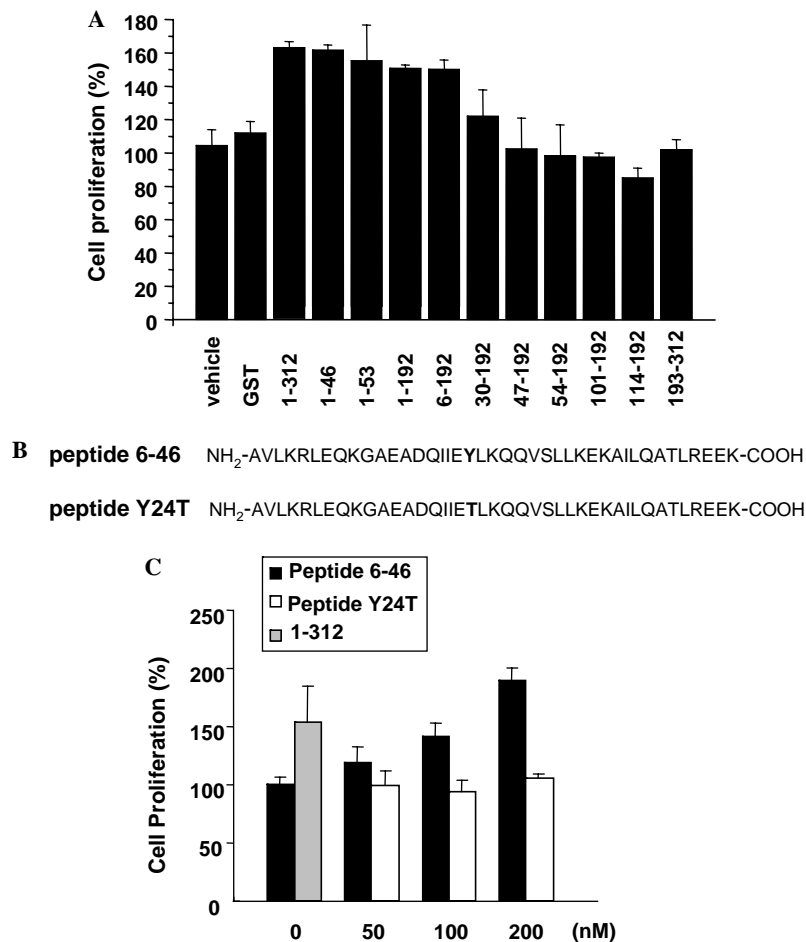


Fig. 4. Identification of fibroblast proliferation-inducing domain of AIMP1. (A) Foreskin fibroblasts were treated with 100 nM AIMP1 mutants for 12 h. At the end of incubation, [<sup>3</sup>H]thymidine was added to each well, and the cells were incubated further for 4 h at 37 °C. [<sup>3</sup>H]Thymidine incorporation was measured as described in Materials and methods. (B) Synthetic peptides of AIMP1-(6–46) used in this work are shown. Y24T indicates the change of 24th tyrosine to threonine. (C) Foreskin fibroblasts were treated with 100 nM AIMP1 or the indicated concentration of AIMP1 peptides as above for 12 h. At the end of incubation, [<sup>3</sup>H]thymidine was added to each well, and the cells were incubated further for 4 h at 37 °C. [<sup>3</sup>H]Thymidine incorporation was measured. Data represent means ± SD.

AIMP1, especially AIMP1-(101–114), might be a cell death-inducing domain on endothelial cells.

#### Identification of growth-stimulating domain of AIMP1

To investigate fibroblast proliferation by AIMP1 mutants, the purified recombinant proteins were added to foreskin fibroblasts. Sixteen hours after incubation, and we determined fibroblast proliferation by [<sup>3</sup>H]thymidine incorporation (Fig. 4A). We found that the N-terminal region of AIMP1 that is different from the cell death-inducing domain increased fibroblast proliferation. AIMP1-(1–312), AIMP1-(1–192), AIMP1-(1–46), AIMP1-(1–53), and AIMP1-(6–192) showed high proliferation-inducing activity, but AIMP1-(30–192), AIMP1-(47–192), AIMP1-(54–192), AIMP1-(101–192), AIMP1-(114–192), and AIMP1-(193–312) did not. These results suggest that the N-terminal region, especially AIMP1-(6–46), might be a proliferation-inducing domain. We then synthesized the peptides corresponding to 6–46 amino acid of AIMP1 and analyzed the effect of these peptides on fibroblast proliferation. The peptide sequences are shown schematically in Fig. 4B. While one contains the wild type sequence, the other was made to contain the substitution of 24th tyrosine to threonine that is highly conserved among AIMP1 sequences of mammalian systems as a control. As shown in Fig. 4C, the wild type 6–46 peptide, but not the Y24T peptide, induced fibroblast proliferation in concentration-dependent manner. These results indicate that the N-terminal region (6–46) of AIMP1 is important and sufficient to fibroblast proliferation and 24th tyrosine residue is critical for its activity. We previously showed that elastase 2 cleaved the peptide bond between 29th and 30th amino acids, resulting in the loss of AIMP1's fibroblast proliferation activity (Fig. 2A). Therefore, the peptide region spanning 24th tyrosine appears to be critical for fibroblast proliferation activity of AIMP1.

