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MUDENG is cleaved by caspase-3 during TRAIL-induced cell death

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ABSTRACT

MUDENG, also known as AP5M1, was originally identified as an adaptin domain-containing gene that induced cell death in lymphoma cell lines. However, little is known of the mechanism responsible for MUDENG-mediated cell death. In this study, we investigated MUDENG changes during TRAIL-induced cell death. We found that MUDENG is rapidly processed in response to TRAIL in Jurkat and BJAB cells with time line similar to that of caspase activation. Caspase-3-mediated MUDENG cleavage was confirmed by an *in vitro* cleavage assay using recombinant active caspase proteins. Caspase cleavage sites (D276 and D290) were located in the adaptin domain of MUDENG, and cleaved MUDENG showed the reduced killing activity. These results suggest that the adaptin domain plays a key role in MUDENG-mediated cell death.
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1. Introduction

MUDENG contains the adaptin domain found in μ subunits of adaptor protein (AP) complexes that transport vesicles from one membrane compartment to another. Previously we have reported that MUDENG can induce cell death when overexpressed in Jurkat cells. MUDENG has been reported to have different molecular weights (54/55 kD, 66 kD, and 80 kD) in different cell lines [1]. In a recent study, MUDENG was found as a medium-sized subunit of a novel AP5 complex. MUDENG appeared to be localized in late endosomes, and the authors suggested that it participates in endosomal trafficking because knock-down of MUDENG expression caused increases of the swollen and emanating tubule-structured multivesicular bodies (MVBs) near the Golgi apparatus [2,3].

In this report, to determine the functional role of MUDENG in TRAIL-induced cell death, we investigated changes in MUDENG expression patterns during TRAIL-induced cell death. The results indicate that MUDENG is cleaved during TRAIL-induced cell death, and the cleaved MUDENG products showed the reduced killing activity in tumor cells.

2. Materials and methods

2.1. Cell culture and reagents

Jurkat, BJAB, and HeLa cells were cultured in RPMI1640 medium or DMEM medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Pan caspase inhibitor z-VAD was purchased from Calbiochem. Recombinant human TRAIL protein was generated in our lab using His bind nickel column chromatography (Novagen, Darmstadt, Germany) as previously described [4].

2.2. Western blotting and antibodies

The cells were prepared with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–Cl, pH 7.5, and 150 mM NaCl) containing protease inhibitors. Total proteins were separated by a 15% SDS–PAGE and transferred to PVDF membrane (BIO-RAD, USA). The membranes were blotted with anti-MUDENG (M3H9, generated in previous study [1]), anti-caspase-8 (Cell Signaling, Danvers, MA), anti-caspase-3 (SantaCruze, Dallas, TX), and anti-actin (Chemicon, Temecular, CA) antibody.

2.3. Introduction of point mutations in MUDENG expression vectors

The human cDNA of MUDENG was cloned into pcDNA6A-HA vector (a modified version of pcDNA6/myc-His A (Invitrogen, Grand Island, NY)). The pcDNA6A-HA-MUDENG plasmid vector was amplified using PCR reactions with upper strand and complementary

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bottom strand primers for indicated residues as following: DDDA63/64/65/67AAAA (cttagattattggcagcagcaaaagcattcgttgagagt), D161A (agccagttgcctgcattgcttctgcag), D174A (ggtactttattagcagccaacttacag), D182A (cagaattcattagcaaataccaatttt), D223A (tccatgcaatatgcaaaacagggtata), D229A (cagggtatagcagcaacatggcaagtt), D242A (acttgcaa gtgtgcattggaaggaatc), D265A (tctccacttcaggcaattctagttcac), D276A (gtaacttctcttgcatctgcaattctg), DDD286/289/290AAA (tctagtagtattgcagcaatggcagcatctgcatttagt), DD289/290AA (attgatgcaa tggcagcatctgcatttagt), DD289/290DA (attgatgcaa tggatgcatctgcatttagt), or DD289/290AD (attgatgcaatggcagactctgcatt tagt). Then, the amplified plasmid vectors were treated with Dpn1 restriction enzyme and were transformed into *Escherichia coli* DH5α. The introduction of point mutations was confirmed by DNA sequencing analysis.

