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Structure, interactions and self-assembly of ASC-dependent inflammasomes

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Abstract

The inflammasome is a multi-protein platform that assembles upon the presence of cues derived from infection or tissue damage, and triggers the inflammatory response. Inflammasome components include sensor proteins that detect danger signals, procaspase 1 and the adapter ASC (apoptosis-associated speck-like protein containing a CARD) tethering these molecules together. Upon inflammasome assembly, procaspase 1 self-activates and renders functional cytokines to arbitrate in the defense mechanism. This assembly is mediated by self-association and protein interactions via Death Domains. The inflammasome plays a critical role in innate immunity and its dysregulation is the culprit of many autoimmune disorders. An in-depth understanding of the factors involved in inflammasome assembly could help fight these conditions. This review describes our current knowledge on the biophysical aspects of inflammasome formation from the perspective of ASC. The specific characteristics of the three-dimensional solution structure and interdomain dynamics of ASC are explained in relation to its function in inflammasome assembly. Additionally, the review elaborates on the identification of ASC interacting surfaces at the amino acid level using NMR techniques. Finally, the macrostructures formed by full-length ASC and its two Death Domains studied with Transmission Electron Microscopy are compared in the context of a directional model for inflammasome assembly.

Keywords

Inflammasome; Inflammasome adapter; ASC; NLRP3; Death domain; PYD CARD; Inflammation; Innate immunity; Protein assembly; NMR; TEM

1. Inflammasomes

Inflammasomes are large multiprotein complexes that assemble in the cytosol of defense cells such as macrophages upon activation by the presence of harmful-related cues [1–7]. Pathogen-associated molecular patterns (PAMPs) [8–13] and danger-associated molecular patterns (DAMPs) derived from endogenous stressors (e.g. changes in K^+ efflux and Ca^{2+} signaling) prompt inflammasome assembly and activation [14–18]. Danger-signal sensing proteins from the NLR (nucleotide-binding oligomerization domain-like receptors), AIM (absent in melanoma 2) families and pyrin [19–25] self-associate and interact with the

inflammasome adapter ASC [26–29], recruiting procaspase 1 [1,23,27–29], which upon oligomerization activates proinflammatory members of the interleukin 1 superfamily [30–32]. The formation of the inflammasome is thus believed to act as a molecular platform for the activation of procaspase 1 through the proximity-induced model of autoactivation [33,34].

The different types of inflammasome sensor proteins, analogously to Toll-like receptors [35–37], become active in response to different stimuli [38–43]. Inflammasomes formed by the sensor NLRP1 (NLR Family Pyrin Domain Containing 1) assemble upon the presence of lethal toxins [44], whereas the NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome is more promiscuous and responds to PAMs from bacterial lipopolysaccharides and DAMPs from membrane rupture caused by different bacterial toxins, lysosomal damage, changes in potassium efflux, reactive oxygen species and mitochondrial disruption [4–6,14–17]. The NLRC4 (NLR family CARD domain-containing protein 4)/NAIP (Baculoviral IAP repeat-containing protein 1) inflammasome responds to bacterial infections through the NLR subfamily NAIP, which detects bacterial flagellin and prompts NLRC4 oligomerization, in turn activating procaspase 1 [45,46]. All in all, the different mechanisms/agents involved in inflammasome activation is a matter of intensive research.

The secretion of proinflammatory cytokines is related to changes in the cell structural integrity that ultimately lead to a form of lytic cell death known as pyroptosis, typically characterized by membrane rupture [47,48]. Recently, pyroptosis and caspase 1 activation have been linked at the molecular level by the identification of the protein gasdermin D as a specific substrate of the protease [49,50]. Upon cleavage, gasdermin D is fragmented in approximately two halves. The C-terminal half is soluble, whereas the N-terminal half self-associates creating large pores (~15–30 nm) in the inner leaflet of the cell membrane [49–55]. Therefore, it is suggested that the secretion of proinflammatory cytokines upon inflammasome assembly happens via lytic death executed by gasdermin D [49–55]. However, other mechanisms for secretion are not ruled out because interleukin release to the extracellular environment in relation to inflammation has been observed in the absence of gasdermin D [56].

Despite the advancement in our understanding of the major players in inflammasome formation; NLRs, procaspase 1 and ASC, new proteins are emerging as important factors for inflammasome regulation and function, such as the protein kinase Nek7 [57] and vimentin [58], a type III intermediate filament protein. In addition, the connections between the inflammasome and other cellular processes such as apoptosis [59,60] and autophagy are areas of increasing interest [61,62].

The three types of proteins that assemble the inflammasome share modular architectures [22,63,64]. All NLRs have a nucleotide-binding and oligomerization domain, and typically also a leucine-rich repeat domain, and one or more Death Domains [65–67]. Inflammasome assembly occurs intra and intermolecularly via protein interactions between domains of the same type [1–4,68,69]. Typical Death Domains are PYDs (pyrin domains) [70–76] and

CARDs (caspase activation and recruitment domains) [77–80] that interact with other PYDs and CARDs, respectively [68,81–85] (Fig. 1).

At the structural level, these domains form six-helix bundles arranged in a Greek-key topology known as the Death fold [86]. Structural differences between subfamilies with this fold, and in particular pertaining to the electrostatic surface, are likely responsible for the observed homotypic binding specificity [87]. Death Domain interactions are classified in three different types (type I, II and III) on the basis of atomic-resolution studies of Death Domain complexes [68,87–89]. These three interaction types are involved in the formation of large molecular platforms such as the inflammasome [1]. Most importantly, the interfaces of these interactions involve different protein regions, which allows for a single Death Domain to interact with several domains for further oligomerization.

2. Structure and dynamics of the inflammasome adapter ASC

The inflammasome adapter ASC (apoptosis-associated speck-like protein containing a CARD) [26,75,89–92] is a member of the Death Domain superfamily, as it contains two Death Domains; an N-terminal PYD and a C-terminal CARD connected by a 23-amino acid long linker [92]. ASC has been shown to self-associate mediating the formation of large molecular assemblies like the inflammasome [1,7,26–28] and the pyroptosome [29]. The latter is described as a supramolecular complex consisting of oligomerized ASC dimers that induces pyroptosis via the activation of procaspase 1 [29]. The adapter ASC functions as a molecular glue via its PYD and CARD domains to bridge the PYD of the sensor protein with the CARD of procaspase 1 [26–28]. ASC is a determinant for the activation of inflammasomes formed by NLRs and AIM sensors, which do not carry a CARD and do require the adapter to become functional. However, even sensors with CARDs, such as NLRP1 and NLRC4, are more active in the presence of ASC [7,93], which is believed to help in procaspase 1 recruitment also for this type of inflammasomes.

Members of the Death Domain family typically have a strong tendency to oligomerize, which often times precludes the formation of high quality, single crystals necessary for x-ray crystallographic analysis, and poses significant challenges for solution NMR studies. In spite of this behavior, our laboratory determined the 3D structure of ASC at atomic resolution by multidimensional nuclear magnetic resonance (NMR) and characterized its interdomain dynamics [92]. The NMR structure of full-length ASC [92] shows two six-helix bundle domains (PYD and CARD) connected by a 23-residue long linker (Fig. 2A). No NMR-derived contacts from nuclear Overhauser effect data (NOE) were observed between domains or connecting each domain with the linker. This result indicates that the PYD and CARD do not interact with each other, in agreement with the previously observed propensity of Death Domains to participate in homotypic interactions, and ASC function as an inflammasome adapter with two protein-protein interacting domains. In the absence of structural information, it would be reasonable to suggest that ASC long linker (residues 90–112) does not adopt any preferred conformations. However, careful analysis of the NMR data show that the linker adopts residual structure [92]. In fact, short-range NOEs (inter-atomic distances $< 5 \text{ \AA}$) were observed for a significant number of amino acids in the linker (Table 1). Moreover, the $^{13}\text{C}_\alpha$ chemical shifts of this region [94] deviate from

tabulated random coil values [95,96]. The linker region from residues 90–94 show positive secondary shifts and NOE data indicative of turn conformations, whereas $^{13}\text{C}_\alpha$ chemical shifts deviate to consistently negative values from residues 95–112, indicating the formation of low populated extended structures (Fig. 3). The presence of two consecutive Pro residues (Pro-103 and Pro-104 in the linker of ASC) favor the polyproline II conformation, which is a common residual extended structure found in protein loops and linkers connecting domains in modular proteins[98].

