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Mature interleukin-33 is produced by calpain-mediated cleavage *in vivo*

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ABSTRACT

Interleukin (IL)-33 is a novel member of the IL-1 family. IL-33 is primarily synthesized as a 30-kDa precursor (pro-IL-33). Pro-IL-33 is cleaved by caspase-1 into an 18-kDa mature form (mature IL-33) *in vitro*. Recombinant mature IL-33 has been known to induce T-helper type-2 (Th2)-associated cytokines and inflammatory cytokines via its receptor, ST2L. However, processing of pro-IL-33 *in vivo* has not been clarified yet. Here, we report that calpain mediates pro-IL-33 processing *in vivo*. Pro-IL-33 was expressed by stimulating human epithelial cells with phorbol 12-myristate 13-acetate. Calcium ionophore induced pro-IL-33 cleavage and mature IL-33 production. This cleavage was inhibited by treatment with a calcium chelator and calpain inhibitors. Moreover, short interfering RNA-mediated knockdown of calpains suppressed pro-IL-33 cleavage. These results indicate that calpains play a critical role in pro-IL-33 processing *in vivo*.

Introduction

Interleukin (IL)-33, also referred to as Nuclear Factor from High Endothelial Venules (NF-HEV), belongs to the IL-1 family [1,2]. IL-33 was recently identified as the specific ligand for ST2L, a member of the IL-1 receptor (IL-1R) family. The functional properties of IL-33 have been well characterized by using recombinant proteins and overexpressed proteins. Mature IL-33 acts as a cytokine and promotes the expression of Th2-associated cytokines and inflammatory cytokines via its receptor complex, consisting of ST2L and IL-1R accessory protein (IL-1RAcP) [2–4]. This is accompanied by the activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPK). Administration of mature IL-33 to mice exacerbates their inflammatory reactions and induces symptoms of asthma [2]. Soluble ST2 blocks IL-33 signaling in allergic airway inflammation [3]. On the other hand, pro-IL-33 localizes in the nucleus with the aid of nuclear localization sequence (NLS) in its N-terminus. Nuclear pro-IL-33 associates with heterochromatin through a homeodomain-like helix-turn-helix motif in its N-terminus and acts as a transcriptional repressor [5]. Thus, these studies indicate that IL-33 is a dual-function protein.

In contrast, the molecular mechanism underlying pro-IL-33 processing has been poorly understood. IL-33 mRNA is primarily translated as pro-IL-33 without a signal sequence for secretion, like IL-1α, IL-1β, and IL-18 [6,7]. Recombinant pro-IL-33 is cleaved by caspase-1 into mature IL-33 containing 112–270 residues *in vitro*, like precursors IL-1β and IL-18 (pro-IL-1β and pro-IL-18) [2,8–10]. However, pro-IL-33 does not possess a definite caspase-1 cleavage site. So far, it has not been confirmed that caspase-1 cleaves pro-IL-33 at the proposed site *in vivo*. On the other hand, precursor IL-1α (pro-IL-1α) is known to be cleaved by calpain, a calcium-dependent cysteine protease [11,12]. Actually, no enzyme responsible for proteolytic cleavage of pro-IL-33 has been identified yet.

In this study, we found that treatment with calcium ionophore induces pro-IL-33 cleavage and mature IL-33 production in human epithelial as well as endothelial cells. Furthermore, we demonstrate that calpain inhibitors and short interfering RNA (siRNA) against calpains inhibit pro-IL-33 cleavage in epithelial cells, suggesting that calpain is the real mediator for pro-IL-33 processing *in vivo*.

Materials and methods

Reagents. Phorbol 12-myristate 13-acetate (PMA), calcium ionophore (ionomycin and A23187), calpain inhibitors (ALLN, calpain inhibitor III, and calpeptin), and caspase inhibitors (caspase inhibitor I, caspase-1 inhibitor I, and caspase-3 inhibitor II) were purchased from Calbiochem. Dimethyl sulfoxide (DMSO) and ethyleneglycol-bis (2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Wako.

