Interaction Patches of Procaspase-1 Caspase Recruitment Domains (CARDs) Are Differently Involved in Procaspase-1 Activation and Receptor-interacting Protein 2 (RIP2)-dependent Nuclear Factor κB Signaling

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Background: The CARD of procaspase-1 regulates activation of procaspase-1 and NF-κB. Results: We show that the CARD procaspase-1 interacts with ASC through a type I interaction, whereas RIP2-mediated NF-κB activation involves a type III interaction.

Conclusion: These findings indicate that the CARD of procaspase-1 is differently involved in inflammasome formation and NF-κB activation.

Significance: These findings may allow development of therapeutics that specifically target a procaspase-1 function.

Protein interaction domains belonging to the death domain-fold superfamily are six-helix bundles that mediate the assembly of large protein complexes involved in apoptotic and inflammatory signaling. Typically, death domains (DDs), a subfamily of the death domain-fold superfamily, harbor six delineated interaction patches on their surfaces that mediate three distinct and conserved types of interaction designated as types I, II, and III. Here, we show that caspase recruitment domains (CARDs), another subfamily of the death domain-fold superfamily, multimerize by employing at least two of the three reported interaction types that were identified in DDs. On the one hand, the CARD of procaspase-1 binds the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) through a type I interaction that involves a patch surrounding residue Asp-27. On the other hand, the CARD of procaspase-1 auto-oligomerizes through a type III interaction involving a patch surrounding residue Arg-45. This oligomerization allows binding of receptor-interacting protein 2 (RIP2). In addition, we show that a 1:1 interaction between ASC and procaspase-1 is sufficient for procaspase-1 to gain proteolytic activity, whereas the formation of a higher order CARD complex involving ASC, procaspase-1, and RIP2 is required for effective procaspase-1-mediated NF-κB activation. These findings indicate that the CARD of procaspase-1 is differently involved in the formation of procaspase-1 activating platforms and procaspase-1-mediated, RIP2-dependent NF-κB activation.

The death domain-fold superfamily is composed of four subfamilies: the death domains (DDs), the death effector domains, the caspase recruitment domains (CARDs), and the pyrin domains (PYDs) (1, 2). These domains are homotypic interaction modules of about 90 amino acids that enable the formation of higher order multimeric complexes, which contribute to cell death and inflammation by activating caspases or protein kinases, the latter of which are predominantly involved in NF-κB signaling pathways (1–4). Despite a very low degree of sequence homology between these domains, their structure is conserved. Six amphipathic α-helices fold into an antiparallel α-helical bundle with Greek key topology to form the designated death domain-fold (5–8). The DD surface was shown to harbor six delineated interaction patches that contribute to the assembly of protein complexes by mediating three conserved interaction types. Type I interactions involve patches Ia and Ib, type II interactions make use of patches IIA and IIB, and type III interactions involve patches IIIa and IIIb (9–11). Whether these interaction types mediate homotypic interactions of other death domain-fold subfamilies, such as CARD–CARD interactions, is currently not known.

The abbreviations used are: DD, death domain; ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase recruitment domain; PYD, pyrin domain; RIP2, receptor-interacting protein 2.
The CARD of procaspase-1 is well suited for analyzing how CARD-containing proteins use these three interaction types. Indeed, procaspase-1 plays two crucial and distinct roles in the innate immune response by engaging different CARD-CARD interacting partners. First, procaspase-1 gains proteolytic activity when it is recruited to the inflammasomes through its CARD (12). The inflammasomes are a group of large multimeric intracellular protein complexes that assemble in response to a wide range of stimuli and confer on procaspase-1 the ability to convert the proforms of interleukin (IL)-1β and IL-18 into bioactive inflammatory mediators (13–17). Proteolytically active caspase-1 also induces an inflammatory type of cell death, called pyroptosis, as a way to eradicate particular intracellular pathogens (18, 19). Depending on the molecular composition of the inflammasome, procaspase-1 is either recruited to the complex by the PYD- and CARD-containing adaptor ASC (PYCARD) or directly by a CARD-containing inflammasome sensor protein, such as NLRC4 (20). Second, we previously reported that the CARD of procaspase-1 can mediate NF-κB activation by recruiting the serine/threonine kinase RIP2 through a CARD-CARD interaction (21). However, it is not known whether the CARD of procaspase-1 uses different interaction types and patches to perform each function.