2.4. In vitro caspase cleavage analysis

The recombinant human active Caspase-8 and caspase-3 protein (R&D System, Minneapolis, MN) were purchased for *in vitro* cleavage assay. The recombinant active caspase-8 (100 ng) or -3 (100 ng) protein was incubated with Jurkat or BJAB total cell lysate (20 or 30 μg) in presence/absence of z-VAD for 1 h at 37 °C. Then, the cleavage of MUDENG was analyzed by Western blotting with anti-MUDENG (M3H9) antibody.

2.5. Viability analysis

Plasmid vectors (0.5 μg) expressing XB:GFP (as a control vector), MUDENG (1–490):GFP, MUDENG (1–290):GFP, or GFP:MUDENG (290–490) were transfected into HeLa or Jurkat cells. Cells were cultured in 96 well plates (2×10^5/well) overnight. Cell viabilities were assessed using XTT-based colorimetric assay kits (Promega, Madison, WI), according to the manufacturer's instructions.

3. Results

We previously reported that MUDENG is an adaptin-containing protein with cell killing activity that is expressed in most tumor cells [1]. Furthermore, we showed that MUDENG produced 80 kD and 66 kD bands in BJAB and Jurkat cells, and 66 kD and 55 kD bands in HeLa and HCT116 cells, although the expected molecular weight of MUDENG is a 55 kD. To investigate whether MUDENG shows any changes in expression patterns during TRAIL-induced cell death, we examined changes in expression patterns of endogenous MUDENG in BJAB cells. MUDENG was rapidly processed in response to TRAIL in BJAB cells (Fig. 1A); its 80 kD band was dramatically decreased, and a new band of 50 kD was detected within 2–4 h of TRAIL treatment in BJAB cells (Fig. 1A). Moreover, active forms of caspase-8 and -3 were detected at round the time of 50 kD MUDENG appearance in BJAB cells, and we also observed similar changes in MUDENG and caspases in Jurkat cells (data not shown). These results suggest that the processing of MUDENG might be mediated by caspases. To investigate whether active caspase-3 and -8 cleave MUDENG, Jurkat and BJAB cells were treated with TRAIL in the presence or absence of the pan-caspase inhibitor z-VAD. The cleavage of MUDENG was completely blocked by z-VAD treatment, suggesting that active caspase-8 and -3 might be responsible for MUDENG cleavage during TRAIL-induced cell death (Fig. 1B and C).

To address this suggestion, we subjected MUDENG to an *in vitro* cleavage assay using Jurkat or BJAB cell lysates and recombinant caspase-3 or caspase-8. In Jurkat cell lysates, recombinant caspase-3 cleaved MUDENG present in cell lysates, and this cleavage was blocked by z-VAD-fmk. Interestingly, recombinant caspase-8 could not cleave MUDENG (Fig. 2A), indicating that caspase-3, but not caspase-8, cleaves MUDENG. On other hand, in BJAB cell lysates, both recombinant caspase-3 and -8 cleaved MUDENG (Fig. 2B), possibly due to the indirect activation of procaspase-3 by recombinant caspase-8, because type I cells, like BJAB cells, can directly activate procaspase-3 with caspase-8. However, in type II cells, such as, Jurkat cells, caspase-8 cannot directly activate procaspase-3 but needs the mitochondrial pathway to activate procaspase-3. Together, these results suggest that caspase-3, and not caspase-8, cleaves MUDENG during TRAIL-induced cell death. To confirm this suggestion, an *in vitro* cleavage assay was performed using *in vitro* transcription and translated (TNT) [S^{35}]

