ASC is an adaptor molecule that mediates apoptotic and inflammatory signals from several Apaf-1-like molecules, including CARD12/Ipaf, cryopyrin/PYPAF1, PYPAF5, PYPAF7, and NALP1. To characterize the signaling pathway mediated by ASC, we established cell lines in which muramyl dipeptide, the bacterial component recognized by another Apaf-1-like molecule, Nod2, induced an interaction between a CARD12-Nod2 chimeric protein and ASC, and elicited cell autonomous NF-κB activation. This response required caspase-8, and was suppressed by CLARP, an inhibitor of caspase-8. The catalytic activity of caspase-8 was required for the ASC-mediated NF-κB activation when caspase-8 was expressed at an endogenous level, although it was not essential when caspase-8 was overexpressed. In contrast, FADD, the adaptor protein linking Fas and caspase-8, was not required for this response. Consistently, ASC recruited caspase-8 and CLARP but not FADD and Nod2 to its speck-like aggregates in cells. Finally, muramyl dipeptide induced interleukin-8 production in MAIL8 cells. These results are the first to indicate that caspase-8 plays an important role in the ASC-mediated NF-κB activation, and that the ASC-mediated NF-κB activation actually induces physiologically relevant gene expression.

Animals and plants have several types of so-called pattern recognition receptors that recognize characteristic molecular structures of microorganisms (pathogen-associated molecular patterns (PAMPs)) and activate innate immune responses. In mammals, Toll-like receptors, homologs of Drosophila’s Toll, recognize PAMPs using their extracellular leucine-rich repeats (LRRs) and play an important role in innate immunity (1). Recently, another class of mammalian PAMPs receptors, homologous to Apaf-1 and plant disease-resistance-related NBLRR proteins, has emerged (2). More than 20 genes encoding Apaf-1-like molecules that have a central nucleotide-binding oligomerization domain (NOD) with homology to Apaf-1 and C-terminal LRRs have been discovered in the human genome. Many of them have a caspase recruitment domain (CARD) or a pyrin-like domain (PYD) at the N-terminal. Among mammalian Apaf-1-like molecules, Nod1 and Nod2 were found to recognize partial structures of bacterial peptidoglycan, namely γ-d-glutamyl-meso-diaminopimelic acid and muramyl dipeptide (MDP), respectively, using their C-terminal LRRs, and to activate NF-κB and caspase-9 through their N-terminal caspase recruitment domains (3–5). Several other members of the Apaf-1-like family induce apoptosis, NF-κB activation, and caspase-1-mediated maturation of IL-1β (6–10), suggesting that these molecules are also cytoplasmic receptors for the innate immune response, although the ligands for these molecules have not been identified. A recent report that CARD12-deficient macrophages do not produce mature IL-1β upon Salmonella typhimurium infection supports this notion (11). Because truncation of the C-terminal LRRs enhances the activity of Apaf-1-like molecules, it has been postulated that the LRRs have a negative regulatory function, and that ligand binding to this region relieves the suppression (6, 12–14).

ASC (also called TMS1) is a 21.5-kDa cytosolic protein consisting of a PYD and a CARD, and was originally identified as (a) a protein that generates speck-like aggregations in apoptotic HL-60 cells treated with chemotherapeutic agents (15) and (b) a gene product whose expression is suppressed in human breast cancers by methylation-induced gene silencing (16). Thus, ASC has been implicated in apoptosis and tumor suppression. PYD and CARD domains belong to the death domain-fold domains that are involved in homophilic protein-protein interactions. In this context, ASC resembles FADD and RAIDD, adaptor proteins involved in the signal transduction of death receptors. In fact, ASC mediates the recruitment of caspase-1 to several PYD-containing members of the Apaf-1-like family (also called the NALP or PYPAF family), and induces caspase-1-mediated IL-1β maturation (6, 8–10). ASC also couples these Apaf-1-like molecules with apoptosis and/or NF-κB activating pathways. In addition, CARD12, another Apaf-1-like molecule, requires ASC to induce apoptosis and NF-κB activation, although CARD12 induces caspase-1-mediated IL-1β processing through direct interaction with caspase-1 in the absence of ASC (7). Apoptosis induced by the expression of CARD12 plus ASC or by forced oligomerization of ASC is mediated by caspase-8 (17). In contrast, it was recently
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reported that the tumor suppressor p53 induces ASC expression, and ASC promotes the translocation of Bax to mitochondria and caspase-9-dependent apoptosis (18). Thus, ASC may activate different apoptotic signaling pathways in different contexts. On the other hand, the molecules that link ASC to the NF-κB activation pathway have not been described.