Interestingly, the ASC^{PYD} domain shows several differences with respect to other PYDs [89,99–103], particularly with those of NLRP1 [99] and NLRP10 (PDB entry 2DO9). The latter show a disordered loop located in the position of helix 3 in ASC^{PYD}. In addition, helices 1 and 6 are also shorter in NLRP1^{PYD}. These structural differences might be related to their different roles in inflammasome formation. In contrast, the PYD-only protein ASC2 [100], which modulates ASC-mediated inflammation through PYD/PYD interactions, is significantly similar to ASC^{PYD} at the structural level. Full-length ASC^{CARD} shows specific structural characteristics that deviate from other known CARDs [68,104,105]. The structures of CARD domains typically show a bend in helix 1 or its separation into two smaller helices, H1a and H1b. The lack of H1a in the CARD of ASC might be related to its plasticity in protein-protein interactions, which would facilitate its role as inflammasome adapter and in apoptotic events. In addition, helices 2 and 3 in ASC^{CARD} show large deviations as compared to other CARDs, which can result from structural changes associated to the binding surface involving the connection between H1a and H1b. The electrostatic surface of full-length ASC^{CARD} is also significantly different from other CARDs such as Apaf-1^{CARD} [104], as it shows positively and negatively charged areas evenly spread across the surface (Fig. 2B), whereas other CARDs typically have two extensive oppositely charged regions. These differences, pertaining to helix length, orientation and electrostatic surface might be necessary to tightly control the binding specificity observed in protein-protein interactions mediated by CARD domains.

At the dynamic level, NMR relaxation of backbone amide nitrogen labeled with spin- $\frac{1}{2}$ NMR active ^{15}N provides insightful information on protein motion [106–110]. Longitudinal [T_1] and transverse [T_2] relaxation times of amide ^{15}N nuclei are affected by N–H bond dynamics and the molecule's rotational diffusion. The noticeably different values of the ^{15}N T_1/T_2 ratios of ASC^{PYD} and ASC^{CARD} domains in the full-length protein [92], indicate that they reorient at different rates. In addition, rotational correlation time (τ_c) values derived from NMR relaxation data for individual ASC domains are significantly larger than the predicted values (Table 2). For globular proteins, the NMR-derived τ_c values are typically in very good agreement with the predicted values.

As shown in Table 2, the NMR-derived τ_c values of both PYD and CARD in ASC are larger than the experimental τ_c of ASC2, with similar size and structure to the ASC^{PYD} and ASC^{CARD} individual domains. This result indicates that the latter do not tumble independently [109,110]. The motion of one domain is restricted by the presence of the other domain, and by the residual structure of the linker. This behavior is illustrated by superimposing each individual domain of the NMR conformational ensemble (Fig. 2C and D). The residual structural preferences of the linker (Fig. 3) define a restricted interdomain

organization in the three-dimensional space that influences the orientation for binding of one domain relative to the other. This architectural arrangement is tuned by the semi-flexible linker to increase the accessible space of each domain, improving the probability to find interacting partners.

It is well-known that protein dynamics is intimately related to protein function [111–113]. Allosteric behavior and enzymatic catalysis strongly depend on protein dynamics and interdomain motion [114,115]. Compelling evidence indicates that protein domain dynamics participate in catalysis, regulation, and the formation of proteins assemblies. Specifically, domain motion resulting from interdomain linker flexibility has been related to protein function in the classic example of the dynamic behavior of the two-domain protein calmodulin [109,110]. Calmodulin's long interdomain linker is highly mobile [110], supporting its promiscuity to bind multiple partners. The flexibility of the linker allows both protein halves to interact simultaneously with other proteins and peptides, and to adopt the different interdomain arrangements required for the formation of each complex [109,110,116]. ASC also needs interdomain flexibility to interact with different partners.

3. Model for ASC functional oligomerization

The structural and dynamics studies on ASC [92] led us to propose a mechanism for its functional oligomerization (Fig. 4). This model proposes that the 23-amino acid linker is structurally dynamic, yet rigid enough to steer the two domains into a back-to-back orientation that favors a specific assembly (Fig. 4A and B). Additionally, the linker dynamics increases the capture radius for effectors, potentially facilitating binding. This study helped to propose a *modus operandi* for ASC dimerization showing that the PYD and CARD are positioned in a confined space, not interfering with the available surfaces for binding of each domain to other partners (Fig. 4B). This interacting mode would lead to a preferred directionality for further self-association, consisting of rows of PYDs and CARDS running parallel to one another (Fig. 4C). This model of assembly growth optimizes the number of homotypic interactions between domains. Other possible modes of oligomerization would be less favorable, as there are less homotypic contacts for macrostructure stabilization (Fig. 4D). However, the interaction of ASC or ASC oligomers with other inflammasome components might lead to different oligomerization architectures.

4. ASC homo and heteromolecular interactions at the amino acid level

Solution NMR techniques can be applied under a large variety of sample conditions, which proves particularly useful to study the structure and dynamics at the atomic level of oligomerizing proteins. More importantly, solution NMR can also be used to study protein-protein, and in general protein-ligand interactions by monitoring the changes of NMR parameters upon binding [117]. One of these parameters is the chemical shift (δ), which is exquisitely sensitive to minute changes in the chemical environment of magnetically active nuclei [118–120]. We have leveraged from this capacity to study the self-association of ASC^{PYD} and its interaction with NLRP3^{PYD}, to develop a better understanding of the initial stages of the assembly of the inflammasome.

We have monitored native ASC^{PYD} self-association by NMR under conditions that favor protein solubility (protein concentration ~0.2 mM and pH 3.7) [89]. The chemical shifts of NMR signals in [¹H–¹⁵N]-sofast-HMQC [121] spectra of ¹⁵N-isotopically enriched ASC^{PYD} change upon the addition of different concentrations of unlabeled ASC^{PYD} (Fig. 5A). At the end of the titration, when NMR signals do not experience any additional changes upon the addition of the unlabeled protein domain, the resulting spectrum perfectly overlaps with that recorded at high concentration of ¹⁵N-labeled ASC^{PYD} (Fig. 5A). From the presence of a single set of signals during the titration, we can conclude that ASC^{PYD} self-association is fast on the NMR chemical shift time scale. Should the process be slower, the spectrum would show several sets of signals corresponding to the different species involved [118].

We also studied the interaction between ASC^{PYD} and NLRP3^{PYD} in solution using a similar procedure [89]. In this case, [¹H–¹⁵N]-sofast-HMQC spectra of ¹⁵N-enriched ASC^{PYD} were acquired upon the addition of increasing amounts of unlabeled NLRP3^{PYD}. Chemical shift changes of ASC NMR signals indicate the binding of both PYDs (Fig. 5B). The amino acids in ASC^{PYD} with the largest variations in chemical shift upon self-association and binding to NLRP3^{PYD} are mapped onto the 3D structure of ASC^{PYD} (Fig. 6A and B) to show that ASC^{PYD} self-associates via two opposing binding interfaces, and that its interaction with NLRP3^{PYD} is mediated by similar binding regions.