#### Identification of migration-inducing domain of AIMP1

AIMP1 shows dual activities on endothelial cells dependent on AIMP1 concentration [14]. While AIMP1 promotes the migration of endothelial cells at low doses, it induces the cell death at high doses. Therefore, using the AIMP1 deletion mutants, we investigated whether the same region of AIMP1 is responsible for its distinct activities on endothelial cells. All of the AIMP1 deletion fragments including 114–192 induced endothelial cell migration at low concentration (1 nM), but AIMP1-(1–46), AIMP1-(1–53), and AIMP1-(193–312) did not (Fig. 5). According to the data obtained in Figs. 3 and 5, the cell death-inducing and migration-inducing domains are different and separated. Therefore, there is a possibility that AIMP1 acts on endothelial cells through two independent receptors. Previously, we reported that both of the N- (1–146) and C-terminal (147–312) domains of AIMP1 showed endothelial cell migration and cell death activities [14], although their activities were lower than the

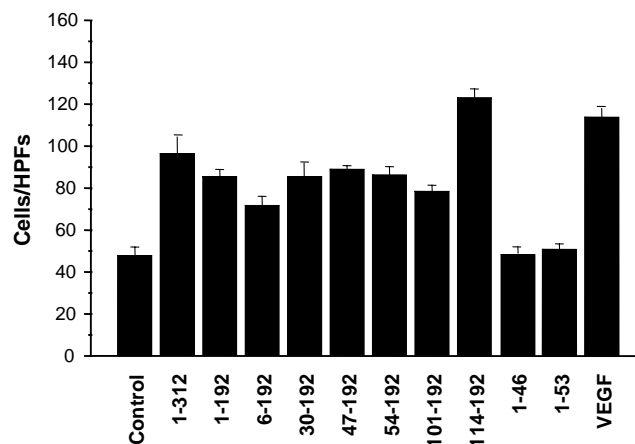


Fig. 5. Identification of endothelial cell migration-inducing domain of AIMP1. The effect of AIMP1 mutants on the endothelial cell migration was assayed using a Transwell chamber with gelatin-coated polycarbonate membrane. BAECs were suspended in the upper chamber, and the indicated concentrations of AIMP1 mutants were filled in the lower chamber. VEGF (0.7 nM) was used as a positive control. The cells migrating to the lower chambers were stained with hematoxylin and counted in high power fields. The data are the averages of the three independent experiments. Data represent means  $\pm$  SD.

full-length AIMP1-(1–312). Thus, in migration-inducing domain (114–192), two subparts (114–146 and 147–192) may contribute to the full activity.

In summary, we dissected the AIMP1 polypeptide to determine the functional domains responsible for each of its complex extracellular activities (Fig. 6). Accumulating evidences have demonstrated that many proteins can exert more than single activity. The multi-functionality of proteins can be achieved at the same region of polypeptide through their structural flexibility [16], and also through the adoption of distinct functional domains. AIMP1 appears to have employed both strategies for its multi-functionality since the C-terminal domain of AIMP1 spanning 146–312 amino acids shows the structural homology to the typical oligonucleotide-binding fold and also to a few cytokines [17], and use different peptide regions for different activities as shown in this work. Structural

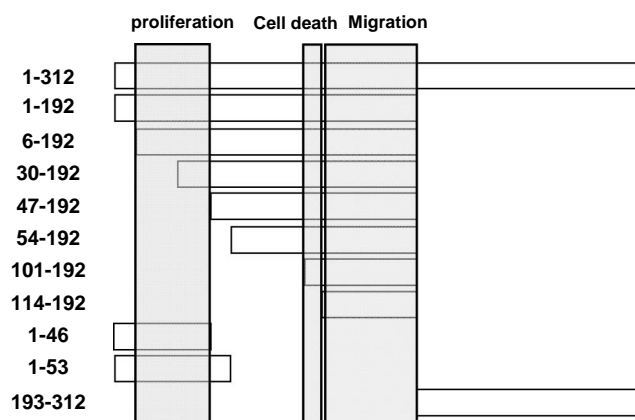


Fig. 6. Summary of functional mapping of AIMP1 activities. Each activity of AIMP1 studied in this work is displayed as box.

separation of the functional domains determined in this work can be further explored to generate various therapeutic peptides for specific indications.

### Acknowledgment

This work was supported by a grant from the National Creative Research Initiatives of the Ministry of Science and Technology of Korea.