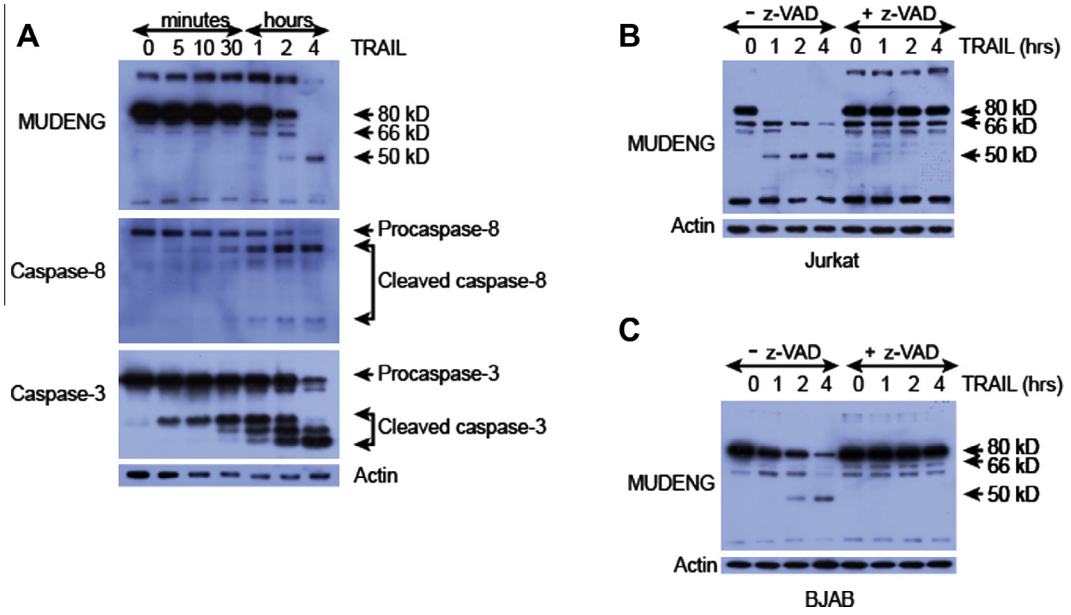

Fig. 1. MUDENG is cleaved in response to TRAIL in BJAB and Jurkat cells. (A) BJAB cells were treated with TRAIL (100 ng/ml) for indicated time. Lysates were subjected to SDS–PAGE, and then analyzed by Western blotting using anti-MUDENG (M3H9 monoclonal antibody), anti-caspase-8, anti-caspase-3, and anti-Actin antibodies. (B) Jurkat cells and (C) BJAB cells were treated with TRAIL (100 ng/ml) in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (10 μM). Lysates were subjected to SDS–PAGE, and then analyzed by Western blotting using anti-MUDENG antibody (M3H9 monoclonal antibody) and anti-Actin antibody.