The human genome encodes three CARD-only proteins (COP, INCA, and ICEBERG) at the caspase-1 locus, which arose from evolutionarily recent duplications of procaspase-1 (22, 23). These three proteins have a high degree of sequence identity with the CARD of procaspase-1 (92, 81, and 53%, respectively). Interestingly, although these procaspase-1-related CARD-only proteins are all negative feedback regulators of caspase-1 activity, only COP has retained the ability to interact with RIP2 and to mediate NF-κB signaling (24–27). These observations indicate that INCA and ICEBERG acquired during evolution additional missense mutations that specifically affect their ability to activate NF-κB and that a few residues within these CARDS determine functionality. Therefore, targeted mutagenesis could be used to identify which residues in the CARD of procaspase-1 are critical for its inflammasome-mediated proteolytic activation and for the RIP2-dependent NF-κB activation.

In this study, we show that the CARD of procaspase-1 can engage at least two of the three designated DD interaction types. Using a targeted mutagenesis approach, we demonstrate that two residues in the CARD of procaspase-1, Asp-27 and Arg-45, are essential for engagement of ASC and auto-oligomerization of the procaspase-1 CARD, respectively. Asp-27 and Arg-45 form an integral part of patches Ib and IIIa, and this classifies their interactions as type I and type III, respectively. Arg-45-mediated procaspase-1 CARD auto-oligomerization is a prerequisite for RIP2 binding, whereas Asp-27-mediated recruitment of the inflammasome adapter ASC has a strong synergistic effect on procaspase-1-mediated RIP2-dependent NF-κB activation. Furthermore, we report that the proteolytic activation of procaspase-1 relies only on a type I interaction between the CARDS of procaspase-1 and ASC and that auto-oligomerization of the procaspase-1 CARD is dispensable for this function.

EXPERIMENTAL PROCEDURES

Plasmids—The PCR-generated cDNA encoding human full-length procaspase-1 was cloned in-frame with an N-terminal FLAG tag epitope in the pCAGGS vector (pCAGGS-FLAG-procaspase-1 WT). The enzymatically inactive human procaspase-1 C285A mutant was made by site-directed mutagenesis PCR and cloned in-frame with a FLAG tag epitope in pCAGGS (pCAGGS-FLAG-procaspase-1 C285A). The CARD and the catalytic domain of human procaspase-1 were amplified from full-length human procaspase-1 by conventional PCR and cloned in-frame with a FLAG tag epitope in pCAGGS (pCAGGS-FLAG-caspase-1 CARD-only and pCAGGS-FLAG-caspase-1 p30). Plasmid pCAGGS-E-caspase-1 CARD-only has been described (21). D27G and R45D mutations were introduced in human procaspase-1 by site-directed mutagenesis PCR in each of the following constructs: pCAGGS-FLAG-procaspase-1 WT, pCAGGS-FLAG-procaspase-1 C285A, pCAGGS-FLAG-caspase-1 CARD-only, and pCAGGS-E-caspase-1 CARD-only. All PCR products were sequenced to ensure that no errors had been introduced.