In this study, we established an experimental system in which MDP induced NF-κB activation causing IL-8 production in cells expressing a CARD12-Nod2 chimeric protein and ASC. Using this system, we have investigated the molecular mechanism of ASC-mediated NF-κB activation.

MATERIALS AND METHODS

Reagents—MDP was purchased from Sigma. Recombinant TNF-α was purchased from Genzyme (Cambridge, MA). Recombinatable soluble mouse Fas ligand was prepared as previously described (19). Z-VAD-fmk, Z-IETD-fmk, and Z-ADD-CMK were purchased from Calbiochem (La Jolla, CA). Anti-p65 mAb (clone 20) was purchased from BD Transduction (Lexington, KY). Anti-FLAG (M2) and anti-HA mAb (HA-7) were purchased from Sigma. Anti-human caspase-8 and anti-glyceraldehyde-3-phosphate dehydrogenase mAbs were purchased from MBL (Nagoya, Japan) and Chemicon (Temecula CA), respectively. Anti-ASC mAb was kindly provided by Dr. Junji Sagara (Graduate School of Medicine, Shinshu University).

Plasmids—Expression plasmids for CARD12, CARD12/ΔLRNs (aa 1–457), ASC, Nod1, Nod2, CLARP-S, CLARP-L, Fas, and IκBα (aa 1–195) were kindly provided by Dr. Ken-ichi Yamamoto (Kanazawa University, Kanazawa, Japan). A FLAG-tagged variant of CARD12 (aa 1–151), and NOD and LRRs from Nod2 (aa 217–1040) were generated in this study. A cDNA for the variant B of caspase-8 was generated from CARD12 (aa 1–151), and NOD and LRRs from Nod2 that transiently expressed C12N2 and ASC alone. Consistent with this, MDP-induced NF-κB activity in HEK293 or human chronic myelogenous leukemia K562 cells that transiently expressed C12N2 in combination with ASC (Fig. 1B). In contrast, MDP did not induce significant NF-κB activation in cells expressing C12N2 or ASC alone.

We next established stable cell lines (MAIL8) expressing FLAG-tagged C12N2 and ASC (Fig. 1, C and D) that were derived from HEK293 cells that had been stably transfected with an NF-κB-responsive GFP-expressing construct. The expression level of ASC in MAIL8 cells was comparable with the endogenous ASC expression levels in the monocyctic THP-1 and promyelocytic HL-60 cell lines. MAIL8 cells expressed GFP upon MDP stimulation (Fig. 1E). Consistent with this, MDP-induced nuclear NF-κB DNA binding activity in MAIL8 cells, as shown by electrophoretic mobility shift assay (Fig. 1F). The detected NF-κB complex contained the p65 subunit, as revealed by a supershift assay using an anti-p65 mAb. Furthermore, the proteasome inhibitor MG132 or a dominant-negative mutant of IκBα inhibited the MDP-induced NF-κB activation in MAIL8 cells (Fig. 1G). To investigate whether MDP induces an interaction between C12N2 and ASC, we performed an immunoprecipitation analysis (Fig. 1H). ASC was coprecipitated with
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Cultured as described in MDP for 16 h, and analyzed by flow cytometry.

Expression plasmid for a dominant-negative mutant of IκBα was transfected with or without an ASC expression plasmid (50 ng) in the presence of luciferase reporter genes and cultured for 24 h. HEK293 or K562 cells were transfected with an empty vector or with expression plasmids for FLAG-C12N2 (5 ng) and/or ASC (50 ng) in the presence of luciferase reporter genes. Cells were stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of the 24-h culture.