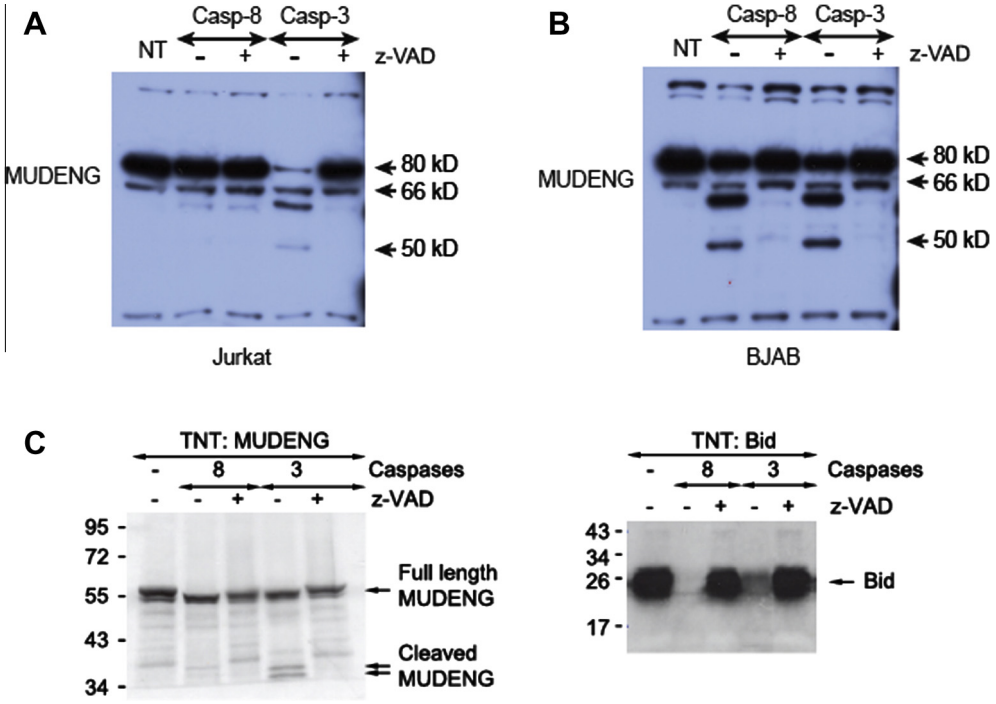

Fig. 2. *In vitro* cleavage of MUDENG by caspase-3. Lysates (20 μg) prepared from Jurkat cells (A) and BJAB cells (B) were incubated with recombinant caspase-8 (100 ng) or recombinant caspase-3 (100 ng) proteins for 1 h at 37 °C in caspase assay buffer. Reaction mixtures were subjected to SDS–PAGE, and then analyzed by Western blotting using anti-MUDENG antibody (M3H9 monoclonal antibody). (C) MUDENG and Bid were *in vitro* transcribed and translated using plasmid vectors (1 μg) containing full length MUDENG cDNA and Bid cDNA, respectively, in the presence of [S^{35}] methionine. These recombinant MUDENG and Bid proteins were then treated with recombinant caspase-3 or caspase-8 protein in the presence or absence of z-VAD-fmk (10 μM) for 1 h or 4 h at 37 °C in caspase assay buffer. Reaction mixtures were subjected to SDS–PAGE, transferred to PVDF membranes, and visualized by exposure to X-ray film.