We made pCAGGS-E-ASC by cloning the human ASC sequence from pCR3.1-FLAG-ASC (produced in the laboratory of Prof. J. Tschopp; University of Lausanne, Epalinges, Switzerland) in-frame with an N-terminal E-tag epitope in the pCAGGS vector. pCR3- VSV-RIP2 and pβact-βgal (encoding β-galactosidase driven by the β-actin promoter) were also kindly provided by Prof. J. Tschopp. pCR3-VSV-RIP2 has been described (28). pNF-ConLuc, encoding the luciferase reporter gene driven by a minimal NF-κB-responsive promoter, was a gift from Prof. A. Israël (Institut Pasteur, Paris, France). pCAGGS-pro-IL-1β has been described (29).

Antibodies and Reagents—The monoclonal RIP2 antibody was obtained from Enzo Life Sciences (Farmingham, NY, catalog no. 804-139). The monoclonal antibody against actin was purchased from Sigma-Aldrich, whereas the E-HRP antibody was obtained from Sigma-Aldrich, whereas the E-HRP antibody was obtained from GE Healthcare (catalog no. 27-9413-01, discontinued). The antibody recognizing the pro and mature forms of IL-1β was obtained from R&D Systems (catalog no. AF-401-NA). Secondary antibodies were purchased from GE Healthcare, except for the anti-goat antibody, which was from Santa Cruz Biotechnology (Santa Cruz, CA; catalog no. sc-2020). siRNA directed against human RIP2 was obtained from Ambion (Foster City, CA; catalog no. AM51331, siRNA ID no. 456, GGAGAAGAAUUUGCCAAAGGtt). As a negative control, we used siCONTROL non-targeting siRNA from Dharmacon (Thermo Fisher Scientific; catalog no. D-001810-10-20). Recombinant human TNF was produced in Escherichia coli in our laboratory and purified to at least 99% homogeneity. Its specific biological activity was 2.58 × 10⁷ units/mg of purified protein, as determined with the international standard code 87/650 (National Institute for Biological Standards and Controls, Potters Bar, UK).

Cell Culture—Human embryonic kidney cells (HEK293T) were maintained in 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf
serum, 2 mM L-glutamine, 0.4 mM sodium pyruvate, and antibiotics. 20 nM siRNA and the indicated amounts of plasmid DNA were transfected by calcium phosphate precipitation. siRNA transfection was performed 1 day before plasmid DNA transfection.

**NF-κB Reporter Assay**—HEK293T cells in 24-well plates were transfected with 5 or 50 ng of the indicated expression vectors in combination with 20 ng of pNF-ConLuc and 20 ng pβact-βgal. Total DNA was equalized with control plasmid. In some experiments, cells were treated with 500 units/ml TNF 6 h before harvesting. Twenty-four hours after transfection, cells were collected, washed in PBS, and lysed in 80 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-cyclohexanediaminetetraacetic acid, 10% glycerol, and 1% Triton X-100). After combining 20 μl of lystate with 40 μl of substrate buffer (658 μM luciferin, 378 μM coenzyme A, and 742 μM ATP), NF-κB activity was quantified with the GloMax™ 96 Plate Luminometer (Promega, Madison, WI). β-Galactosidase activity in cell lysates was assayed by colorimetry to normalize for transfection efficiency. In brief, 20 μl of cell lysate was incubated for 5 min at room temperature with 200 μl of a solution containing 5 mM chlorophenol red-β-β-galactosidase (Roche Applied Science; catalog no. 884308), 10 mM KCl, 1 mM β-mercaptoethanol, and 60 mM NaHPO₄ pH 7.0. Absorbance was measured at 595 nm. Results are expressed as relative luciferase units/second per optical density of β-galactosidase activity.

**Immunoprecipitation and Immunoblotting Assays**—For the co-immunoprecipitation assays, HEK293T cells in 10-cm dishes were transfected with 1 μg of the indicated plasmids. They were lysed in Nonidet P-40 lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 1% Nonidet P-40) supplemented with a protease inhibitor tablet (Roche Applied Science; catalog no. 11-873-580). Clarified lysates were immunoprecipitated by using specific antibodies in combination with protein G-Sepharose beads (GE Healthcare; catalog no. 17-0618-01) or FLAG antibody-coupled beads (Sigma-Aldrich; catalog no. A2220). Immune complexes and total lysates were analyzed by SDS-PAGE and immunoblotting.