Cell culture and transfection. Human gastric carcinoma cells, AGS, and human colon carcinoma cells, CW-2, were kindly provided by

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Dr. T. Mutoh (Jichi Medical University, Tochigi, Japan). Human umbilical vein endothelial cells (HUVEC) were purchased from Cell Applications. AGS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone). CW-2 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS. HUVEC were cultured in Basal medium supplemented with growth supplements (Cell Applications). All cells were grown at 37 °C in a humidified atmosphere under 5% CO_2. Expression of endogenous IL-33 in AGS and CW-2 cells was induced with treatment of 10 nM PMA for 3 or 12 h. In the expression of exogenous IL-33, cells were transfected with IL-33 expression vectors for 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After a change to a serum-free medium, 5 μM ionomycin or A23187 was added to induce pro-IL-33 cleavage for 1 h. Inhibitors were added 1 h prior to stimulation with calcium ionophore.

Reverse transcription (RT)-PCR analysis. Total RNAs were isolated from cells using TRI reagent (Sigma). First-strand cDNA synthesis and PCR amplification were performed as described previously [13]. After cDNAs were treated at 94 °C for 10 min, PCR was carried out for 25 or 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by treatment at 72 °C for 10 min. The nucleotide sequences of primers used were as follows: IL-33 (forward 5′-GGCC TTCACTGAAAACAGGTAGAA-3′, reverse 5′-CAGTTGGAGTGCATATT ATGAAGGA-3′), calpain 4 (forward 5′-ATTACTCCAACATTGAGGCC-3′, reverse 5′-TCTGAGTAGCGTCGGATGAT-3′), calpain 1 (forward 5′-ACATGGAGACTATTGGCTTC-3′, reverse 5′-ATCTCGTAGGCACTC ATGCT-3′), calpain 2 (forward 5′-AGGAGAGTACATTCTCGTGC-3′, reverse 5′-ATGATGAGCTGGTCATCTGC-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward 5′-CTGGCGTCTTCAC CACCATGGAGAA-3′, reverse 5′-GTCAGAGGAGACCACCTGGTGCTC A-3′). PCR products were developed by electrophoresis on 2% agarose gels, and then the gels were stained with ethidium bromide.

Construction of plasmids. Construction of pEF6-IL-33-FL and pEF6-IL-33–112 proceeded as follows. The pEF6-IL-33-FL contains a Flag-tag in the N-terminus and V5-His-tags in the C-terminus. The pEF6-IL-33–112 contains V5-His-tags in the C-terminus. The fragments of human IL-33 were obtained from cDNA derived from HUVEC by PCR amplification. The nucleotide sequences of primers were as follows: a forward primer for pro-IL-33 (5′-GCGGCCGCAAA GAGGAGTGCAGCCACCATGGACTACAAGGATGACGATGACAAGAAGC CTAAAATGAAGTATTC-3′), a forward primer for mature IL-33 (5′-GGTACCATGAGTATCACAGGAATTTCA-3′), and a reverse primer for pro- and mature IL-33 (5′-TCTAGAAGTTTCAGAGAGCTTAAACA-3′). PCR products were digested with NotI and XbaI (pro-IL-33 fragment) or KpnI and XbaI (mature IL-33 fragment), then ligated into a NotI/XbaI-digested or KpnI/XbaI-digested pEF6-V5-His (Invitrogen).

Measurement of cytotoxicity. Cytotoxicity was determined by measuring the level of lactate dehydrogenase (LDH) released from cells. AGS cells were transfected with IL-33 expression vectors for 24 h. After changing to a serum-free medium, the cells were untreated or treated with 5 μM ionomycin. After 1 h, the culture media were collected. LDH activity in the culture media was measured using an LDH Cytotoxicity Detection Kit (Takara Bio). One-hundred-percent cytotoxicity was defined as the level of LDH activity released from the Triton X-100-treated cells, according to the manufacturer's instructions. Data are presented as the average ±SD of three experiments.

Western blotting. Cells were lysed in lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS) containing protease inhibitor cocktail (Roche Applied Science). The protein samples were separated by electrophoresis on SDS-10% or 12.5% polyacrylamide gels and transferred to PVDF membranes (Millipore). The PVDF membranes were probed with mouse monoclonal anti-human IL-33 (MBL), mouse monoclonal anti-calpain 4 (Santa Cruz Biotechnology), goat polyclonal anti-calpain 1 (Santa Cruz Biotechnology), rabbit polyclonal anti-calapin 2 (Abcam), or mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology) as the primary antibody. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (IgG) (Bio-Rad Laboratories), anti-goat IgG (Santa Cruz Biotechnology), or anti-rabbit IgG (Amersham Biosciences) as the secondary antibody. Can Get Signal (Toyobo) was used to detect endogenous IL-33. Proteins were visualized using Immobilon western detection reagents (Millipore), and the membranes were exposed to X-ray films (Fuji Photo Film).