ASC^{PYD} regions mediating self-association include: helices H1, H4 and H5 N-terminus for one side, and helices H2, H3 and H5 C-terminus for the other (Fig. 6A). This result agrees with previous mutational studies on the oligomerization of ASC^{PYD} [122,123]. However, several mutations found to abolish ASC^{PYD} self-association do not show particularly large chemical shift perturbation upon interaction (E13, K21 and D48). This discrepancy could result from an overall destabilization of the protein structure caused by the mutations.

Electrostatic interactions have been traditionally considered as the main driving force in PYD-PYD complex formation [102,122–124]. However, the NMR titration studies shed light on the critical role of hydrophobic interactions in PYD self-association [89], as several hydrophobic residues show the largest chemical shift perturbations, including A4, L25, V30, L44, L45 and L68. Particularly, L25, V30 and L45 are sufficiently exposed to participate in side chain-side chain interactions. Interestingly, these residues are all located in the interface formed by H2 and H3 (Fig. 6A).

The change of chemical shift upon binding can be used to obtain apparent values of the dissociation constant (K_D) for the PYD-PYD interactions (Fig. 7) [117–120]. NMR data indicate that both surfaces in ASC^{PYD} (H1, H4, N–H5 and H2, H3, C–H5) self-associate with similar affinity, showing K_D values in the micromolar range (~40 μ M – ~100 μ M) (Fig. 7). The determination of the dissociation constants is less accurate for the ASC^{PYD}-NLRP3^{PYD} interaction because NLRP3^{PYD} has a stronger tendency to aggregate, which precluded reaching the plateau. However, several accurate values were obtained for R5, T16, E19, D54 and E67 (Fig. 7B). The average value for these residues is 22 μ M, which indicates higher affinity for the ASC^{PYD}-NLRP3^{PYD} oligomer.

These results agree with a previous study indicating that upon mutation of certain residues in the ASC^{PYD} binding interfaces identified by our NMR data, both ASC^{PYD}-ASC^{PYD} and ASC^{PYD}-NLRP3^{PYD} interactions were affected [122,123]. In addition, the importance of hydrophobic interactions in ASC^{PYD}-NLRP3^{PYD} binding is also highlighted by the large changes in chemical shifts observed in L25, V30 and L45 (Fig. 5B).

The influence of both hydrophobic and electrostatic interactions in ASC^{PYD}-ASC^{PYD} and ASC^{PYD}-NLRP3^{PYD} oligomers was studied by monitoring the effect of NaCl concentration and pH [89]. The addition of 100 mM NaCl results in a [¹H-¹⁵N]-sofast-HMQC spectrum of ASC^{PYD} with characteristics indicating that the protein has already oligomerized (Fig. 8A). In fact, spectra obtained at the beginning of the titration in the presence of salt are more similar to spectra acquired at the end of the titration in the absence of salt, which suggests that the fraction of oligomers is larger at lower protein concentrations under the former conditions. The effect of pH was also monitored by NMR. [¹H-¹⁵N]-sofast-HMQC spectra of labeled ASC^{PYD} at pH 5.8 were acquired upon the addition of unlabeled ASC^{PYD} and NLRP3^{PYD}, showing changes in chemical shift values, despite significant protein precipitation observed at this pH value. The chemical shift perturbation mapped onto the 3D structure of ASC^{PYD} indicates that the binding interfaces at pH 5.8 are equivalent to those identified at the more acidic pH value (Fig. 8B).

Mutants of ASC^{PYD} designed to improve its solubility have been used to study the PYD-PYD interaction. In particular, the L25A mutant shows significantly enhanced solubility [102,125]. Using analogous NMR experimental procedures as those described above, we have determined an average K_D value for ASC^{PYD} L25A self-association of 165 μM, which is significantly larger than the value obtained for the wild-type (65 μM). The binding interfaces still include H1, H4, N-term H5 and H2, H3, C-term H5 (interface with the L25A substitution).

It is worth noting that the NMR data indicate that the mutation results in structural changes in helix 3, as chemical shifts are significantly perturbed for residues in this region, far apart from the mutated site (Fig. 9). Other residues showing chemical shift perturbations are located in the interface formed by helices H2, H3 and H5 C-terminus, particularly in the vicinity of L25 and L45 (Fig. 9), and around residue T16, also positioned between the two interfaces. Residue K24 seems to be important for the stabilization of H3 [125], thus the L25A mutation could lead to a destabilized H3, in turn reducing binding affinity.

Moreover, the structural perturbation on the H2-H3 interface of the mutant protein could explain previous studies indicating that the binding of the L25A mutant with the PYD-only protein POP1 is exclusively mediated by the H1-H4 interface of ASC^{PYD} [125]. In addition, the L25A mutant was also used to test the interaction between ASC^{PYD} and NLRP4^{PYD} with negative results [102]. PYDs are capable of polymerizing into fibrils and, importantly, the L25A mutant has been shown unable to form these supramolecular structures [123]. The interaction through the H2-H3 interface is possibly impeded due to the mutation and thus, only dimers interacting through H1-H4 interface can be formed. Altogether, this evidence points to the effects mutants can cause in the protein electrostatic surface and overall fold,

which are often times neglected when deriving conclusions on results obtained with mutated proteins.

The NMR studies described above unveiled the *in vitro* interaction between ASC^{PYD} and NLRP3^{PYD} that *in vivo* evidence had identified [123]. However, it is also important to identify the binding surfaces of NLRP3^{PYD} towards other PYDs. The 3D structure of intact NLRP3^{PYD} was determined by NMR [89] with significant difficulties resulting from its high tendency to aggregate, which rendered spectra with severe signal overlap in the amide central region. These signals correspond to monomeric and oligomerized species, posing great challenges for the NMR titration. Nonetheless, chemical shift changes upon binding of up to 76% of total number of signals (Fig. 10) were monitored [89].

Two opposing interfaces were identified to be involved in the association with ASC^{PYD}. One region includes helix H1, the N-terminus of H2 and H4 (residues R9, Y10, E13, D14, V18, D19, L20, A47, D48, V50, D51, K84 and D88); whereas the other region also involves helix H5 (residues T4, G35, I37, F59, G61, E63, W66, A67, V70, W71, A74, E89 and K91). Interestingly, as already observed for ASC, NMR signals shift in equivalent directions to those observed at higher NLRP3^{PYD} concentration (Fig. 10), indicating that NLRP3^{PYD} self-associates using similar interfaces for binding ASC^{PYD}. In addition, these interfaces also match those in Pyrin involved in the interaction with ASC^{PYD} [126]. The presence of opposing interfaces allows ASC^{PYD}-NLRP3^{PYD} oligomer formation, which is the basis for inflammasome assembly.

5. Supramolecular models of ASC^{PYD} self-association and ASC^{PYD}-NLRP3^{PYD} oligomeric structures

Protein docking was used in combination with the NMR data on the specific residues critical for interaction to generate starting models of the dimeric complexes. The ASC^{PYD}-ASC^{PYD} dimer was calculated using the H1–H4 surface of one protomer with the H2–H3 surface of the other (Fig. 11A). Both PYDs would still contain the opposing surfaces that were used as template for the structural alignment of additional ASC^{PYD}-ASC^{PYD} pairs to generate a fibril (Fig. 12).

The electrostatic surface of each domain clearly shows differently charged interfaces that prompt polymerization (Fig. 11A). The resulting structure is an intertwined helical fibril that resembles the ASC^{PYD} helical tube described by cryo-Electron Microscopy data [124]. Although the model obtained from computational docking is structurally different from the helical tube, the latter could grow from protofibrils with small dimensions to larger assemblies. The helical tube is stabilized by three types of interactions between the PYD subunits, with the type I dominating in each strand, whereas the type II and III are highly variable and are found between the strands, supposedly stabilizing the final helical tube (Fig. 13).