**Molecular Modeling**—The procaspase-1 CARD was modeled on the Apaf-1 CARD (Protein Data Bank ID code 1CY5) with the FoldX plugin in PyMOL (34). The electrostatic potential of the proteins was mapped on their individual solvent-accessible surfaces using the APBS plug-in in PyMOL (33). Structures were aligned with the CEAlign plug-in in PyMOL (34).

**RESULTS**

**Asp-27 and Arg-45, Which Are Located in Differently Charged Surface Patches on the CARD of Procaspase-1, Are Crucial for Procaspase-1-mediated NF-κB Activation**—The only crystal structure of a CARD-CARD interaction reported so far revealed that the CARDs of Apaf-1 and procaspase-9 interact through surface patches complementary in charge and shape (35). The acidic, convex surface patch of the Apaf-1 CARD recognizes the basic, concave surface patch of the procaspase-9 CARD (35). We found that the CARD of procaspase-1 also harbors two differently charged surface patches on opposite sides (Fig. 1A). To study the contribution of each surface patch of procaspase-1 to procaspase-1-mediated NF-κB activation, we performed a mutation-based structure-function analysis in which a single point mutation was introduced at the differently charged procaspase-1 CARD surface patches. The aspartic acid at position 27 (Asp-27) was selected for the acidic patch of procaspase-1 CARD because the crystal structure of the interaction between Apaf-1 and procaspase-9 revealed that this residue forms an integral part of the acidic interaction patch of Apaf-1 (Fig. 1A) (35). For the basic patch, on the other hand, the arginine at position 45 (Arg-45) was selected empirically (Fig. 1A). To mimic the amino acid sequences of INCA and ICEBERG, the two procaspase-1-related CARD-only proteins that fail to activate NF-κB (24–26), we mutated Asp-27 and Arg-45 into a glycine (D27G) and an aspartic acid (R45D), respectively (Fig. 1B). Using NF-κB reporter assays, we then compared the ability of the mutants to activate NF-κB when they are ectopically expressed in HEK293 cells. We found that both mutations abrogated the NF-κB-activating potential when full-length procaspase-1 or the CARD-only portion of the protein (caspase-1 CARD-only) was expressed (Fig. 1C). These results indicate that effective procaspase-1-mediated NF-κB signaling requires CARD-CARD interactions involving the two differently charged surface patches on opposite sides of the CARD of procaspase-1.

**Residue Arg-45 in the Basic Patch of Procaspase-1 CARD Is Important for Auto-oligomerization of Procaspase-1 CARD and Recruitment of RIP2**—To characterize better the nature of the CARD-CARD interactions that contribute to procaspase-1-mediated NF-κB signaling, we performed co-immunoprecipitation experiments in HEK293T cells ectopically expressing the caspase-1 CARD-only or one of the mutants. Arg-45 in the basic surface patch of procaspase-1 CARD seems to be essential for recruitment of the downstream NF-κB mediator RIP2 because the caspase-1 CARD-only R45D mutant failed to interact with RIP2 (Fig. 2A). In contrast, Asp-27 in the acidic surface patch of procaspase-1 CARD does not seem to be required for RIP2 recruitment (Fig. 2A).