### References

- [1] S. Quevillon, F. Agou, J.C. Robinson, M. Mirande, The p43 component of the mammalian multi-synthetase complex is likely to be the precursor of the endothelial monocyte-activating polypeptide II cytokine, *J. Biol. Chem.* 272 (1997) 32573–32579.
- [2] S. Park, K. Ewalt, S. Kim, Functional expansion of aminoacyl-tRNA synthetases and their interacting factors: new perspectives on housekeepers, *Trends Biochem. Sci.* 30 (2005) 569–574.
- [3] J. Kao, J. Ryan, J. Brett, J. Chen, H. Shen, Y.-G. Fan, G. Godman, P. Familletti, F. Wang, Y.-C. Pan, D. Stern, M. Clauss, Endothelial monocyte-activating polypeptide II. A novel tumor-derived polypeptide that activates host-response mechanisms, *J. Biol. Chem.* 267 (1992) 20239–20247.
- [4] L. Old, Tumor necrosis factor (TNF), *Science* 230 (1985) 630–632.
- [5] L. Old, B. Benacerraf, D. Clarke, E. Carswell, E. Stockert, The role of the reticuloendothelial system in the host reaction to neoplasia, *Cancer Res.* 21 (1961) 1281–1300.
- [6] J. Kao, K. Houck, Y. Fan, I. Haehnel, S.K. Libutti, M.L. Kayton, T. Grikscheit, J. Chabot, R. Nowygrod, S. Greenberg, W.-J. Kuang, D.W. Leung, J.R. Hayward, W. Kisiel, M. Heath, J. Brett, D.M. Stern, Characterization of a novel tumor-derived cytokine. Endothelial-monocyte activating polypeptide II, *J. Biol. Chem.* 269 (1994) 25106–25119.
- [7] J. Kao, Y.-G. Fan, I. Haehnel, J. Brett, S. Greenberg, M. Clauss, M. Kayton, K. Houck, W. Kisiel, R. Seljelid, J. Burnier, D. Stern, A peptide derived from the amino terminus of endothelial-monocyte-activating polypeptide II modulates mononuclear and polymorphonuclear leukocyte functions, defines an apparently novel cellular interaction site, and induces an acute inflammatory response, *J. Biol. Chem.* 269 (1994) 9774–9782.
- [8] H.A. Behrendorf, M. van de Craen, U.E. Knies, P. Vandenamee, M. Clauss, The endothelial monocyte-activating polypeptide II (EMAP II) is a substrate for caspase-7c, *FEBS Lett.* 466 (2000) 143–147.
- [9] G. Barnett, A.M. Jakobsen, M. Tas, K. Rice, J. Carmichael, J.C. Murray, Prostate adenocarcinoma cells release the novel proinflammatory polypeptide EMAP-II in response to stress, *Cancer Res.* 60 (2000) 2850–2857.
- [10] Y.-G. Ko, H. Park, T. Kim, J.-W. Lee, S.G. Park, W. Seol, J.E. Kim, W.-H. Lee, S.-H. Kim, J.E. Park, S. Kim, A cofactor of tRNA synthetase, p43, is secreted to up-regulate proinflammatory genes, *J. Biol. Chem.* 276 (2001) 23028–23033.
- [11] H. Park, S.G. Park, J.W. Lee, T. Kim, G. Kim, Y.-G. Ko, S. Kim, Monocyte cell adhesion induced by a human aminoacyl-tRNA synthetase-associated factor, p43: identification of the related adhesion molecules and signal pathways, *J. Leukoc. Biol.* 71 (2002) 223–230.
- [12] H. Park, S.G. Park, J. Kim, Y.-G. Ko, S. Kim, Signaling pathways for TNF production induced by human aminoacyl-tRNA synthetase-associated factor, p43, *Cytokine* 20 (2002) 148–153.
- [13] S.Y. Chang, S.G. Park, S. Kim, C.Y. Kang, Interaction of the C-terminal domain of AIMP1 and the alpha subunit of ATP synthase. Its functional implication in endothelial cell proliferation, *J. Biol. Chem.* 277 (2002) 8388–8394.
- [14] S.G. Park, Y.S. Kang, Y.H. Ahn, S.H. Lee, K.R. Kim, K.W. Kim, G.Y. Koh, Y.-G. Ko, S. Kim, Dose-dependent biphasic activity of tRNA synthetase-associating factor, p43, in angiogenesis, *J. Biol. Chem.* 277 (2002) 45243–45248.
- [15] S.G. Park, H. Shin, Y.K. Shin, Y. Lee, E.C. Choi, B.J. Park, S. Kim, The novel cytokine p43 stimulates dermal fibroblast proliferation and wound repair, *Am. J. Pathol.* 166 (2005) 387–398.
- [16] P. Tompa, C. Szasz, L. Buday, Structural disorder throws new light on moonlighting, *Trends Biochem. Sci.* 30 (2005) 484–489.
- [17] Y. Kim, J. Shin, R. Li, C. Cheong, K. Kim, S. Kim, A novel anti-tumor cytokine contains an RNA binding motif present in aminoacyl-tRNA synthetases, *J. Biol. Chem.* 275 (2000) 27062–27068.