Cells were transfected with an empty vector and HEK293 cells transfected with expression plasmids for CARD12α and ASC and HEK293 cells transfected with an NF-κB-responsive luciferase gene construct (NF-κB-Luc) (Fig. 2). When the same cells were transfected with the CARD12α, ASC, and the reporter gene, NF-κB activity was detected. However, when the reporter gene and the mixture of CARD12α and ASC genes were introduced into cells separately, no NF-κB activation was observed. Thus, the NF-κB activation induced by CARD12α + ASC was cell autonomous. Similarly, MDP induced only weak NF-κB activation when MAIL8 cells transfected with an empty vector and HEK293 cells transfected with an NF-κB reporter construct were cocultured. In contrast, MDP-induced potent NF-κB activation when MAIL8 cells were transfected with the CARD12α + ASC reporter construct (Fig. 2B). These results indicated that the MDP-induced NF-κB activation in MAIL8 cells was a mainly cell-autonomous response, although upon MDP stimulation, MAIL8 cells produced some paracrine factor(s) that could induce significant NF-κB activation in HEK293 cells.

Caspase-8 Is Involved in the ASC-mediated NF-κB Activation—Others and we recently found that caspase-8 plays an essential role not only in apoptosis but also in the NF-κB activation induced by Fas ligand (21, 25). Another recent finding indicated that caspase-8 interacts with ASC and is involved in the ASC-induced apoptosis (17). These results prompted us to investigate whether caspase-8 is involved in the ASC-induced NF-κB activation. On the other hand, Nod2-induced NF-κB activation is mediated by RICK (14, 26). Therefore, we tested the effect of caspase-8- and RICK-targeting siRNAs on

![Fig. 1. MDP induces interaction between C12N2 and ASC, and elicits NF-κB activation in MAIL8 cells.](Image)

![Fig. 2. ASC-mediated NF-κB activation is cell-autonomous.](Image)
CARD12Δ + ASC- and Nod2-induced NF-κB activation. These siRNAs specifically inhibited caspase-8 and RICK expression, respectively (Fig. 3A). Furthermore, the caspase-8 targeting siRNA suppressed the expression level of endogenous caspase-8 (Fig. 3B). Importantly, the caspase-8 targeting siRNA inhibited the CARD12Δ + ASC-induced but not the Nod2-induced NF-κB activation, whereas the RICK-targeting siRNA did not (Fig. 3C). The caspase-8 targeting siRNA also inhibited the NF-κB activation induced with MDP but not TNF-α in MAIL8 cells (Fig. 3D).

To further confirm the requirement for caspase-8 in ASC-mediated NF-κB activation, we used a subline (293-K) of the HEK293 cell line, which expressed caspase-8 at a level at least 10 times lower than HEK293 cells based on Western blot analyses (Ref. 23, and Fig. 3E and data not shown). Importantly, the 293-K cells did not show NF-κB activation in response to Nod2 (100 ng/ml MDP for 16 h or with 10 ng/ml TNF-α) (Fig. 3F). When exogenous caspase-8 was expressed in 293-K cells, they became responsive to CARD12Δ + ASC expression. These results indicate that caspase-8 is required for the ASC-mediated NF-κB activation.