Several CARD-containing proteins undergo CARD-dependent auto-oligomerization to recruit downstream effectors and activate signaling pathways (36–38). To examine whether auto-oligomerization of procaspase-1 CARD is necessary for NF-κB activation, we analyzed the ability of caspase-1 CARD-only mutants to form auto-oligomers and compared these results with their ability to recruit RIP2. In agreement with a critical role for Arg-45 in RIP2 recruitment, mutation of this residue precluded procaspase-1 CARD auto-oligomerization (Fig. 2B). Conversely, mutation of Asp-27, located in the acidic patch, did not hamper procaspase-1 CARD auto-oligomerization (Fig. 2B) or RIP2 recruitment. Together, these results indicate that procaspase-1 CARD auto-oligomerization correlates with RIP2 recruitment and that these processes rely on residue Arg-45. However, the observation that Asp-27 and Arg-45 of procaspase-1 CARD are both necessary for efficient NF-κB activation points to a model in which procaspase-1 CARD needs to engage CARD-CARD interactions at opposite sides of its surface to induce NF-κB activation. Although CARD-CARD...
Interactions involving Arg-45 of procaspase-1 CARD allow assembly of procaspase-1 oligomers that most likely enable recruitment of RIP2, the resultant protein complex is incapable of triggering NF-κB signaling. In addition, the Asp-27-containing surface patch of procaspase-1 CARD has to recruit other unknown proteins to the complex.

ASC Potentiates Procaspase-1-mediated NF-κB Activation by Docking at the Asp-27-containing Patch of Procaspase-1 CARD—The bipartite PYD-CARD adaptor protein, ASC, is a recognized interaction partner of procaspase-1 CARD that enables recruitment of procaspase-1 to ASC-containing inflammasomes to allow procaspase-1 activation (20, 39). Because the involvement of ASC in NF-κB activation is still debated, we assessed the effect of ASC on procaspase-1-mediated NF-κB activation. To this end, we ectopically expressed in HEK293T cells modest amounts of full-length procaspase-1 or the isolated CARD of procaspase-1 in the absence or presence of ASC and monitored NF-κB activity by using an NF-κB-dependent reporter system. Addition of ASC resulted in a 10-fold increase in NF-κB activity compared with expression of procaspase-1, the isolated CARD of procaspase-1, or ASC alone (Fig. 3A and supplemental Fig. 1A). In agreement with experiments utilizing dominant negative RIP2 mutants (21), transcriptional down-regulation of RIP2 with specific siRNA oligonucleotides selectively abrogated procaspase-1-mediated NF-κB activation (Fig. 3A and supplemental Fig. 1A). Importantly, RIP2 repression had no
Differential Signaling by Procaspase-1–CARD Patches

**FIGURE 2.** Arg-45-containing surface patch of procaspase-1 CARD is involved in auto-oligomerization and RIP2 binding. *A*, HEK293T cells were transiently transfected with plasmids encoding VSV-RIP2 and CARD mutants of FLAG-caspase-1 CARD-only, as indicated. After 24 h, cells were lysed, and the caspase-1 CARD-only domain mutants were immunoprecipitated with FLAG-antibody coupled to beads. Co-immunoprecipitated RIP2 was detected by immunoblotting with a VSV tag antibody. Data are representative of at least three independent experiments. *B*, HEK293T cells were transiently transfected with plasmids encoding FLAG-caspase-1 CARD-only mutants and E-caspase-1 CARD-only mutants, as indicated. After 24 h, cells were lysed, and co-immunoprecipitation assays were performed in both directions. Data are representative of two independent experiments.

...effect on the modest NF-κB induction obtained when expressing ASC alone, but it completely inhibited the synergistic effect of ASC on procaspase-1-mediated NF-κB activation (Fig. 3A and supplemental Fig. 1A). The specificity of these effects is illustrated by the observation that TNF-induced NF-κB activation, which proceeds independently of RIP2 (40), was unaffected by either RIP2 knockdown or ASC co-expression (Fig. 3A and supplemental Fig. 1A). Collectively, these results indicate that ASC has a strong synergistic effect on procaspase-1-mediated, RIP2-dependent NF-κB activation and suggest that the CARD of procaspase-1 functions as a bridging moiety that enables recruitment of RIP2 to ASC-containing protein complexes to allow NF-κB signaling.