Immunocytochemistry. AGS cells were cultured on a μ-Dish (Ibidi GmbH) and transfected with IL-33 expression vectors. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with PBS containing 0.1% (w/v) Triton X-100 for 20 min. After the cells on the dish were blocked with PBS containing 5% goat serum for 1 h, the cells were stained with anti-human IL-33 (MBL) and rabbit polyclonal anti-FLAG (Sigma) in PBST (PBS containing 0.05% (w/v) Tween) containing 5% goat serum for 1 h. After the cells were washed with PBST, the cells were stained with Alexa Fluor 488-conjugated goat anti-mouse IgG, or Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes). Finally, ProLong Gold antifade reagent with DAPI (Molecular Probes) was dropped on the stained cells. Stained cells were observed using a BioZERO BZ8000 (Keyence).

RNA interference (RNAi). Predesigned Stealth Select siRNAs against calpain 4 (HSS101350 and HSS101352), calpain 1 (HSS101345 and HSS188702), calpain 2 (HSS101347 and HSS188705), and negative controls (Low GC Duplex#2 and Medium GC Duplex #2) were purchased from Invitrogen. Transfection with siRNA was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

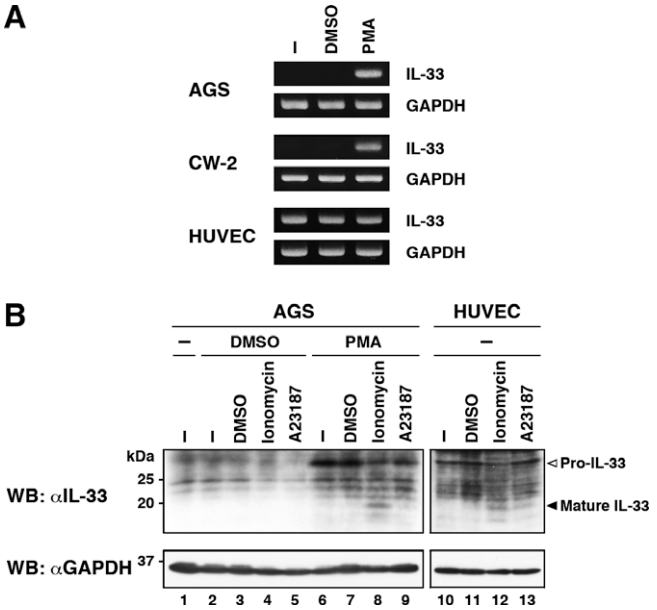

Fig. 1. Calcium ionophores induce pro-IL-33 cleavage *in vivo*. (A) AGS cells, CW-2 cells, and HUVEC were unstimulated (–) or stimulated with DMSO and PMA for 3 h, and then expression of IL-33 and GAPDH mRNAs was detected by RT-PCR. (B) After AGS cells were stimulated with PMA for 12 h, the cells were untreated (–) or treated with DMSO, ionomycin (5 μM), and A23187 (5 μM) for 1 h. IL-33 and GAPDH proteins in cell lysates were detected by Western blotting (WB) with the indicated antibodies. Detection of endogenous IL-33 was used with a mouse monoclonal anti-human IL-33 antibody (αIL-33), which recognizes human mature IL-33 (residues 112–270). White and black arrowheads indicate endogenous pro-IL-33 and mature IL-33, respectively.