MDP-induced NF-κB activation in MAIL8 Cells requires the catalytic activity of caspase-8 —We next investigated whether the catalytic activity of caspase-8 is required for the ASC-mediated NF-κB activation. Pan-caspase inhibitor Z-VAD as well as a caspase-8-specific inhibitor, Z-IETD, inhibited the NF-κB activation induced by CARD12Δ + ASC but not Nod2, whereas a caspase inhibitors Z-AAD inhibited neither of these responses (Fig. 4A). Z-VAD completely inhibited the MDP- but not TNF-α-induced NF-κB activation in MAIL8 cells (Fig. 4B).
To further investigate the role of caspase-8 activity in the ASC-mediated NF-κB activation, we tested whether a catalytically inactive mutant of caspase-8 (C377S) could complement the responsiveness of 293-K cells to CARD12Δ + ASC. Surprisingly, both the wild-type and mutant caspase-8 complemented the CARD12Δ + ASC-induced NF-κB activation in 293-K cells (Fig. 4C). Furthermore, Z-VAD did not inhibit the NF-κB activation in this system (Fig. 4D). Because caspase-8 might be overexpressed by transient transfection, we then generated stable transfectants constitutively expressing the wild-type or mutant caspase-8 (Fig. 4E). The wild-type caspase-8 at levels close to normal restored the CARD12Δ + ASC-induced NF-κB activation in 293-K cells. In contrast, stable transfectants expressing caspase-8 (C377S) at normal levels exhibited no or weak NF-κB activation upon CARD12Δ + ASC expression. There was a trend that cells expressing higher levels of the mutant caspase-8 showed significant NF-κB activation in response to CARD12Δ + ASC expression, but there was no such trend in the Nod2-induced response. Consistent results were obtained when these stable transfectants were transiently transfected with expression plasmids for C12N2 and ASC and then stimulated with MDP (see Supplemental Materials Fig. S2A). As expected, Z-VAD inhibited the ASC-mediated NF-κB activation in 293-K stable transfectants expressing the wild-type caspase-8 but not the weak NF-κB activation observed in cells expressing the mutant caspase-8 (see Supplemental Materials Fig. S2, B and C). Taken together, these results suggest that the catalytic activity of caspase-8 is essential for the ASC-mediated NF-κB activation when the expression of caspase-8 is at the endogenous level in MAIL8 cells, but is dispensable when caspase-8 is overexpressed.

**FADD Is Not Required for the ASC-induced NF-κB Activation**—Because FADD plays an important role in Fas-mediated NF-κB activation (21, 25), we investigated whether FADD is also involved in the ASC-mediated NF-κB activation. A dominant-negative mutant of FADD significantly inhibited the Fas ligand-induced but not the CARD12Δ + ASC-induced NF-κB activation (Fig. 5A). Furthermore, a FADD targeting siRNA that specifically attenuated FADD expression (Fig. 5B) inhibited the Fas ligand-induced NF-κB activation in KBG cells but not the MDP-induced NF-κB activation in MAIL8 cells (Fig. 5C). Consistent with this, FADD interacted with caspase-8 but not with ASC in 293T cells, as revealed by immunoprecipitation assays (Fig. 5D). These results indicate that FADD is not involved in ASC-mediated NF-κB activation.

**CLARP Inhibits NF-κB Activation Mediated by ASC**—Because caspase-8 was involved in NF-κB activation mediated by ASC, we investigated whether CLARP, the cellular inhibitor of caspase-8, inhibits this response. CLARP is mainly expressed in two isoforms, either the short form (CLARP-S) that consists of two death effector domains or the long form (CLARP-L) that consists of the death effector domains and one caspase-like domain (27). CLARP-S inhibited NF-κB activation that was induced by CARD12Δ + ASC or a high dose of ASC alone in HEK293 cells (Fig. 6A). Western blot analysis indicated that CLARP did not suppress the expression of ASC (Fig. 6A, inset). Another Apaf-1-like molecule, cryopyrin (also called PYPAF1 or NALP3) induces NF-κB activation in an ASC-dependent manner, and deletion of the LRRs from cryopyrin (cryopyrinΔ) enhances this activity (6). CLARP-S also inhibited cryopyrinΔ + ASC-induced NF-κB activation. In contrast, CLARP-S did not inhibit NF-κB activation that was induced by the expression of Nod2, MEKK1, Bcl-10, MyD88, or stimulation with TNF-α. Induction of the nuclear NF-κB DNA-binding activity by CARD12Δ + ASC was also inhibited by CLARP-S expression (Fig. 6B). Titration of the plasmid expressing FLAG-tagged CLARP-S indicated that a small amount of CLARP-S strongly inhibited the CARD12Δ + ASC-induced NF-κB activation (Fig. 6C). Transfection of plasmid expressing CLARP-L also inhibited the CARD12Δ + ASC-induced NF-κB activation, but less efficiently than CLARP-S (Fig. 6D). Consistent with previous reports, a high dose of CLARP induced NF-κB activation by itself (28).