Under the NMR experimental conditions, only the type I interaction was observed (Fig. 11A). The structure of the ASC^{PYD} domain inside the mature fibrils shows significant conformational changes on H3, which the authors attribute to structural rearrangements

[124]. In addition, the type I interaction characterized by NMR [89] slightly differs from the one described in the helical tube. Most differences result from the rotation of one protomer relative to the other. In the fibril generated by the NMR data in combination with computational docking, the residues responsible for the type II and III interactions (Fig. 13) remain accessible for binding, thus with potential capability to laterally stack other fibrils. It is noteworthy that among the 12 residues mutated in the cryo-EM study with effects in helical tube formation [124], 7 participate in the dimer interface identified by NMR (E13, K21, K22, K26, R41, L50 and D51). Based on mutational studies, another ASC^{PYD}-ASC^{PYD} homodimer model was proposed [122] that significantly deviates from the NMR dimer (Fig. 11B). The discrepancy might arise from the low number of restraints used in the mutational study to drive the docking calculations and in potential perturbations of the PYD structure caused by the mutations.

For ASC/NLRP3 oligomerization, NMR data combined with docking experiments generate four different types of dimers between the PYDs of ASC and NLRP3, in agreement with the interaction interfaces: ASC H1–H4/NLRP3 H1–H4, ASC H1–H4/NLRP3 H5, ASC H2–H3/NLRP3 H1–H4 and ASC H2–H3/NLRP3 H5 (Fig. 14). The oligomeric structure is modeled by structural alignment of consecutive pairs, resulting in an hexameric ring (Fig. 15). This macromolecular platform is analogous to those formed by other members of the Death Domain superfamily [127]. The rotational symmetry of the ASC^{PYD}-NLRP3^{PYD} is of 6th order, whereas the apoptosomes from *C. elegans*, human and *D. melanogaster* display four-fold, seven-fold and eight-fold symmetry, respectively [127]. Double-ring structures have been described for NLRP1 with five-fold and seven-fold symmetries [7], and NLRC4 has been shown to form rings of 11 protomers [128]. ASC^{CARD} has also been proposed to form rings based on these structures [129]. The topology of the ring (Fig. 15) leaves ASC^{CARD} domains in an accessible space for further interaction facilitated by the flexibility of the ASC linker [92], which could in turn serve as a mechanism for oligomerization into other ring structures. Importantly, mutations associated with hereditary periodic autoinflammatory syndromes found in the PYD of Pyrin protein [130,131] correspond to residues Y11, V18, K24, R41 and D80 in NLRP3, which are located in the ASC^{PYD}-NLRP3^{PYD} interfaces described above. It follows that those mutations could hamper the formation of the ring.

NMR data show that the PYD-PYD interaction is low affinity, which seems to be a characteristic of the Death Domain superfamily [85,127] that could potentially be involved in the formation of oligomeric structures with the capacity of recruiting other components that ultimately arrange into large multimeric ensembles. Even though the ASC^{PYD}-NLRP3^{PYD} interaction is of higher affinity than ASC^{PYD} self-association, it is plausible that self-association and heteromeric interaction occur simultaneously. Thus, different combinations would result in various types of supramolecular complexes. The formation of these oligomeric structures is further convoluted by other factors, such as the autoinhibitory mechanism proposed for NLRs that controls oligomerization [132]. All in all, the NMR studies discussed shed light into our understanding of PYD domains in the ASC-NLRP3 inflammasome, by representing the initial stages of the activation cascade. This information might prove critical to gear efforts into therapeutic targets for the treatment of a variety of diseases related to inflammation [133]. NMR spectroscopy, combined with

other techniques, is a powerful approach to analyze inhibitors of inflammasome complex formation at the atomic level.

Cryo-EM studies have revealed that the PYD of ASC forms a right-handed helical tube with outer and inner diameters of ~9 and ~2 nm, respectively [124]. These studies were repeated with the mouse version of the PYD of ASC, resulting in similar structural characteristics [134]. The latter work added solid-state NMR data of the filaments of full-length mouse ASC suggesting that ASC^{CARD} is unfolded in the filaments. In light of previous studies, this result is striking as the NMR solution structure of full-length human ASC shows a properly folded CARD [92]. It is not clear why cryo-EM studies were not performed on filaments formed by the intact protein. Based on the cryo-EM data on ASC^{PYD}, it was suggested that the CARD does not form part of the filament core. The cryo-EM structure of the PYD [124] and the solution NMR structure of the intact protein [92] were used in concert to propose a model for the assembly of ASC-dependent inflammasomes. This model proposes that ASC^{PYD} domains form the helical tube, whereas the folded CARDS protrude out from the helix available for interaction with the CARDS of procaspase 1 molecules [124].

6. ASC^{CARD} and ASC^{PYD} self-associate into different macrostructures

The CARD of the inflammasome adapter ASC is key in procaspase 1 recruitment within the inflammasome and was shown using fluorescence spectroscopy to have an important role in ASC foci formation [135].

Detailed TEM studies revealed that ASC^{CARD} forms highly organized bundles of fibrils or filaments (Fig. 16). These bundles are very directional, with dimensions of ~150 nm in width and ~0.5–1 µm in length, from which narrower bundles branch out [136]. ASC^{CARD} assembles into two types of fibrils that are 3.4 ± 0.5 nm in width and self-organize into thicker structures, 10.0 ± 0.8 nm wide (Fig. 16). Seemingly unorganized protein material forms the transition between the thin and the thicker fibrils. The dimensions of the latter, including some inter-fibril space, suggest that they might be composed of two thin fibrils.

Other CARDS, including procaspase 1^{CARD}, ASC^{CARD}, and NLRC4^{CARD} have been studied by cryo-EM, showing that the three CARDS oligomerize as helical tubes of approximately four protomers per turn with an outer diameter of 7–8 nm (Table 3) [137–139]. One of these studies proposes a mechanism for the heteromolecular oligomerization of different CARDS that is based on structure complementarity of tetrameric surfaces [139]. In addition, two cryo-EM studies of the CARD of the protein MAVS also show slightly different filament widths (8.5 and 9.0 nm in outer diameter, [140–142]) and up to 2 nm different from other CARDS (Table 3). The width of ASC^{CARD} type A fibrils is significantly smaller than the width of these helical tubes. The discrepancy is larger than the expected experimental error. One possible explanation for this difference is the presence of additional domains used to solubilize CARDS that could affect the oligomerization process. In addition, it might also indicate that CARDS of different proteins could assemble into macrostructures with different features. It has been observed experimentally that N- and C-terminal tags help to control oligomerization in CARDS [139]. It is worth noting that

protein constructs of CARDs or other Death Domains lacking additional proteins and/or domains for solubilization might better represent the natural self-association process.

TEM studies have also revealed the dimensions of filaments and filament bundles formed by ASC^{PYD}. This domain tends to form thick filament bundles of varying widths (Fig. 17). Individual filaments within the bundles are hard to distinguish, which suggests a very different polymerization mechanism as compared to ASC^{CARD} (Fig. 16). In addition, ASC^{PYD} filaments do not show additional ultrastructure.

7. NMR identification of ASC^{CARD} interacting surfaces

TEM unequivocally indicates that CARD-CARD interactions in human ASC are involved in filament formation. Solution NMR titrations were used to study this process at the atomic/ amino acid level [136]. The observed chemical shift perturbation data are relatively small as compared to the PYD-PYD interaction of ASC and ASC-NLRP3 [89], which suggests that most protein molecules have already oligomerized at the initial stages of the titration. These NMR data were mapped onto the structure of ASC^{CARD} (Fig. 18), showing that the amino acids involved in ASC^{CARD} self-association are located in the turn preceding helix 1, and mainly in helices 2, 3, 5 and 6. Three contact regions in the surface of ASC^{CARD} are identified from the NMR data; one formed by the N- and C-terminus of H1 and H6, respectively, a second one composed of the C-terminus of H5 and the N-terminus of H6, and the third region includes helices H2 and H3. These regions participate in the three canonical Death Domain interaction types I, II and III.

