Three-dimensional Structure of the NLRP7 Pyrin Domain

**INSIGHT INTO PYRIN-PYRIN-MEDIATED EFFECOR DOMAIN SIGNALING IN INNATE IMMUNITY**

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The innate immune system provides an initial line of defense against infection. Nucleotide-binding domain- and leucine-rich repeat-containing protein (NLR or (NOD-like)) receptors play a critical role in the innate immune response by surveying the cytoplasm for traces of intracellular invaders and endogenous stress signals. NLRs themselves are multi-domain proteins. Their N-terminal effector domains (typically a pyrin or caspase activation and recruitment domain) are responsible for driving downstream signaling and initiating the formation of inflammasomes, multi-component complexes necessary for cytokine activation. However, the currently available structures of NLR effector domains have not yet revealed the mechanism of their differential modes of interaction. Here, we report the structure and dynamics of the N-terminal pyrin domain of NLRP7 (NLRP7 PYD) obtained by NMR spectroscopy. The NLRP7 PYD adopts a six-α-helix bundle death domain fold. A comparison of conformational and dynamics features of the NLRP7 PYD with other PYDs showed distinct differences for helix 3 and loop α2–α3, which, in NLRP7, is stabilized by a strong hydrophobic cluster. Moreover, the NLRP7 and NLRP1 PYDs have different electrostatic surfaces. This is significant, because death domain signaling is driven by electrostatic contacts and stabilized by hydrophobic interactions. Thus, these results provide new insights into NLRP signaling and provide a first molecular understanding of inflammasome formation.

Eukaryotes have evolved an array of strategies, collectively referred to as the innate immune system, to directly detect pathogens and “danger signals.” This efficient pathogen-associated molecular pattern recognition enables eukaryotes to quickly eliminate intruders, thereby increasing the chance of survival. Inflammatory reactions via the innate immune system and cell death are related processes that utilize parallel signaling mechanisms and employ common molecular effectors. Many of these molecular effectors share a common structural fold, the death domain fold. The death domain superfamily consists of: 1) the death domain (DD) itself (1, 2), the death effector domain (3), 3) the caspase activation and recruitment domain (CARD) (4), and 4) the pyrin domain (5–7) (PYD, formerly called PAAD (8) or DAPIN (9)).

The most recently discovered family of proteins that are essential for the regulation of the innate immune system are the NLRs. These intracellular proteins act as receptors and regulators of innate immunity and apoptosis (10–12). NLRs have three distinct domains: 1) a C-terminal leucine-rich repeat domain, which is responsible for pathogen-associated molecular pattern recognition, 2) a central NOD-WH-SH domain (nucleotide-binding and oligomerization-winged helix-superhelical domain), also known as the NACHT domain, which is essential for multimerization of NLR receptors upon pathogen-associated molecular pattern recognition, and 3) an N-terminal effector domain, which links the NLR proteins to distinct downstream signaling pathways via adaptor proteins, such as RICK/RIP2 and the apoptosis-associated speck-like protein containing a CARD (ASC). The largest NLR subfamily, comprising NLRP1–NLRP14, uses a PYD as its N-terminal effector domain (13).

Some members of the NLRP family activate innate immune responses via direct interactions with adaptor proteins. These multi-component complexes are commonly referred to as inflammasomes. Inflammasomes, in turn, activate inflammatory caspases, which then process proinflammatory cytokines like pro-IL-1β and pro-IL-18 into their mature, secreted forms (14). To date, only NLRP1 and NLRP3 have been shown to form inflammasomes. These NLRPs bind the adaptor protein ASC, and the resulting inflammasomes activate procaspase-1. However, it was recently recognized that other members of the NLRP subfamily exhibit significantly different functions. Namely, they inhibit NF-κB and/or pro-caspase-1 activation (15–18). In particular, NLRP7 (also known as Nalp7 or PYPAF3) inhibits the processing of pro-IL-1β and pro-

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2 The abbreviations used are: DD, death domain; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; RMSD, root mean square deviation; NLR, nucleotide-binding domain and leucine-rich repeat-containing proteins; PYD, pyrin domain; CARD, caspase activation and recruitment domain; ASC, apoptosis-associated speck-like protein containing a CARD; FAF-1, Fas-associated factor 1; IL, interleukin; hetNOE, 1H-1H-NOE; FID, Fas-interacting domain.