Because pyrin, the product of the causative gene for familial Mediterranean fever (FMF), also inhibits ASC-mediated NF-κB activation (17), we compared the efficacy of the inhibitory activity of pyrin and CLARP-S on the CARD12Δ + ASC-induced NF-κB activation. Whereas 10 ng of plasmid expressing CLARP-S/FLAG completely inhibited CARD12Δ + ASC-induced NF-κB activation, 100 ng of FLAG-pyrin-expressing
plasmid inhibited it only partially in HEK293 cells (Fig. 6E). Immunoprecipitation of the FLAG-tagged proteins followed by Western blotting using an anti-FLAG mAb indicated that the amount of CLARP-S-FLAG was much lower than the amounts of FLAG-pyrin under these experimental conditions (Fig. 6F), indicating that CLARP-S inhibited the ASC-mediated NF-κB activation more efficiently than pyrin.

CLARP-S also inhibited MDP-induced NF-κB activation in MAIL8 cells in a dose-dependent manner (Fig. 6G), although a larger amount of CLARP-S expression plasmid was required in this experiment to increase the transfection efficiency. Considering that the transfection efficiency was about 70% in control experiments using GFP-expressing plasmids, the inhibition of MDP-induced NF-κB activation by CLARP-S was almost complete. As reported previously (6, 7), the expression of CARD12Δ (without ASC), a large amount of ASC (without CARD12Δ), or cryopyrinΔ + ASC induced caspase-1-mediated IL-1β expression; however, both CLARP-S and -L failed to inhibit caspase-1-mediated IL-1β secretion under these conditions (see Supplemental Materials, Fig. S3), cryopyrinΔ + ASC induced caspase-1-mediated IL-1β expression; however, both CLARP-S and -L failed to inhibit caspase-1-mediated IL-1β secretion under these conditions (see Supplemental Materials, Fig. S3).

Caspase-8 and CLARP Co-localize with ASC Specks—To investigate the intracellular localization of ASC, caspase-8, and CLARP, COST cells transiently expressing these proteins were stained with fluorescent antibodies and examined under a confocal laser-scanning microscope. When ASC was transiently expressed in HEK293 cells, it formed specks (Fig. 7A). In contrast, caspase-8 and CLARP were distributed throughout the cytoplasm under similar conditions (Fig. 7, B and D). However, when caspase-8, CLARP, FADD, or Nod2 was co-expressed with ASC, caspase-8 and CLARP colocalized with the ASC specks, whereas FADD and Nod2 were distributed throughout the cytoplasm and did not colocalize with ASC (Fig. 7, C and E–G). Importantly, when ASC, caspase-8, and CLARP (either S or L) were expressed together, they all colocalized as specks (Fig. 7, H and I).

We next examined the localization of ASC and caspase-8 in MAIL8 cells before and after MDP stimulation. ASC generated specks in MAIL8 cells upon MDP stimulation (Fig. 7, J and K). Under these conditions, endogenous caspase-8 colocalized with ASC in specks. Endogenous CLARP was not detectable using an anti-CLARP mAb in these experiments (data not shown). These results indicate that caspase-8 is recruited to ASC when ASC is aggregated. Our results also indicate that although both caspase-8 and CLARP interact with ASC, CLARP does not interfere with the interaction between ASC and caspase-8.

**MDP Induces NF-κB-dependent IL-8 Production in MAIL8 Cells**—The biological significance of the ASC-mediated NF-κB activation had not yet been shown. Therefore, we investigated whether MDP induces the production of IL-8, an NF-κB-responsive gene product, in MAIL8 cells. We found that MDP induced potent IL-8 production (Fig. 8A). Both the proteasome inhibitor MG132 and a dominant-negative mutant of IκBκ that inhibited NF-κB activation suppressed the IL-8 production (Fig. 8, B and C). Z-VAD and CLARP-S also inhibited MDP- but not TNF-α-induced IL-8 production in MAIL8 cells (Fig. 8, D and E). These results indicate that MDP-induced ASC-mediated NF-κB activation led to IL-8 production in MAIL8 cells. This is the first demonstration that NF-κB activated by ASC induces biologically significant gene expression.