Results

Cleavage of pro-IL-33 is induced by treatment with calcium ionophore

IL-33 is constitutively expressed in HUVEC, but no inducible system of IL-33 expression has been established in cultured cells. We found that PMA was able to induce IL-33 expression in AGS and CW-2 cells (Fig. 1A). In addition, pro-IL-33 was detected in PMA-stimulated AGS cells (Fig. 1B, lane 6). Furthermore, we found that calcium ionophores induce pro-IL-33 cleavage. In PMA-stimulated AGS cells and unstimulated HUVEC, cleaved pro-IL-33 corresponding to mature IL-33 was detected as a doublet band (approximately 20 and 18 kDa) after treatment with calcium ionophores (Fig. 1B, lanes 8, 9, 12, and 13). The 20 kDa band was a major product rather than the 18 kDa band. Pro-IL-33 and mature IL-33 were also detected in CW-2 cells (data not shown). These results suggest that the modulation of intracellular calcium levels is required for pro-IL-33 cleavage. Taken together, the results indicate that AGS, CW-2 cells, and HUVEC primarily have expression and cleavage systems of pro-IL-33.

Pro-IL-33 and mature IL-33 are released to culture medium

To further study pro-IL-33 cleavage *in vivo*, we constructed human IL-33 expression vectors, pEF6-IL-33-FL and pEF6-IL-33–112. IL-33-FL is a 35 kDa protein, which is composed of FLAG-tag (1 kDa), pro-IL-33 containing full-length (31 kDa), and V5-His-tags (3 kDa). IL-33–112 is a 21 kDa protein, which is composed of mature IL-33 containing 112–270 residues (18 kDa) and V5-His-tags (3 kDa). After AGS cells were transfected with these vectors, the cells were stained with anti-IL-33 antibody. IL-33-FL was localized in the nucleus, while IL-33–112 was located in both the cytoplasm and the nucleus (Supplementary figure). Next, we performed an ionomycin-induced cleavage assay in transfected cells (Fig. 2A).