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caspase-1. Recent reports have also shown that this inhibition is mediated by a direct interaction with both pro-IL-1β and procaspase-1 (17). Furthermore, the NLRP7 PYD has also been reported to bind Fas-associated factor 1 (FAF-1), a protein previously recognized to activate apoptosis and inhibit NF-κB activation (19, 20).

NLRP7 regulates multiple important biological processes, especially during embryonic development (21). Various NLRP7 mutations were linked to familial recurrent hydatidiform moles, which is a rare embryonic misdevelopment resulting in early pregnancy loss (22–28). Abnormal regulation of NLRP7 was also detected in endometrial cancer tissues (29) and testicular seminomas (30). Most recently, mutations in NLRP7 were identified in patients with autoimmune conditions such as Hashimoto disease, lupus, vitiligo, and Crohn disease (28). The cause(s) of these disorders remain elusive. Understanding how NLRP7 mediates signal transduction at a molecular level may provide new therapeutic approaches to these diseases. Despite the abundance of PYDs in several protein families, structural information about PYDs is very limited (31–34). Currently, only a few structures of effector domains involved in NLR signaling are known. Furthermore, no structural data are available for complexes formed between NLRs and their effector proteins (i.e. ASC or caspases). Both are essential for understanding the molecular mechanisms of NLR signaling.

Here we report the three-dimensional structure of the NLRP7 PYD, as well as an analysis of its dynamics using NMR spectroscopy. Importantly, the NLRP7 PYD shows structural and dynamic differences when compared with the PYDs of NLRP1, as well as ASC and ASC2. Recently, a completely disordered helix (helix α3) was reported in the NLRP1 PYD structure (34). This has subsequently inspired speculation about the possibility that large conformational changes might occur upon PYD-PYD binding. Canonical PYD-PYD and CARD-CARD (35–37) interactions are driven primarily by strong electrostatic interactions and likely stabilized by the subsequent formation of hydrophobic interactions. For example, based on the total buried surface area in the APAF-1 CARD-caspase-9 CARD complex (35), it was estimated that 70% of the interaction was due to electrostatic interactions, and 30% was due to hydrophobic interactions. Here we identify structural, hydrophobic, and electrostatic surface charge differences between the different NLRP PYDs. These observed differences now begin to explain the differential downstream signaling of these NLRPs, critical for understanding the formation of inflammasomes. We also probe these and other protein-protein interactions of the NLRP7 PYD by NMR titration interaction studies in vitro. Taken together, we provide the first critical insights into PYD signaling and fundamentally enhance our molecular understanding of the NLR-based innate immune system.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

Protein expression and purification of the human NLRP7 PYD (residues 1–96) was recently described (38).

**ASC PYD**—ASC PYD was cloned into pET28 (Novagen). The plasmids were transformed into *Escherichia coli* BL21 (DE3) cells. The cultures were grown to an *A*₆₀₀ of ~0.8 at 37 °C, and expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside. Proteins were expressed for 5 h at 25 °C. Cells were harvested by centrifugation, resuspended in 30 ml of buffer (50 mM Tris, pH 8.0, 100 mM NaCl), transferred to a 50-ml Falcon tube, and stored at ~20 °C overnight. For purification, the cells were lysed by sonication (5 pulses of 70 W for 35 s) on ice. Immediately following sonication, 5 µl of DNase (1 unit/µl) was added to the lysed sample, and it was incubated for 20 min on ice. The cell debris was harvested by centrifugation (50,000 × g, 20 min, 4 °C). The ASC-containing pellet was resuspended in 25 ml of urea buffer (50 mM Tris, pH 8.0, 8 M urea) and incubated overnight at room temperature. Following centrifugation (50,000 × g, 20 min, 4 °C), the supernatant containing denatured ASC was loaded onto a nickel-nitrilotriacetic acid column. The column was then washed with 20 ml of wash buffer 1 (20 mM Tris, pH 8.0, 6 M urea, 5 mM imidazole) followed by 20 ml of wash buffer 2 (20 mM Tris, pH 8.0, 1 M NaCl, 20 mM imidazole). The protein was eluted three times with 1.2 ml of elution buffer (50 mM citric acid, pH 4.0, 1 M NaCl, 5 mM dithiothreitol). The purity of ASC was verified by SDS-PAGE, and its folded state was verified by NMR spectroscopy.