Analysis of the procaspase-1 CARD mutants in this ASC synergism model revealed that the defect in NF-κB activation by the Arg-45 mutant can be partially rescued by ASC co-expression (Fig. 3B and supplemental Fig. 1B). However, NF-κB activation was not induced by the Asp-27 mutant despite co-expression of ASC (Fig. 3B and supplemental Fig. 1B). These observations correlate with the ability of the procaspase-1 CARD mutants to interact with ASC. Whereas residue Asp-27 in the CARD of procaspase-1 appears to be indispensable for ASC binding, residue Arg-45 does not seem to be crucial because its mutation reduced but did not prevent the ability of procaspase-1 to co-immunoprecipitate with ASC (Fig. 3C). As a result, ASC co-expression only partially restored the NF-κB signaling capacity in the case of Arg-45 mutation. Remarkably, this restoration of NF-κB activation was blocked by depletion of RIP2 (Fig. 3B and supplemental Fig. 1B), suggesting that ASC co-expression enables RIP2 to bind to the complex, even though our results show that residue Arg-45 in the CARD of procaspase-1 is important for RIP2 binding (Fig. 2A). By these results, we indicate that residue Asp-27 of the procaspase-1 CARD forms an essential component of the ASC docking site.

Asp-27-mediated ASC Binding Is Crucial for the Proteolytic Activation of Procaspase-1—Having identified two residues, Asp-27 and Arg-45, located on opposite sides of the procaspase-1 CARD as being critical for procaspase-1-mediated NF-κB activation, we next assessed their contribution to induction of procaspase-1 activation and maturation of IL-1β, the well recognized caspase-1 function. For this purpose, we developed a system that adequately circumvents two important technical issues: expressing large amounts of wild-type procaspase-1 is cytotoxic, and it induces procaspase-1 activation regardless of its CARD because a CARDless caspase-1 variant also attains proteolytic activity when it is overexpressed in large amounts (data not shown). Therefore, we used an intricate cellular system in which minute amounts of procaspase-1 gain proteolytic activity only when expressed in the presence of ASC (Fig. 4). As a read-out to monitor caspase-1 activity, we followed the conversion of pro-IL-1β into mature IL-1β and found that mutating Asp-27 of procaspase-1 precluded activation of procaspase-1 (Fig. 4). These findings are in line with the ability of the procaspase-1 CARD mutants to interact with ASC. Indeed, mutating Arg-45 in the CARD of procaspase-1 had only a minor effect on ASC recruitment (Fig. 3C), and consequently, it did not affect the proteolytic activation of procaspase-1 (Fig. 4). These data suggest that procaspase-1 is recruited by ASC into ASC-containing inflammasomes through CARD-CARD interactions involving a surface patch that contains Asp-27 and is located on the acidic surface region of procaspase-1 CARD. Notably, auto-oligomerization of procaspase-1 CARD, mediated by an Arg-45-containing surface patch on the basic surface region, does not appear to be required to gain proteolytic activity. The inability of a CARDless caspase-1 variant (caspase-1 p30) to induce proteolytic maturation of IL-1β further supported the validity of this system (Fig. 4).

Recruitment of Procaspase-1 by ASC and Procaspase-1 CARD Auto-oligomerization Comprise Type I and Type III Interactions, Respectively.—Finally, we determined whether the CARD-CARD interactions of procaspase-1 comply with the death-domain fold interaction types characterized for DDs (9–11, 41). More precisely, can they be categorized as type I, II, or III interactions. Superposition of the six defined interaction patches of RAIDD DD, previously identified by x-ray crystallography (10), onto the procaspase-1 CARD revealed that Asp-27 and Arg-45 reside in patches Ib and IIIa, respectively (Fig. 5). This finding implies that Asp-27-dependent ASC binding involves a type I interaction and that Arg-45-dependent procaspase-1 CARD auto-oligomerization involves a type III interaction.
DISCUSSION

The contribution of procaspase-1 to host-mediated inflammatory responses depends on two traits. First, there is the well-established proteolytic activity which enables IL-1β and IL-18 processing, and leads to pyroptosis (12, 18). Second, overexpression studies also indicate that procaspase-1, as is the case for caspase-8 and caspase-2, may function as a scaffold and is implicated in the activation of a specific NF-κB signaling pathway (21). Although the CARD of procaspase-1 is crucial for the homotypic interactions in the execution of both functions, little is known on the precise interaction mechanisms that form the basis of these two distinct functions.