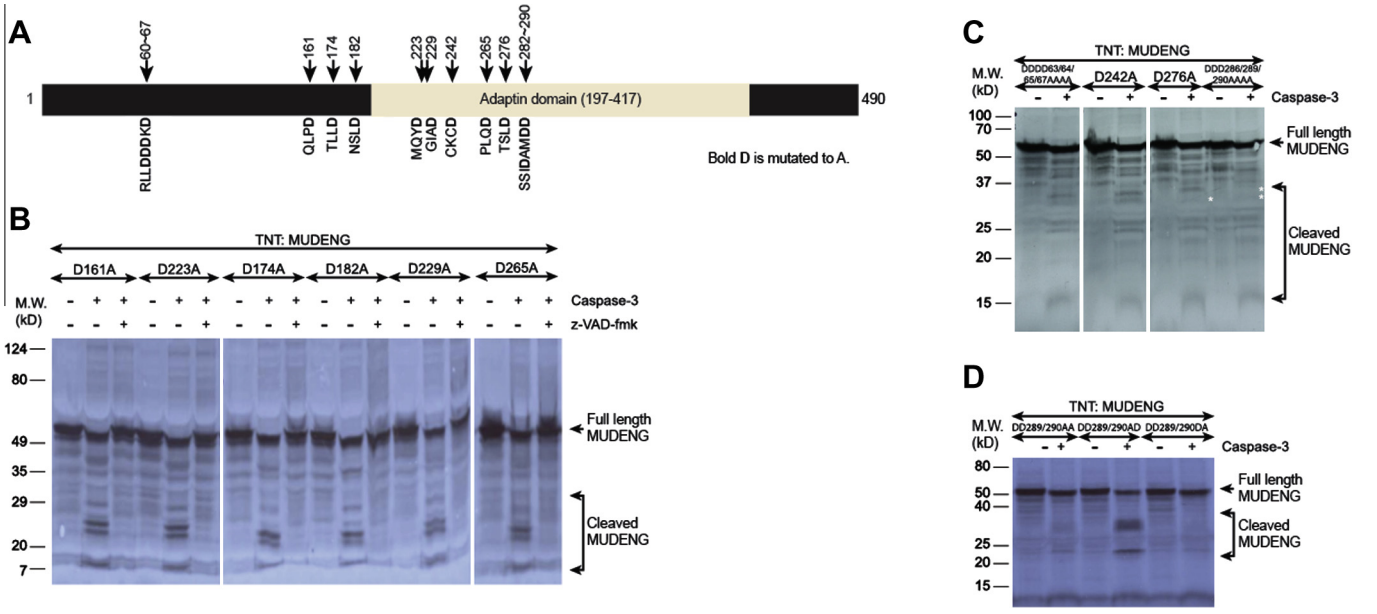

Fig. 3. Identification of caspase-3 cleavage sites in MUDENG. (A) Diagram of plausible caspase-3 cleavage sites in MUDENG. Bold D (Asp) letters indicates plausible cleavage sites. These D (Asp) residues were mutated to A (Ala) residues for *in vitro* cleavage assays (B–D). Mutant forms of MUDENG at 161, 223, 174, 182, 229, or 265 (B), 63/64/65/67 (quadruple mutant), 242, 276, or 286/289/290 (triple mutant) (C), 289/290 (double mutant), 289, or 290 (D) were *in vitro* transcribed and translated as described in Fig. 2. These mutant forms of MUDENG proteins were then treated with recombinant caspase-3 protein in the presence or absence of z-VAD-fmk (10 μM) for 4 h at 37 °C in caspase-3 assay buffer. Reaction mixtures were subjected to SDS–PAGE, transferred to PVDF membranes, and visualized by exposure to X-ray film.

Met-labeled MUDENG and recombinant active forms of caspase-3 and caspase-8. Recombinant caspase-3, but not caspase-8, cleaved [S^{35}] Met-labeled MUDENG, whereas both recombinant caspase-3 and -8 cleaved [S^{35}] Met-labeled Bid, a well-known substrate for caspase-3 and -8 (Fig. 2C). These results confirm that caspase-3 cleaves MUDENG *in vitro* and *in vivo* during TRAIL-induced cell death.

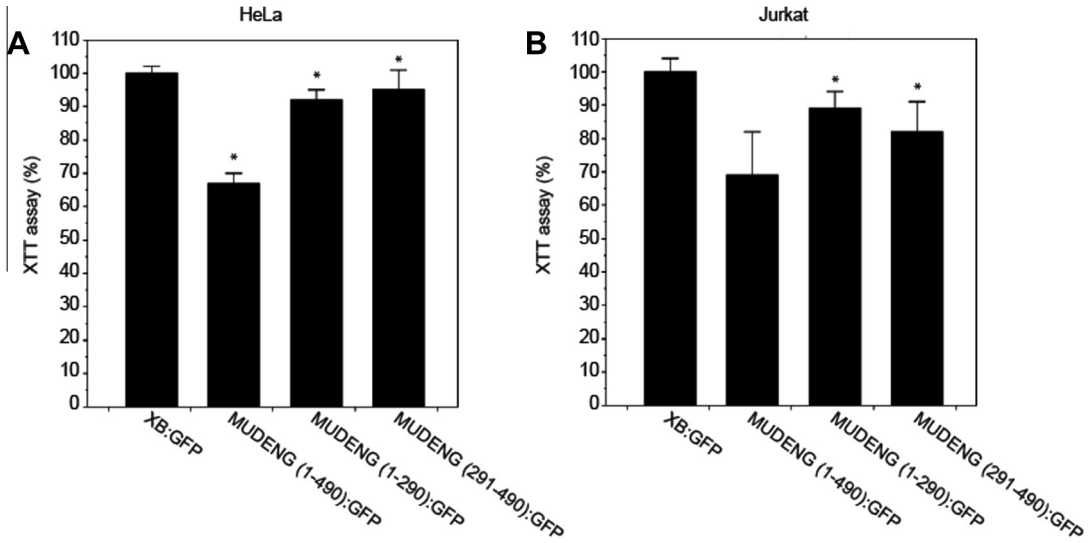

Fig. 4. Loss of cell death-inducing activity by cleaved MUDENG. Plasmid vectors (0.5 μg) expressing XB:GFP (as a control vector), MUDENG (1–490):GFP, MUDENG (1–290):GFP, or GFP:MUDENG (290–490) were transfected into HeLa (A) or Jurkat (B) cells. Cell viabilities were monitored by XTT assay at 24 h after transfection.