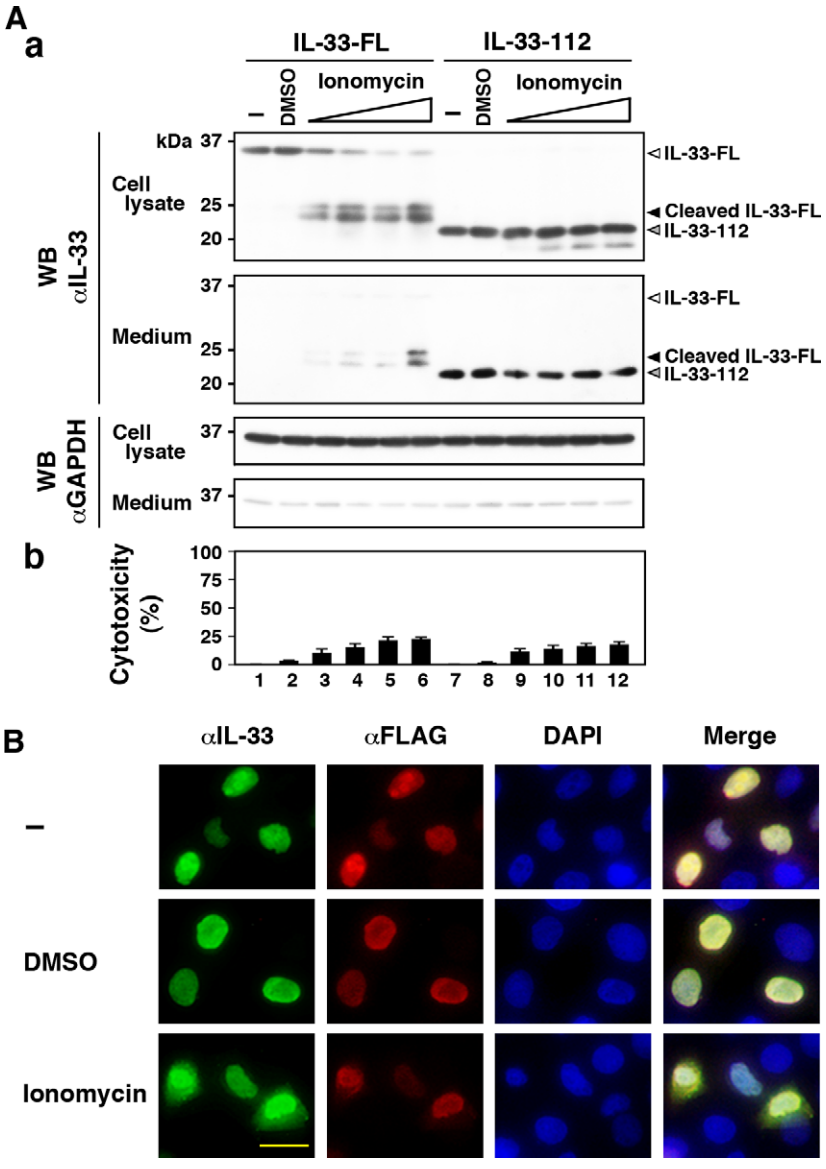

Fig. 2. Pro-IL-33 and mature IL-33 are released from ionomycin-treated AGS cells. (A) AGS cells were transfected with pEF6-IL-33-FL or pEF6-IL-33–112. After 24 h, the transfected cells were untreated (–) or treated with DMSO and ionomycin (0.625, 1.25, 2.5, 5 μM) for 1 h. (a) The cell lysates and medium were probed with the indicated antibodies. White and black arrowheads indicate IL-33-FL and cleaved IL-33-FL, respectively. Gray arrowhead indicates IL-33–112. (b) Cytotoxicity was estimated by measurement of LDH activity of the culture medium using an LDH cytotoxicity detection kit. The thin bars indicate the standard deviation of three independent experiments. (B) AGS cells were transfected with pEF6-IL-33-FL, and then the cells were untreated (–) or treated with DMSO and ionomycin (5 μM). After 1 h, the cells were stained with the indicated antibodies. Nuclear DNA was stained with DAPI. Scale bar indicates 20 μm.

IL-33-FL decreased as the ionomycin concentration increased; on the other hand, cleaved IL-33-FL corresponding to the mature form was increased in cell lysates (Fig. 2A, a, lanes 3–6). Cleaved IL-33-FL was also detected as a doublet band (24 and 23 kDa), and their molecular weights were larger than that of IL-33–112. Also, IL-33–112 was slightly degraded in the presence of ionomycin (Fig. 2A, a, lanes 9–12). Interestingly, cleaved IL-33-FL and IL-33–112 were detected in the medium. Cleaved IL-33-FL in the medium was observed only in the presence of ionomycin, and the releasing pattern was identical with the production pattern in cell lysates. On the other hand, IL-33–112 in medium was detected regardless of treatment with ionomycin. Under the experimental conditions, cytotoxicity was elevated with an increasing concentration of ionomycin (Fig. 2A, b). Furthermore, we observed localization of IL-33 in IL-33-FL-transfected cells (Fig. 2B). After treatment with ionomycin, IL-33 distribution was diffused to the cytoplasm (lower panel). These results indicate that pro-IL-33 cleavage is promoted in damaged cells. In addition, our results suggest that pro-IL-33 is released only from damaged cells, while the release of mature IL-33 is independent of cytotoxicity.

Calpain mediates cleavage of pro-IL-33 in vivo

Pro-IL-1α and pro-IL-1β are cleaved by calpain and caspase-1 *in vivo*, respectively [8,9,11,12]. To investigate candidate enzymes responsible for pro-IL-33 cleavage *in vivo*, we carried out an ionomycin-induced cleavage assay in the presence of calpain and caspase inhibitors (Fig. 3). IL-33-FL cleavage was inhibited by the calpain inhibitors but not by the caspase inhibitors (Fig. 3, lanes 6–11). In addition, a specific calcium chelator, EGTA, also inhibited IL-33-FL cleavage (Fig. 3, lane 5). These results suggest that pro-IL-33 cleavage is dependent on calpain activity, not on caspase activity.

To further confirm whether or not calpain mediates pro-IL-33 cleavage, we used an RNAi approach to ablate calpain. AGS cells were treated with siRNA for a regulatory subunit (calpain 4) and for catalytic subunits (calpain 1 and calpain 2). Expression of calpain 4, calpain 1, and calpain 2 mRNAs was suppressed by treatment with the corresponding siRNA (Fig. 4A). First, we tested IL-33-FL cleavage in siRNA-treated cells (Fig. 4B). AGS cells were treated with siRNAs prior to transfection with pEF6-IL-33-FL. Treatment of siRNA against calpain 1 and calpain 2 led to the reduction of calpain 4 levels in protein in addition to reductions in the calpains themselves, but they were not reduced in mRNA (compare Fig. 4A and B). Ionomycin-induced IL-33-FL cleavage was suppressed in calpain-ablated cells (Fig. 4B, lanes 5–10). Furthermore, we confirmed that cleavage of endogenous pro-IL-33 was not also cleaved in siRNA-treated cells (Fig. 4C, lanes 5–10). These results indicate that calpain mediates pro-IL-33 cleavage *in vivo*.