Accumulating evidence indicates that DDs, another subfamily of the death domain-fold superfamily of homotypic interaction modules, can engage up to six interaction partners through six different surface patches to mediate three distinct interaction types (9–11, 41). Here, we demonstrate that the interaction mechanisms used by the CARD of procaspase-1 fits with the model reported for DDs. Our mutagenesis-based study, initially designed in accordance to the crystal structure of the

![Graphs and images related to the discussion]

FIGURE 3. Procaspase-1-mediated NF-κB activation is potentiated by ASC, which binds to the Asp-27-containing surface patch of procaspase-1 CARD. A, HEK293T cells were transfected with control or RIP2 siRNA and then with 5 ng of plasmid DNA encoding FLAG-procaspase-1 C285A, FLAG-caspase-1 CARD-only, or E-ASC in combination with the NF-κB-dependent luciferase reporter plasmid. One setup encompassed TNF stimulation as a control. Cells were lysed 24 h after the second transfection, and NF-κB activity was measured. See supplemental Fig. 1A for Western blot analysis of this experiment. Data are representative of at least five independent experiments. B, HEK293T cells were transfected with control or RIP2 siRNA and then with a plasmid encoding the NF-κB-dependent luciferase reporter and 5 ng of plasmids encoding E-ASC, and CARD mutants of FLAG-procaspase-1 C285A, and FLAG-caspase-1 CARD-only, as indicated. One setup encompassed TNF stimulation. Cells were lysed 24 h after the second transfection, and NF-κB activity was measured. See supplemental Fig. 1B for Western blot analysis of this experiment. Data are representative of at least five independent experiments. C, HEK293T cells were transiently transfected with plasmids encoding E-ASC, and CARD mutants of FLAG-procaspase-1 C285A and FLAG-caspase-1 CARD-only, as indicated. After 24 h, cells were lysed, and the procaspase-1 C285A and caspase-1 CARD-only mutants were immunoprecipitated with α-FLAG-antibody coupled to beads. Co-immunoprecipitated ASC was detected by immunoblotting with an α-E-tag antibody. Data are representative of two independent experiments.
charge-dependent CARD-CARD interaction between Apaf-1 and procaspase-9 (35), revealed that two important residues in the procaspase-1 CARD, Asp-27 and Arg-45, localize at the center of patch Ib and IIIa and engage type I and type III interactions, respectively. Functionally, the type I interaction enables recruitment of procaspase-1 by the inflammasome adaptor protein ASC, whereas the type III interaction mediates procaspase-1 CARD auto-oligomerization and contributes to the recruitment of the NF-κB signal transducer RIP2.