To identify the cleavage site(s) in MUDENG used by caspase-3, various aspartic acid residues at expected cleavage sites were mutated to alanine residues as indicated in Fig. 3A. These mutant forms of MUDENG were then subjected to *in vitro* cleavage assay using recombinant caspase-3. MUDENG bearing aspartic acid to alanine replacements at 63, 64, 65, 67, 161, 174, 182, 223, 229, 242, or 265 showed a caspase-3 cleavage pattern similar to that of wild type MUDENG (Fig. 3B and C). However, some bands were missing from the caspase-3 cleavage pattern of MUDENG bearing a mutation at 276 or mutations at 286, 289, and at 290 (indicated by the asterisks in Fig. 3C), indicating that caspase-3 cleaves MUDENG at these sites. To identify the actual cleavage site(s) a triple mutation DDD286/289/290AAA, double mutation (DD289/290AA), and a single mutation (DD289/290AD or DD289/290DA) were introduced in MUDENG. Recombinant caspase-3 cleaved MUDENG bearing the DD289/290AD mutation, but not MUDENG bearing the DD289/290AA or the DD289/290DA mutation (Fig. 3D), indicating that the aspartic acid at 290 of MUDENG was the caspase-3 cleavage site. Together, these results reveal that caspase-3 cleaves MUDENG at aspartic acid residues 276 and 290.

To determine the functional consequences of the caspase-3-mediated cleavage of MUDENG, we constructed plasmid vectors expressing cleaved MUDENG (1–290) or MUDENG (291–490) fused with GFP. HeLa or Jurkat cells were then transfected with MUDENG (1–490):GFP, MUDENG (1–290):GFP, or MUDENG (291–490):GFP, and cell viabilities were monitored using an XTT assay. MUDENG (1–490):GFP reduced the viabilities of HeLa cells and Jurkat cells, as we previously reported [1], but the cleaved forms of MUDENG (1–290):GFP and MUDENG (291–490):GFP showed reduced MUDENG (1–490):GFP-mediated cytotoxicity in HeLa cells and Jurkat cells. Together, these results indicate that the caspase-3-mediated cleavage of MUDENG reduces MUDENG-induced cytotoxicity in HeLa or Jurkat cells.

4. Discussion

Many proteins have known to be cleaved by caspases during apoptosis induced by various stimuli. Some proteins, like gelsolin, are cleaved by caspases during apoptosis to produce functionally active cleaved products that may promote or inhibit apoptosis [5], whereas other proteins simply lose their activities after caspase cleavage [6]. This study shows that caspase-3 cleaves MUDENG during TRAIL-induced cell death and that this results in a reduction of MUDENG-induced cytotoxicity (Fig. 4). Furthermore, two caspase-3 cleavage sites in MUDENG were identified (Fig. 3); however, we did not exclude the possibility that caspases other than caspase-8 could also cleave MUDENG during apoptosis. It is likely that MUDENG is not a good substrate for recombinant caspase-3, because recombinant caspase-3 could not completely cleave MUDENG, but completely cleaved Bid and ICAD under the same conditions *in vitro* (Fig. 2 and data not shown, respectively). Although MUDENG (1–290) and MUDENG (291–490) cleavages were associated with the reduction of cytotoxicity in HeLa cells and Jurkat cells, the cleavages of MUDENG (e.g., MUDENG (1–276), MUDENG (276–490) or MUDENG (276–290)) may result in different activities of cytotoxicity or endosome membrane trafficking characteristics. The results emphasize that the adaptin domain of MUDENG may play a key role in its cytotoxicity, because caspase-3 cleavage sites are located in this domain. The molecular mechanism whereby the adaptin domain of MUDENG is involved in cytotoxicity remains elusive; but, it is probably associated with the role of MUDENG in endosome trafficking.

Acknowledgments

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