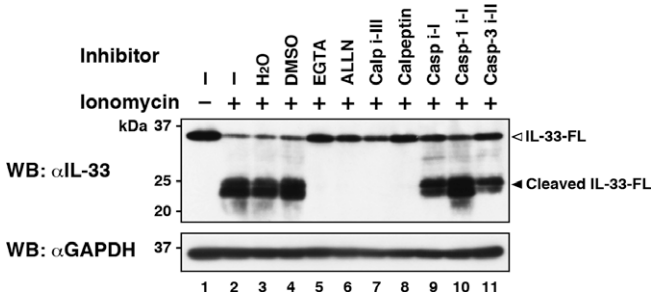

Fig. 3. Calcium chelator and calpain inhibitors inhibit pro-IL-33 cleavage in AGS cells. AGS cells were transfected with pEF6-IL-33-FL. After 24 h, the transfected cells were untreated (−) or treated with vehicles (H_2O and DMSO), EGTA (5 mM), calpain inhibitors (50 μM): ALLN, calpain inhibitor III (calp i-III), and calpeptin, and caspase inhibitors (50 μM): caspase inhibitor I (casp i-I), caspase-1 inhibitor I (casp-1 i-I), and caspase-3 inhibitor II (casp-3 i-II) for 1 h. The cells were then untreated (−) or treated with ionomycin (5 μM) for 1 h. The cell lysates were probed with the indicated antibodies.

Discussion

This study showed that pro-IL-33 processing is mediated by calpain in human epithelial cells. We developed an inducible system for expression and cleavage of pro-IL-33 using AGS and CW-2 cells. In our system, although we also observed IL-33 expression in human embryonic kidney HEK293T cells and human promyelocytic leukemia HL-60 cells, PMA failed to induce expression of IL-33 mRNA in these cells (data not shown). PMA has been known to activate protein kinase C (PKC). Therefore, in addition to PKC activation, specific factor(s) acting in epithelial cells may be required for transcriptional regulation of the IL-33 gene.

We also tried to cleave pro-IL-33 by treatment with IL-1β, TNF-α, IFN-γ, or lipopolysaccharide (LPS) besides calcium ionophore. In the processing of pro-IL-1α and pro-IL-1β, calcium ionophore and LPS have been known to activate calpain and caspase-1, respectively [11,14]. Interestingly, only calcium ionophores induced pro-IL-33 cleavage. Furthermore, the results of our inhibitor and RNAi experi-