Changes in chemical shifts upon ASC^{CARD} self-association show a hyperbolic dependence up to values of 400 μM (Fig. 19). An additional increase in chemical shift perturbation is observed at higher concentrations, revealing that ASC^{CARD} polymerization might involve other more complicated oligomerization processes. The fitting of the data (excluding the last titration point) leads to apparent values for the dissociation constant (K_D) that range from 6 to 94 μM , with an average value of 50 μM (Fig. 19). The self-association of ASC^{PYD} shows similar values ($K_{D_{av}} = 65 \mu\text{M}$) and NMR perturbation data indicate a major involvement of helices 1, 4 and 2, 3, pointing to a predominance of the type I interaction [89].

Three ASC^{CARD} mutants were designed to test the validity of the NMR results on the amino acids involved in self-association [136]. ASC^{CARD} mutant 1 with seven mutations to Ala out of the 10 residues shown in Fig. 19 (E130A, W131A, D134A, S184A, S186A, D191A, D193A) could not be expressed in *E. Coli*, likely because of protein instability caused by the large number of mutations. The second mutant was designed with three mutations; E130A, W131A, and D134A and was expressed and purified. ASC^{CARD} mutant 2 forms short and thin filaments with no apparent macrostructural organization and no specific bundles and are significantly different in appearance (Fig. 20) as compared to wild-type ASC^{CARD} (Fig. 16). This result indicates that the mutations significantly alter the oligomerizing capabilities of ASC^{CARD}, although self-association is not completely abolished. A third mutant was designed to include non-conservative mutations on negatively charged residues with the largest NMR perturbation data; E130R, W131A, D134R, D191R, E193R. Mutant 3 is not capable of forming filaments or any other regular structure [136]. The TEM results

on ASC^{CARD} mutants support the NMR findings on the role of specific amino acids in ASC^{CARD} filament formation.

Because solution NMR studies on Death Domains are typically performed at low pH, it is important to discuss the physiological relevance of the interactions identified at acidic pH. It has been reported that ASC^{PYD} shows a stronger tendency to oligomerize in the presence of NaCl [89] according to NMR and analytical ultracentrifugation studies, indicating that hydrophobic interactions play an important role in Death Domain oligomerization. Acidic pH values are not expected to specifically affect hydrophobic interactions. Thus, hydrophobic residues Trp 131 and Thr 142 in ASC^{CARD}, with some of the largest chemical shift perturbations, are probably involved in self-association at low and physiological pH values. However, acidic pH will result in the protonation of different populations of the side chains of acidic residues (Glu and Asp). At pH 3.8, the conditions used in the NMR titration experiments of ASC^{CARD}, approximately 56% of the Asp side chains (generic pK_a = 3.9), and ~33% of the Glu side chains (generic pK_a = 4.1) are expected to be negatively charged. Therefore, these residues will be able to participate in electrostatic interactions at Ph 3.8, which can be detected by NMR. All of these considerations suggest that the interactions responsible for ASC^{CARD} self-association, identified by NMR at pH 3.8 are also relevant at neutral pH.

To generate a minimal model of ASC self-association, all NMR chemical shift perturbation data for both ASC^{CARD} and ASC^{PYD} homotypic interactions were simultaneously incorporated as experimental restraints in protein docking protocols [136,143]. A representative model of the resulting dimer is shown in Fig. 21. The largest dimensions of the ASC dimer are ~6 nm [136]. This experimentally-derived, minimal oligomerization unit will have implications to understand ASC self-association and the formation of macrostructures by the full-length protein (*vide infra*), as only truncated versions containing the extricated PYD and CARD domains have been studied [124,138].

The TEM results on ASC^{CARD} fibril and bundle dimensions point to a role of ASC^{CARD} in the integral assembly of the ASC filament, in addition to its binding to procaspase 1 via recruitment by CARD-CARD interaction. These results also indicate that truncated versions of ASC (PYD-only and CARD-only) self-assembly into different macro-structures. In addition, the NMR results show that Death Domains operate differently at the atomic-molecular level, despite sharing a common fold. This conclusion is inferred from the participation of three interacting regions in ASC^{CARD} self-association, whereas the type I interaction has a prominent role in PYD oligomerization [89]. Insight into the subtle differences governing Death Domain interactions is key to understand inflammasome inhibition and regulation by PYD- and CARD-only proteins POPs and COPs [69,144].

8. Full-length human ASC forms filaments with integral participation of its two Death Domains, PYD and CARD

Electron Microscopy has played a key role in determining the structural characteristics of inflammasomes formed by sensors with CARDs. The first one to be studied, NALP1, arranges into nanometer-size discs with an outer diameter of ~13 nm [7]. Another example,

the NAIP5-NLRC4 inflammasome, also assembles into double-stacked discs and helical arrangements with a diameter of ~28 nm [128,145]. NAIP5 and NAIP2 act as nucleators of NLRC4 self-association into these disc-like structures [128,145–148]. The molecular bases for the formation these discrete assemblies have been unveiled by x-Ray crystallography and cryo-EM studies [128,145–148]. NLRC4 becomes activated by the relative rotation of two domains that results in the exposure of several binding surfaces, prompting in turn self-assembly. In contrast, inflammasome sensors have also been shown to form large micrometer-size filamentous rings in the cytosol, concentrically bound to analogous rings formed by the adapter ASC, and oligomerized caspase in the center of the micrometer-size structure [149].

Several studies with truncated versions of these proteins (inflammasome sensors, ASC and procaspase 1) show that their PYD and CARD domains self-associate to form filamentous structures and filament bundles [124,136–139,150]. These structures could be relevant to understand the formation of inflammasomes that assembly into micrometer-size filamentous rings > 100 nm in width [149].

One EM study reports detail structural characteristics of the filamentous macrostructures formed by full-length ASC [136]. This study shows evidence indicating that full-length ASC forms filament bundles of dimensions that can only be explained if both CARD and PYD form integral part of the filament. The results from this work deviate from the model based on the cryo-EM data of the truncated ASC^{PYD} that assumes the CARDS of ASC protrude from the polymerized PYDs forming the filament [124]. In addition, it is now known that ASC^{CARD} is as capable as ASC^{PYD} to form filaments [136,138].

Full-length ASC filaments show a high tendency to stack laterally forming bundles, and appear in isolation less frequently. Bundles are typically composed of 2–7 filaments, being the 3- and 4-filament arrangements more abundant (Fig. 22) [136].

Table 4 shows average dimensions of bundles composed of different number of laterally stacked filaments. Individual filaments in isolation and in bundles are approximately 7 nm wide. The total width of the different bundles coincides with the width of one filament multiplied by an integer number that equals the number of filaments in the bundle. These data deviate from the model for ASC filament formation derived from the cryo-EM data of ASC^{PYD}.

The presence of the CARD would give rise to a larger width for an individual filament formed by the full-length protein. However, if the CARD is mobile within the filament as a result of the semi-flexible linker, the EM images most likely would reflect only the PYD tube, and thus ASC^{PYD} and full-length ASC filaments would share similar width. When considering the width of the stacked filaments, this value should be larger than the sum of the width of the individual filaments because of the space occupied by the CARDS, as the filaments are assumed to bind via CARD-CARD interactions (Fig. 23C, D, E). Therefore, the bundle width should reflect the presence of the CARDS and linkers.