**FAF-1 FID**—The FAF-1 FID domain (residues 1–180) was cloned into pET1B (39). Freshly transformed *E. coli* BL21-Codon-Plus (DE3)-RIL (Stratagene) cells were grown at 37 °C to an *A*₆₀₀ of ~0.6, and expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside. The proteins were expressed for 18 h at 18 °C. The cultures were harvested by centrifugation, and the cell pellets were stored at ~80 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM imidazole, 500 mM NaCl, 0.1% Triton X-100) supplemented with a Complete EDTA-free protease inhibitor tablet (Roche Applied Science). The cells were lysed by high pressure homogenization (Avestin C-3 Emulsiflex), and the cell debris was removed by centrifugation (50,000 × g, 40 min, 4 °C). The clarified lysate was loaded onto a His-Trap HP Column (GE Healthcare), and the protein was eluted with a 5–500 mM imidazole gradient. Fractions containing the FAF-1 FID domain were pooled, incubated with TEV protease to remove the N-terminal Thiò-His₆ tag, and dialyzed against fresh buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl). The cleaved sample, containing the now untagged FAF-1 FID domain, was loaded onto a nickel-nitrilotriacetic acid column (Invitrogen), from which it eluted in the flow through. For the final purification step, the protein was loaded onto a Superdex 75 26/60 size exclusion column (GE Healthcare) equilibrated with 20 mM sodium phosphate, pH 6.0, 200 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine. Fractions containing the pure FAF-1 FID domain, as confirmed by SDS-PAGE, were pooled and concentrated.

**NMR Spectroscopy**

NMR measurements were performed at 298 K on a Bruker AvanceCell 500 MHz spectrometer. A 13C- and 1H NOESY spectrum was recorded on a Bruker AvanceCell 800 MHz spectrometer. Both spectrometers are equipped with TCI HCN z-gradient cryoprobes. All of the NMR experiments were performed with either a 15N- or a 15N/13C-labeled NLRP7 PYD sample at a final temperature of 298 K.
concentration of 1 mM dissolved in 20 mM sodium phosphate, pH 6.0, 50 mM NaCl, 0.25 mM tris(2-carboxyethyl)phosphine.

Established NMR methods were employed for the sequence-specific backbone and side chain assignments of NLRP7 PYD (40). Semi-automated programs were used to evaluate the data (CARA). We used the following spectra for the structure calculation: three-dimensional 15N-resolved 1H,1H NOESY, three-dimensional 13C-resolved 1H,1H NOESY (mixing time, 80 ms), and two-dimensional 1H,1H NOESY (mixing time, 80 ms; D2O solution). NOE peak picking, NOE peak assignment, and three-dimensional structure calculation were performed automatically using the ATNOS/CYANA software package (41–43). The input for the structure calculations of NLRP7 PYD was the amino acid sequence, the complete chemical shift lists, and the three- and two-dimensional NOE spectra. Constraints for backbone dihedral angles derived from 13C chemical shifts were used only in the initial structure calculation.

A total of 1670 NOE-derived distance constraints (~17.5 NOE constraints/residue) were used for the structure calculation of NLRP7 PYD. The 20 conformers from the final CYANA cycle with the lowest residual CYANA target function values were energy-minimized in a water shell with CNS (44) using the RECOORD (45) script package (Table 1). The NLRP7 PYD model has excellent stereochemistry, with 98.1% of the residues in the most favored and additionally allowed regions of the Ramachandran diagram, 1.3% in the generously allowed region, and 0.6% in the disallowed region. The quality of the structures was assessed by the programs WHATCHECK (46), AQUA (47), NMR-PROCHECK (47), and MOLMOL (48). All of the structure comparisons throughout the manuscript were performed using the lowest energy conformer of the bundle of structures.