**DISCUSSION**

ASC is an adaptor protein that mediates apoptotic and inflammatory signals from several Apaf-1-like molecules including CARD12, cryopyrin, PYPAF5, PYPAF7, and NALP1 (6–10).
ASC and FLAG-Nod2; caspase-8-C377S-HA (50 ng). Cells were fixed 24 h later, and proteins nanograms of any given plasmid were added to a single well, except for I FLAG; S-FLAG; E.

Using Lipofectamine and Plus reagents (Invitrogen) as follows: 6-well plates, and transfected with one or more expression plasmids

These Apaf-1-like molecules are likely to be cytoplasmic receptors for PAMPs, because of their structural similarity to Nod1, Nod2, and plant NBS-LRR proteins. However, the lack of information regarding the PAMPs recognized by CARD12, cryopyrin, PYPAF5, PYPAF7, and NALP1 has precluded us from investigating the functions of these Apaf-1-like molecules or of ASC under physiological conditions. To overcome this problem, in this study, we established a novel experimental system in which the C12N2 chimeric protein transduces the MDP signal through ASC to activate NF-κB and eventually induces IL-8 production. A similar system is applicable for other Apaf-1-like molecules, because a chimeric protein consisting of the PYD from cryopyrin and the NOD and LRRs from Nod2 worked just like C12N2.2

In Drosophila, DREDD, the homolog of caspase-8, plays an essential role in the activation of Relish, a homolog of NF-κB (29). The DREDD-Relish pathway plays an important role in the activation of the innate immune response to Gram-negative bacteria and is mediated by peptidoglycan recognition protein-LC (30). In mammalian cells, overexpression of caspase-8 induces NF-κB activation (28, 31). Furthermore, others and we recently demonstrated that endogenous caspase-8 plays an important role in Fas ligand-induced NF-κB activation and IL-8 production (21, 25). Because Apaf-1-like molecules and ASC have been implicated in the innate immune response, our observation may be the first evidence that caspase-8 plays a role in the mammalian innate immune system. In other words, it is possible that caspase-8 plays an important role in a novel NF-κB activation pathway that is evolutionally conserved.

It has been reported that the catalytic activity of caspase-8 is dispensable for the NF-κB activation induced by caspase-8 overexpression (28, 31). However, we recently found that the Fas ligand-induced NF-κB activation is partly dependent on and partly independent of the catalytic activity of caspase-8 (21). Here, we showed that pan-caspase inhibitor Z-VAD and caspase-8 inhibitor Z-IETD inhibited the MDP-induced NF-κB activation in MAIL8 cells (Fig. 4, A and B). Further study involving caspase-8-defective 293-K cells reconstituted with either the wild-type or an enzymatically inactive caspase-8 suggested that the caspase activity is indispensable for the ASC-mediated NF-κB when the caspase-8 expression level is low, but it is dispensable when caspase-8 is overexpressed (Fig. 4, C–E). Interestingly, DREDD directly cleaves Relish (29). In mammals, caspases cleave several molecules involved in the NF-κB activation pathway including NF-κB (23). However, CLARP-L inhibited caspase-8 activation induced by caspase-8 (32–35). The N-terminal fragment of CLARP-L processed by caspase-8 has been shown to induce NF-κB activation (23). However, CLARP-L inhibited rather than promoted ASC-mediated NF-κB activation in our system. Thus, the caspase-8 substrate that plays an important role in the ASC-mediated NF-κB activation remains to be determined.

CIAS1 (the gene for cryopyrin) and MEFV (the gene for pyrin) are causative genes for the cold-induced autoinflammatory syndrome and FMF, respectively (36–38). Pyrin inhibits caspase activity and NF-κB production (21, 25). Because Apaf-1-like molecules and ASC have been implicated in the innate immune response, our observation may be the first evidence that caspase-8 plays a role in the mammalian innate immune system. In other words, it is possible that caspase-8 plays an important role in a novel NF-κB activation pathway that is evolutionally conserved.