ASC^{CARD} has a diameter of ~4 nm according to the NMR structure. Thus, a three-filament bundle that arranges laterally by CARD-CARD interactions, as proposed by the current

model, should be ~26–29 nm thick with a filament width of 6–7 nm (Table 4). Analogously, four-filament (Fig. 23E) and five-filament arrangements should show a total width of ~36–40 nm, and ~46–51 nm, respectively (Table 4). Measurements of 7-filament arrangements are also significantly lower (~53 nm) than the expected values (73–80 nm), again pointing to a different structural architecture of the filament formed by the full-length protein relative to the truncated construct (Table 4). Thus, the expected dimensions according to the originally proposed model are significantly larger than the observed values in the TEM images of the full-length protein, and the difference increases with the number of filaments in the bundle (Table 4). Moreover, it is worth noting that the estimated bundle widths are minimum values, as the CARDS are assumed to stack vertically upon filament interaction, occupying only the size of one CARD (Fig. 23).

The discrepancy between the observed and expected values (Table 4) suggests that there is no room for the CARDS in the structural model for the ASC filament proposed on the basis of the cryo-EM structure of the truncated protein. Thus, the filament macrostructure formed by full-length ASC is different from the reported cryo-EM structure of ASC^{PYD} filaments.

9. Stacking of particles with the size of ASC dimers form ASC filaments

Bulges of protein material protruding from the individual filaments are observed frequently in TEM images of full-length ASC (Fig. 22) [136]. Because the size of these bulges is close to the filament width (~7 nm), it is possible to argue that the protein material corresponds to CARDS protruding from the filament, as previously suggested on the basis of the cryo-EM structure of the ASC^{PYD} helical tube. However, several considerations indicate that this is not a reasonable explanation: 1) the size of the protein material does not match that CARD size (~4 nm); 2) the protruding CARDS should populate the whole filament length (Fig. 23C and D), instead of appearing only at certain positions along existing filaments (Fig. 22); 3) the possibility of observing the CARD by TEM would imply that it is structurally fixed relative to the PYD, although the proposed model suggests it is flexible [124]. Importantly, the protein material protruding from the filaments is clearly ring-shaped with a diameter similar to the filament width (Fig. 22B and C). The rings appear to stack in line forming the filaments (Fig. 22D), and are also bound to pre-existing filaments (Fig. 22B, C, D), which suggests a mode for the lateral stacking and for the formation of filament bundles.

A careful analysis of the profile intensities along the long axis of the filaments allows to identify maximum and minimum intensity values (Fig. 24). The lowest intensities likely correspond to the spacing between monomers and also to the spacing between the holes of the rings, whereas the maximum intensities are associated to the presence of protein material, constituting the actual ring. The profile intensities provide a measurement of the repeating distance between maxima of 5.0 ± 0.6 nm, which indicates the size of the repeating unit (Fig. 24). This value is slightly smaller than the measurement of the rings attached to preexisting filaments, probably reflecting some compression happening upon filament formation. The bottom of panel B in Fig. 22 shows a ring that appears to be detached from the end of the filament (highlighted with the arrow at the far left of panel B in Fig. 22), which is larger than the other protomers within the filament. This image illustrates the compression of the ring upon stacking to form the filament. The different

types of measurements used; particle size from micrographs, intensity profiles and particle distances can also contribute to the deviation of these values.

Importantly, the size of the ring-shaped structures agrees with the size of the ASC dimer determined based on docking results of the NMR solution structure of full-length human ASC and the NMR data for the CARD-CARD and PYD-PYD interactions (Fig. 21). The ASC dimer was proposed originally as building block for ASC self-assembly based on cross-linking studies on the pyroptosome [29], thus in agreement with the TEM data and the NMR-based docking structure. Altogether, these results suggest a potential role of the ASC dimer in filament formation. The TEM and NMR-computational data suggest that the ASC dimer can be the structural building block of the filament, and participate in bundle formation by attaching to pre-existing filaments. The formation of the dimer and concomitant participation of the CARD and PYD of ASC in filament assembly will result in mobility decrease of one domain relative to the other. Recent work using Förster Resonance Energy Transfer to study conformational changes in ASC associated to its polymerization indicate that the PYD and CARD domains approximate to one another upon oligomerization [151]. The ASC rings observed in the TEM images (Fig. 22) also point to a reduced flexibility of the linker.

Full-length human ASC forms filaments and bundles with structural features that significantly deviate from those of the ASC^{PYD} helical tube and ASC^{CARD} bundles. Thus, individual Death Domains in ASC can oligomerize into different architectures. The specific structural arrangement of ASC filaments could have important implications in inflammasome assembly, particularly in relation to the binding of ASC to NLR proteins and procaspase 1.

The simultaneous participation of both PYD and CARD in ASC filament formation implies that the filament contains two sides, each occupied with PYDs and CARDS, respectively, and each capable of interacting with the PYDs of NLRP3 and the CARDS of procaspase 1. This structural arrangement in the ASC filament will lead to a directionally-controlled oligomerization of ASC with NLRP3 and procaspase 1. This model for inflammasome formation is illustrated in Fig. 25. The directionality resulting from the ASC filament macrostructure potentially agrees with the presence of concentric rings of NLRP3 bound to ASC, and oligomerized procaspase 1 in the center, as observed in the cytosol of activated macrophages [149]. The specific arrangement of the different protein filaments forming these rings still needs to be investigated.

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Fig. 1. Types of inflammasomes.

Schematic representation of three different types of inflammasomes, showing the modular protein components (domain names in red and black) and full-length proteins (vertical protein names in blue), including the adapter ASC.

Fig. 2. Three-dimensional atomic-resolution solution structure of the inflammasome adapter ASC.

A. Ribbon diagram representing the secondary structure arrangement of the 3D structure of ASC (N and C termini as well as helix numbers are indicated). **B.** Electrostatic surface of ASC (red and blue denote negatively and positively charged surfaces, respectively).

C. Conformational space available to the CARD domain relative to the PYD. **D.**

Conformational space available to the PYD domain relative to the CARD. Image created with MOLMOL [97]. This research was originally published in the Journal of Biological Chemistry. E. de Alba. Structure and Interdomain Dynamics of Apoptosis-associated Speck-like Protein Containing a CARD (ASC). J. Biol. Chem. 2009; 284:32932-32941. © E. de Alba.

Fig. 3. ASC semi-structured linker.

Deviations of $^{13}\text{C}\alpha$ chemical shifts of ASC [94] relative to random coil values [95,96].

Positive values indicate α -helical conformation, whereas negative values indicate the presence of extended conformation. Most residues in the 95–112 fragment are clustered in the extended structure region except G99 and G111. This research was originally published in the Journal of Biological Chemistry. E. de Alba. Structure and Interdomain Dynamics of Apoptosis-associated Speck-like Protein Containing a CARD (ASC). J. Biol. Chem. 2009; 284:32932-32941. © E. de Alba.

Fig. 4. Model for ASC functional oligomerization.

A. Available space for CARD relative to the PYD domain shown in its volume. The type I, II and III interactions are possible for the PYD in the presence of CARD motion, as helices 1 and 4 (red), 2 and 3 (blue), and the loops connecting them are accessible for binding.

B. Structural dimer of ASC based on the CARD-CARD dimer complex between Apaf-1 CARD and caspase-1 CARD [104,105]. **C.** Schematic representation of ASC preferred directionality for functional oligomerization. **D.** Unfavored modes for ASC self-association.

Fig. 5. ASC^{PYD} interacts with other PYDs through two equivalent interfaces.

Superimposed sofast-HMQC spectra resulting from the titration of ^{15}N -labeled ASC^{PYD} with increasing concentrations of unlabeled ASC^{PYD} (A) and NLRP3^{PYD} (B). Some signals (e. g., L12 NH amide) remain unperturbed, whereas others show significant changes in δ upon protein interaction. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 6. ASC^{PYD} self-association and interaction with NLRP3^{PYD} is mediated by similar opposing binding surfaces.