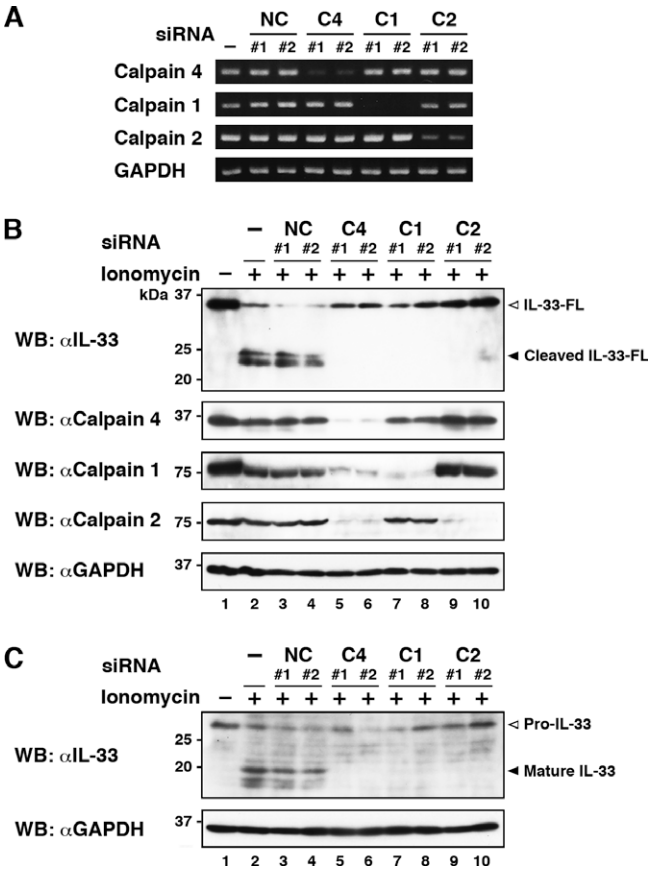

Fig. 4. siRNA treatment against calpains suppresses mature IL-33 production in AGS cells. (A) AGS cells were transfected with siRNA against calpain 4 (C4), calpain 1 (C1), or calpain 2 (C2). NC indicates negative controls. RNAi experiment was carried out using two siRNAs (#1 and #2) against each gene. After 96 h, expression of calpains and GAPDH mRNAs was detected by RT-PCR. (B) After AGS cells were treated with the indicated siRNAs for 96 h, the cells were transfected with pEF6-IL-33-FL. After 24 h, the cells were untreated (−) or treated with ionomycin (5 μM) for 1 h. The cell lysates were probed with the indicated antibodies. (C) After siRNA-treated cells were stimulated with PMA to induce endogenous pro-IL-33 for 12 h, the cells were untreated (−) or treated with ionomycin (5 μM) for 1 h. The cell lysates were probed with the indicated antibodies.

ments strongly suggest that pro-IL-33 cleavage was mediated by calpain, not caspase-1. The most recent study demonstrated that pro-IL-33 is not a direct substrate for caspase-1 *in vivo* [15]. Activated caspase-1 cleaved pro-IL-1β, but not pro-IL-33, in human monocytic leukemia THP-1 cells. Another recent study showed that caspase-1 cleaved recombinant pro-IL-33 not at Serine 111 but at Asparagine 178 *in vitro* [16]. Thus, there is no definite evidence that caspase-1 cleaves pro-IL-33 *in vivo* or *in vitro*. In cellular distribution and biological activity, pro-IL-33 is similar to pro-IL-1α rather than pro-IL-1β. Pro-IL-33 and pro-IL-1α localize in the nucleus because they possess the NLS and affect transcriptional regulation [5,17,18]. In addition, pro-IL-33 and pro-IL-1α possess biological activity as cytokines [15,19]. Therefore, pro-IL-33 processing may also resemble that of pro-IL-1α.

In our study using IL-33 expression vectors, cleaved IL-33-FL was clearly detected as a doublet band in ionomycin-treated cells. Two products had larger molecular sizes (24 and 23 kDa) than IL-33–112 (21 kDa). The results suggest that pro-IL-33 possesses two cleavage sites, which are different from a previously proposed cleavage site by caspase-1 *in vitro* [2]. Based on the molecular weight of the cleaved IL-33-FL, we deduced that two calpain-mediated cleavage sites might be located within 30 residues upstream from Serine 111. We will need to determine the precise cleavage site. Interestingly, both IL-33-FL and cleaved IL-33-FL were released to the medium from ionomycin-damaged cells. A previous study also showed pro-IL-33 and mature IL-33 in a culture medium of THP-1 cells [20]. The release of functional IL-33 has been shown also in murine glia by stimulation with both LPS and ATP [21]. Recently, the release of IL-33 has been suggested to function as an alarmin such as IL-1α and HMGB1 [22–24]. Our and other results may support this suggestion.

We speculate that pro-IL-33 is processed *in vivo* as follows. Pro-IL-33 is expressed in endothelial and epithelial cells and localized in their nuclei. When the cells are seriously damaged by external stimulation such as inflammatory stimuli, the level of intracellular calcium ion is raised by an influx of extracellular ion or a release from an intracellular store. ATP may be needed to change the level of calcium ion. Excess calcium ion activates calpain, which then cleaves pro-IL-33. So far, we do not know whether pro-IL-33 cleavage occurs in the nucleus, the cytoplasm, or both.

In summary, we demonstrated that pro-IL-33 is expressed by stimulation with PMA in epithelial cells and that pro-IL-33 cleavage requires calcium-dependent calpain activity. Thus, our findings indicate that calpain is a convincing candidate to mediate pro-IL-33 processing *in vivo*. Further study should investigate the physiological stimulation by which pro-IL-33 processing is switched on.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.018.

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