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CIAS1 (the gene for cryopyrin) and MEFV (the gene for pyrin) are causative genes for the cold-induced autoinflammatory syndrome and FMF, respectively (36–38). Pyrin inhibits caspase-mediated apoptosis and NF-κB activation, and competes

Fig. 7. Caspase-8 and CLARP but not FADD or Nod2 co-localize with ASC. A–J, COS7 cells were cultured on glass coverslips in 6-well plates, and transfected with one or more expression plasmids using Lipofectamine and Plus reagents (Invitrogen) as follows: A, ASC; B, caspase-8-C377S-HA; C, ASC and caspase-8-C377S-HA; D, CLARP-S-FLAG; E, ASC and CLARP-S-FLAG; F, ASC and FLAG-FADD; G, ASC and FLAG-Nod2; H, ASC, caspase-8-C377S-HA and CLARP-S-FLAG; I, ASC, caspase-8-C377S-HA and CLARP-L-FLAG. Five hundred nanograms of any given plasmid were added to a single well, except for caspase-8-C377S-HA (50 ng). Cells were fixed 24 h later, and proteins were detected using anti-ASC (red), anti-caspase-8 (blue), and anti-FLAG (green) mAb. J and K, MAIL8-3 cells were cultured on glass coverslips in 6-well plates, and left untreated in J, or stimulated with 10 ng/ml MDP in K, for 16 h. ASC and endogenous caspase-8 were detected using anti-ASC (red) and anti-caspase-8 (blue) mAb. The left panels show merged images of the fluorescence and transmitted light images. Bar: 50 μm for A–E, H, J, and K; 70 μm for F, G, and I.

2 M. Hasegawa and T. Suda, unpublished observation.
mediated NF-κB and inhibit the Fas ligand-induced proteolytic maturation of caspase-8. Because CLARP was reported to inhibit the interaction between ASC and caspase-8, in this study, we demonstrated that CLARP-S inhibited the ASC-mediated NF-κB activation more efficiently than did pyrin (Fig. 6, E and F). CLARP-S and -L co-localized with caspase-8 at ASC specks (Fig. 7, H and I). Thus, unlike pyrin, CLARP does not inhibit the interaction between ASC and caspase-8. Because CLARP was reported to inhibit the Fas ligand-induced proteolytic maturation of caspase-8 (39), it is possible that CLARP inhibited the ASC-mediated NF-κB activation by inhibiting the processing of caspase-8. However, it was also reported that dimerization of pro-caspase-8 without proteolytic processing is sufficient to gain its caspase activity (40). If this is true for the ASC-mediated activation of caspase-8, CLARP might inhibit the formation of enzymatically active caspase-8 dimers. In addition, we previously demonstrated that CLARP inhibits both caspase activity-dependent and -independent pathways of Fas ligand-induced NF-κB activation (21). As described above, the catalytic activity of caspase-8 was not essential for the ASC-mediated NF-κB activation when caspase-8 was overexpressed. In such a case, CLARP might inhibit interaction between caspase-8 and a downstream signaling molecule for NF-κB. In any case, CLARP-S is a strong inhibitor of ASC-mediated NF-κB activation. These findings may have important implications for the molecular pathology of and/or therapeutic strategies for cold-induced autoinflammatory syndrome, FMF, and other related genetic diseases.

MDP induced IL-8 production in MAIL8 cells (Fig. 8). This is the first demonstration that ASC-mediated NF-κB activation causes physiological gene expression. One of the most striking features of MDP attack is the massive infiltration of neutrophils into the site of inflammation. It has been suggested that ASC is activated in FMF patients because of a mutation in the pyrin gene (41, 42). Because IL-8 is a chemotactic factor for neutrophils, the finding that the activation of ASC induces IL-8 production may be important for understanding the molecular mechanism of FMF pathogenesis.

In this study, we mainly focused on ASC-mediated NF-κB activation, because our HEK293 cell line, and most HEK293-derived cell lines, does not exhibit significant apoptosis upon ASC expression. However, MDP induces both NF-κB activation and apoptosis in a subline of 293T cells transiently expressing C12N2 and ASC (2), indicating that NF-κB activation and apoptosis can occur simultaneously or sequentially upon ASC activation. Furthermore, some subclones of the MAIL8 cell line showed apoptotic morphology upon MDP stimulation (2). Using these experimental systems, we are currently investigating the molecular mechanism of the apoptosis mediated by Apaf-1-like proteins and ASC. These studies will also produce important knowledge for the physiological and pathological roles of ASC.

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