ASC^{PYD} structure showing exposed residues with δ changes above the threshold value upon ligand binding for self-association (A) and interaction with NLRP3^{PYD} (B). (ASC structure PDB code 2KN6 [92]). This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 7. Dissociation constant values indicate stronger affinity of ASC^{PYD} for NLRP3^{PYD} vs. self-association.

Changes in chemical shift vs. protein concentration for ASC^{PYD} self-association (A) and ASC^{PYD}-NLRP3^{PYD} interaction (B). K_D values are shown for each graph. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pysin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 8. Influence of NaCl and pH on ASC^{PYD} interacting mode.

A. sofast-HMQC spectra of ASC^{PYD} at different concentrations in the absence and presence of NaCl. **Top:** In blue 50 μM ^{15}N ASC^{PYD}; green: 50 μM ^{15}N ASC^{PYD} + 500 μM ASC^{PYD}; red: 50 μM ^{15}N ASC^{PYD}/100 mM NaCl. **Bottom:** In red: 50 μM ^{15}N ASC^{PYD}/100 mM NaCl (same as in top), black: 50 μM ^{15}N ASC^{PYD} + 250 μM ASC^{PYD}/100 mM NaCl. **B.** Chemical shift perturbation data mapped onto the 3D structure for the ASC^{PYD}-ASC^{PYD} and ASC^{PYD}-NLRP3^{PYD} interaction at pH 5.8 (In red: ASC^{PYD}-ASC^{PYD}; in blue: ASC^{PYD}-NLRP3^{PYD}). This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 9. Structural perturbations in ASC^{PYD} caused by the L25A mutation.

A. Chemical shift differences between wild type and mutant L25A ASC^{PYD} vs. residue number. **B.** Largest chemicals shift variations mapped onto the ASC^{PYD} structure. L25 is shown in red. The largest structural effects (shown in blue) involve H2–H3. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 10. NLRP3^{PYD} interacts with ASC^{PYD} via similar interfaces.

A. Superimposed sofast-HMQC spectra acquired in the titration of ^{15}N -labeled NLRP3^{PYD} with ASC. NLRP3^{PYD} severe aggregation precluded titration at molar ratios > 1:1.

NMR signal changes upon titration with ASC match those observed at high NLRP3^{PYD} concentration, indicating that self-association of the latter and its interaction with ASC^{PYD} are mediated by identical interfaces. **B.** Chemical shift perturbation data for the NLRP3^{PYD}-ASC^{PYD} interaction. **C.** Mapping of NLRP3^{PYD} residues undergoing above-threshold δ

changes upon ASC binding. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 11. Electrostatic complementarity of the ASC^{PYD} dimer compared with proposed dimer based on mutational studies.

A. Structural model of the ASC^{PYD} dimer from NMR-based docking experiments. Side chains of residues involved in the interaction are shown in blue (positive charge) and red (negative charge). The interaction interface and the free interfaces show complementary charges for the polymerization. **B.** Comparison of dimer in A with dimer proposed on the basis of mutational studies [122] (in red). Protomers at the left are superimposed to show the different orientation of the remaining protomer in both dimer models. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 12. ASC^{PYD} fibril formation based on NMR data.

The polymerization of the ASC^{PYD} dimeric complex formed by the type I interaction results in a helical fibril. The ribbon diagram represents the dimer. The side chains of the residues that drive the docking calculation are shown. The colored circles represent the type I interaction taking place for fibril formation. Each replicate is axially rotated $\sim 38^\circ$. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 13. Death Domain interaction types in the cryo-EM structure of the helical tube formed by ASC^{PYD} polymerization.

Top and lateral views of human ASC^{PYD} helical tube [124] with the 15 ASC^{PYD} domains of the PDB entry (PDB 3j63). Each group of 5 ASC^{PYD} domains is colored in yellow, red and blue to show the helical arrangement. Bottom figure shows the interaction types that participate in polymerization.

Fig. 14. ASC^{PYD}-NLRP3^{PYD} oligomeric structures.

A. Models of four different complexes between ASC and NLRP3 PYDs based on NMR data: ASC H1–H4/NLRP3 H5, ASC H1–H4/NLRP3 H1–H4, ASC H2–H3/NLRP3 H5 and ASC H2–H3/NLRP3 H1–H4. ASC^{PYD} and NLRP3^{PYD} are shown as ribbon diagrams, colored in green and purple, respectively. Side chains of residues used in the docking are highlighted. ASC^{PYD} and NLRP3^{PYD} are depicted as green circles and purple squares, respectively, showing the corresponding type of interaction with numbers. **B.** Combination of alternative interactions based on the dimeric structures shown in A. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 15. Macromolecular complex formed by ASC^{PYD}-NLRP3^{PYD} oligomerization.

Top (left) and side (right) views of the hexameric ring modeled using as template the resulting docking pairs of the different dimeric units shown in Fig. 14. ASC^{PYD} and NLRP3^{PYD} are depicted in green and purple, respectively. ASC^{CARD} is shown in blue. The C-terminal domains of NLRP3 point to the bottom of the side view of the ring (right). The inner and outer dimensions of the ring are shown. This research was originally published in the Journal of Biological Chemistry. J. Oroz et al. ASC Pyrin Domain Self-associates and Binds NLRP3 Protein Using Equivalent Binding Interfaces. J. Biol. Chem. 2016; 291:19487–19501. © J. Oroz et al.

Fig. 16. ASC^{CARD} self-associates into filaments and filament bundles.

ASC^{CARD} forms ~0.5–1 μm long bundles with preferred directionality (**A, B**). Narrower bundles branch out from the thicker bundles. ASC^{CARD} forms thin filaments or fibrils of ~4 nm width (labeled as type A) and wider filaments likely composed of two narrower filaments (labeled as type B) (**C, D, E**). This research was originally published in the *Journal of Biological Chemistry*. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. *J. Biol. Chem.* 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 17. Filaments and filament bundles formed by ASC^{PYD}.

A, B, C, Representative micrographs with scale bar of 200 nm, and **D**, 0.5 μm. This research was originally published in the Journal of Biological Chemistry. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. J. Biol. Chem. 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 18. Interacting surfaces in ASC^{CARD} self-association.

ASC^{CARD} structure [92] is shown as ribbon diagram in two different views. The amino acids that are exposed to the solvent and with the largest chemical shift perturbations are depicted in purple and labeled in black. Helices are numbered in purple. This research was originally published in the *Journal of Biological Chemistry*. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. *J. Biol. Chem.* 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 19. ASC^{CARD} self-associates with $K_D \sim 50 \mu\text{M}$.

Chemical shift perturbations of ^{13}C , ^{15}N -uniformly labeled ASC^{CARD} vs. unlabeled ASC^{CARD} concentration. Representative residues exposed to the solvent with largest perturbations are shown, together with the corresponding K_D values. This research was originally published in the *Journal of Biological Chemistry*. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. *J. Biol. Chem.* 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 20. Filaments formed by ASC^{CARD} mutant 2.

Mutations include E130A, W131A, and D134A. This research was originally published in the Journal of Biological Chemistry. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. J. Biol. Chem. 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 21. Dimensions of the ASC dimer.

Contact surface of the ASC dimer structure derived from NMR-based protein docking [136]. The PYD and CARD domains are labeled. Dimensions were calculated with MOLMOL [97]. This research was originally published in the Journal of Biological Chemistry. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. J. Biol. Chem. 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 22. Full-length ASC forms filament bundles with individual filaments formed by the stacking of ring-shaped particles of the size of ASC dimers.

A. Full-length ASC filaments. Rectangles show zoomed areas that compose panels (**B**), bottom rectangle and (**C**), upper rectangle. Arrows point to ring-shaped structures (possibly ASC dimers) laterally attached to pre-existing filaments. **D.** An additional zoom of the upper-central region of (**C**). The circle shows stacked rings forming the filaments. This research was originally published in the *Journal of Biological Chemistry*. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. *J. Biol. Chem.* 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 23. Dimensions of stacked filaments of full-length human ASC deviate from the model based on the cryo-EM structure of truncated ASC^{PYD}.