Our data indicate that these interaction types contribute differently to the two procaspase-1 functions. The type I CARD-CARD interaction between procaspase-1 and ASC not only enables procaspase-1 to gain proteolytic activity in ASC-containing inflammasomes but can also participate in the formation of the signaling complex for procaspase-1-mediated NF-κB activation. Indeed, we found that co-expression of ASC with procaspase-1 has a strong synergistic effect on RIP2-dependent NF-κB induction. Whether the latter property con-
cerns a physiological ASC function in an inflammasome context is currently unknown and requires further research. The concept of an ASC-containing complex performing both functions is supported by the observation that a single receptor, the mitochondria-associated virus-sensing RIG-I receptor, can mediate both NF-κB activation as well as formation of a procaspase-1 activator (42). Still, the functional outcome of procaspase-1 signaling, cytokine processing, or NF-κB activation has been reported to rely on the ASC/RIP2 ratio (43). Strong ectopic expression of ASC or RIP2 favors proteolytic activation of procaspase-1 and NF-κB activation, respectively (43). At first sight, this model contradicts our observation of a strong synergistic effect of ASC on procaspase-1-mediated, RIP2-dependent NF-κB activation, but these authors also demonstrated that expressing modest amounts of ASC enhances NF-κB induction by procaspase-1 (43). Further increasing the ASC levels, on the other hand, tips the balance to the proteolytic activation of procaspase-1 (43). Taken together, these findings suggest that both procaspase-1 functions cannot be executed simultaneously and that they would rely on the formation of different complexes. Notably, such a model has already been elucidated for TNF receptor signaling, which induces the formation of complexes whose molecular composition depends on the required function: activation of NF-κB or induction of apoptotic or necroptotic cell death (44).

Whereas the type I CARD–CARD interaction between procaspase-1 and ASC is crucial for activation of both procaspase-1 and NF-κB, type III CARD–CARD interactions mediated by procaspase-1 CARD are only needed for effective NF-κB signaling. Although our co-immunoprecipitation experiments demonstrate that mutating Arg-45 in patch IIa preclude procaspase-1 CARD auto-oligomerization and RIP2 binding, our data also suggest that the recruitment of RIP2 is most likely a consequence of procaspase-1 CARD auto-oligomerization and would involve a yet undefined interaction type. ASC co-expression partially restored the inability of the patch IIa procaspase-1 mutant to induce RIP2-dependent NF-κB activation indicating that RIP2, despite the mutation in patch IIa of procaspase-1, was recruited to the procaspase-1 complex that signals to NF-κB. Therefore, we rather propose that the type III interaction is important only for the auto-oligomerization of procaspase-1 CARD and that this auto-oligomerization forms a prerequisite for RIP2 recruitment via a newly created or freed docking site on the surface of the procaspase-1 CARD. By interacting with procaspase-1 through a type I interaction, ASC probably forces the patch IIIa mutant to form auto-oligomers that enable RIP2 recruitment and result in a partial rescue of NF-κB induction. Still, a more intricate interaction pattern can be envisioned for the CARDs within the ASC–procaspase-1–RIP2 NF-κB signaling complex. In analogy to the PIDDosome, Fas/FADD-DISC, and MyDDosome, a single CARD would simultaneously interact with up to six other CARDs by employing each of the three interaction types twice (9–11). The hierarchical and sequential assembly of the helical MyDDosome, in which MyD88 first recruits IRAK4 and then binds to IRAK2, might represent a relevant model for the assembly of the ASC–procaspase-1–RIP2 complex (9). However, our results do not provide any experimental support for such a model. Only a crystal structure of this complex or maybe of another CARD complex, such as the NOD2–RIP2–CARD9 complex (45), would indisputably clarify this issue. Nonetheless, our results provide indications that the CARD of procaspase-1, by engaging a single or multiple interactions, determines the functional outcome of procaspase-1 signaling. Moreover, CARDs can, in general and in contrast to previous assumptions, engage interactions that exceed 1:1 charge-dependent interactions.

The structural information we provide here could be of value in fundamental research and for the development of novel therapies. The notion that residue Arg-45 is implicated only in NF-κB activation makes it an ideal target for the generation of a mutant procaspase-1 knock-in mouse specifically defective in procaspase-1-mediated NF-κB signaling. This mouse would be particularly suited to identify (patho-)physiological settings for this enigmatic procaspase-1 function. At the moment, procaspase-1 has only been attributed a physiological role in LPS- and Malp2-induced NF-κB activation (43, 46). From a therapeutic point of view, the identification of residue Asp-27 as part of the ASC interaction patch might be important for the development of new inflammasome-targeting compounds for treatment of IL-1β-driven, inflammasome-dependent pathologies (47–50).

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