A&B. Top and lateral views of human ASC^{PYD} helical tube (cryo-EM PDB 3j63) with the dimension reported in the published work [124] and containing the 15 ASC^{PYD} domains of the PDB entry. Each group of 5 ASC^{PYD} domains is colored in yellow, red and blue to show the helical arrangement (**A**, **B**). Top (**C**) and side (**D**) views of the ASC^{PYD} tube with protruding ASC^{CARD} domains and linker to show CARD availability for potential interactions. The PYDs of 15 full-length NMR structures of ASC molecules ([92]; PDB 2KN6) are superimposed to the 15 PYD domains of the cryo-EM structure. After superposition, the NMR PYDs are deleted and only the cryo-EM PYDs and the NMR CARDs are shown. One side of the helical tube is colored in red and the corresponding CARDs in orange. Dimension shown was calculated with MOLMOL [97] (**C**, **D**). **E** Modeled bundle of an arrangement of four ASC^{PYD} helical filaments with protruding CARDs and linker from **D**. The red side of the PYD tube and corresponding CARDs in orange (from **C** and **D**) are shown. CARD domains in filament interfaces are assumed to stack vertically (overlapping as worse-case scenario), thus occupying approximately the dimension of the ASC^{CARD} (~3.5 nm). The dimensions shown are; 1) the minimum width of the 4-filament bundle, which is estimated by assuming a filament width of 9 nm from the cryo-EM structure; 2) the estimated width range according to the model shown in (**D**) for the 4-filament bundle (**E**), which is calculated assuming a filament width of 6–7 nm from our TEM studies. This research was originally published in the Journal of Biological Chemistry. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. J. Biol. Chem. 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 24. Protomer spacing in bundled human ASC filaments.

A. Subsection of TEM micrograph of ASC filaments shown in panel A of Fig. 22. Line cuts of 3pxl (corresponding to 3 nm) using ImageJ along the long axis of each filament in the region of interest. **B.** Line cut along filament number 3 is shown in cyan. **C.** Average values of intensity profiles over the 3 nm width. Red arrowheads indicate locations of pixel intensity values above the mean intensity along the filament (green-dashed line). **D.** Mean values for the separation of maximum peaks from averaged intensity profiles for each of the filaments/lines cuts across a total number of 22 filaments. The mean protomer separation is $5.0 \text{ nm} \pm 0.6 \text{ nm}$. Scale bar in (A) is 100 nm. This research was originally published in the *Journal of Biological Chemistry*. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. *J. Biol. Chem.* 2019; 294:439–452. © R.J.T. Nambayan et al.

Fig. 25. Directionality model for ASC-dependent inflammasome assembly.

ASC dimer shown as the minimal building block. The ASC filament presents two interacting sides, one for recruiting procaspase 1 CARD and the other for interaction with the PYD of NLRs sensor proteins. The binding direction propagated by the ASC filament results in micrometer-size inflammasomes with a concentric distribution of inflammasome components; NLRs, ASC, and procaspase 1.

Table 1

Short-range NOEs observed in ASC interdomain linker.

Residue (i)	Atom (i)	Residue (j)	Atom (j)	Distance (Å)
A ₈₇	H ^N	H ₉₀	H ^N	4.4
A ₈₈	H _α	H ₉₀	H ^N	3.5
A ₈₈	H _β	H ₉₀	H ^N	3.3
A ₈₈	H ^N	H ₉₀	H ^N	3.4
T ₈₉	H ^N	Q ₉₁	H ^N	4.4
H ₉₀	H _α	G ₉₂	H ^N	4.0
H ₉₀	H _{β2}	G ₉₂	H ^N	4.8
Q ₉₁	H _{β1}	S ₉₃	H ^N	4.9
Q ₉₁	H _{β2}	S ₉₃	H ^N	4.9
G ₉₉	H ^N	Q ₁₀₁	H _{β2}	4.7
P ₁₀₄	H _{β1}	S ₁₀₆	H ^N	4.2
P ₁₀₄	H _{β2}	S ₁₀₆	H ^N	3.3
Q ₁₀₅	H _{β2}	A ₁₀₇	H ^N	4.1
K ₁₀₉	H _δ	G ₁₁₁	H ^N	4.5

This research was originally published in the Journal of Biological Chemistry. E. Alba. Structure and Interdomain Dynamics of Apoptosis-associated Speck-like Protein Containing a CARD (ASC). J. Biol. Chem. 2009; 284:32932-32941. © E. de Alba.

Table 2Rotational correlation times (τ_c).

System	Number of structured residues	τ_c (ns) ^a	τ_c (ns) ^b
Full-length ASC	168	9.8	12.0
ASC-PYD	87	10.5	6.5
ASC-CARD	78	9.2	5.8
ASC2	84	6.2 ^c	6.3
calmodulin N-terminal domain	73	7.1 ^c	4.1
calmodulin C-terminal domain	66	6.3 ^c	3.7

^aExperimental values from magnetic relaxation experiments. (NMR relaxation experiments for full-length ASC were performed at 0.2 mM protein concentration and pH 3.8).

^bTheoretical values obtained with a spherical model using the number of residues specified and the temperature corresponding to each case: ASC (298 K), ASC2 (298 K), calmodulin (308 K).

^cReported experimental values: ASC2 [100], calmodulin [109,110]. This research was originally published in the Journal of Biological Chemistry. E. de Alba. Structure and Interdomain Dynamics of Apoptosis-associated Speck-like Protein Containing a CARD (ASC). J. Biol. Chem. 2009; 284:32932-32941. © E. de Alba.

Table 3

Filament dimensions of different CARDS.

System	Tags or solubilization domains	Filament width (nm)
ASC ^{CARD} [136]	N-terminal-His ₆	3.5/10
ASC ^{CARD} [138]	N-terminal-His ₆ -MBP/C-terminal SUMO	8.0
NLRC4 ^{CARD} [138]	N-terminal-GFP	8.0
NLRC4 ^{CARD} [139]	N-terminal-MBP/C-terminal GFP	7.0
MAVS ^{CARD} [140]	N-terminal tag (19 amino acids)	8.5
MAVS ^{CARD} [141,142]	N-terminal Flag tag	9.0
Caspase 1 ^{CARD} [137]	N-terminal-His ₆ -MBP/C-terminal SUMO	8.0

Table 4

Full-length human ASC filament and bundle dimensions.

Filament Number in Bundle	Average Bundle Width (nm) ^a	Expected Bundle Width (nm) ^b	Average Filament Width (nm) ^c
1	6.4 ± 0.8 ^d	6–7	6.4 ± 0.8
2	14.0 ± 1.1 ^e	16–18	7.0 ± 0.6
3	22.5 ± 1.3 ^f	26–29	7.5 ± 0.4
4	29.9 ± 1.8 ^g	36–40	7.5 ± 0.4
5	35.9 ± 1.2 ^h	46–51	7.2 ± 0.2
7	52.9 ± 1.7 ⁱ	73–80	7.6 ± 0.2

^a Measured average and standard deviation from a population of d: 208; e: 34; f: 42; g: 16; h: 6; i: 3.

^b The expected bundle width according to model derived from ASC^{PYD} helical tube is calculated by assuming filament width values of 6–7 nm, and 7–8 nm in the case of the 7-filament bundle (different staining conditions for 7-filament bundle resulted in filaments 7–8 nm wide).

^c Calculated average filament width from bundle width divided by number of filaments. This research was originally published in the Journal of Biological Chemistry. R.J.T. Nambayan et al. The inflammasome adapter ASC assembles into filaments with integral participation of its two Death Domains, PYD and CARD. J. Biol. Chem. 2019; 294:439–452. © R.J.T. Nambayan et al.