**Auto-correlated and Cross-correlated Relaxation Measurements**

15N longitudinal ($R_1$) and transverse ($R_2$) relaxation rates and the 15N(1H)-NOE (hetNOE) were measured as described previously (49–51) at 500 MHz. All of the spectra were recorded with 2048 points in the direct dimension and 256 points in the indirect dimension. The sweep widths of the direct and indirect dimensions were 6010 and 1242 Hz. $T_2$ was sampled at 20, 80, 110, 140, 180, 220, 250, and 350 ms; $T_1$ was measured at 20, 100, 180, 250, 320, 380, 450, and 650 ms. For the hetNOE measurement, an experiment with proton presaturation was interleaved with an experiment without proton presaturation. The relaxation delay for all $T_1$, $T_2$, and hetNOE experiments was 3 and 5 s for all of the hetNOE experiments. For accurate error estimations, two complete sets for $T_1$ and hetNOE and three complete sets of $T_2$ spectra were recorded.

One set of $T_2$ spectra was recorded at 800 MHz ($T_2$ sampled at 20, 80, 110, 140, 180, 220, 250, and 350 ms). Cross-correlation rates between the 15N chemical shift anisotropy and 15N-1H dipole-dipole interactions ($\eta_{xy}$; mixing time, 60 ms) were measured at 500 MHz (52). One set of $T_1$ spectra (on-resonance, 2 kHz field) was recorded at 500 MHz ($T_1$ sampled at 12, 24, 48, 72, 96, 120, 144, and 180 ms).

All of the relaxation spectra were processed using NMRPipe version 97.027.12.56 (53), and peak intensities were analyzed with NMRView version 5.2.2.01 (54). The exponential decay function $I = Ae^{-(t/r)}$ (where $I$ is the peak intensity, $A$ is the amplitude, $t$ is the relaxation delay, and $r = R_1$ or $R_2$) was fit to the peak intensities using the jitter function in NMRView to determine the $R_1$, $R_{1\mu}$, and $R_2$ relaxation rates. hetNOEs were calculated by dividing the intensity of the peaks in the spectra without presaturation by the intensity of the peaks in the presaturated spectra. The data from overlapped peaks were excluded from the analysis. A Modelfree relaxation analysis (isotropic diffusion) was performed using the program TEN-SOR (55). 15N chemical shift anisotropy and 15N-1H dipole-
dipole cross-correlation rates $\eta_{n\beta}$ were calculated as described in Refs. 52 and 56; values of $r_{NH} = 1.02 \text{ Å}$, $\Delta \sigma_N = -160 \text{ ppm}$, and $\beta = 22^\circ$ were used for the calculation of $\kappa$.

Chemical Shift Assignments and Coordinates

Chemical shift assignments of NLRP7 PYD were deposited in the Biological Magnetic Resonance Data Bank under accession number 16263 (38), and the coordinates were submitted to the Protein Data Bank under accession code 2KM6.

RESULTS

Protein Expression and Purification—Numerous NLRP7 PYD constructs with different C-terminal lengths were tested for overexpression. NLRP7_{1-96} (NLRP7 PYD) showed the highest overexpression of soluble and folded protein and was subsequently used for all structural studies. NLRP7 PYD is a monomer in solution as verified by size exclusion chromatography, where a sharp peak containing NLRP7 PYD corresponding to a molecular mass of a ~12 kDa was detected, as identified by SDS-PAGE analysis. The monomeric state of NLRP7 PYD was further confirmed by dynamic light scattering, which showed an $R_g$ of 1.77 nm, an expected radius for a 12.7-kDa globular protein. The ASC PYD domain was expressed and refolded as described under “Experimental Procedures.” The FAF-1 FID domain (residues 1–180) was produced in a similar manner to the NLRP7 PYD. The FAF-1 FID domain is a monomer in solution as verified by size exclusion chromatography.

NLRP7 PYD Structure Determination and Analysis—The NLRP7 PYD three-dimensional structure adopts a typical death domain fold (Fig. 1, A and B), which is comprised of six $\alpha$-helices, $\alpha_1$–$\alpha_6$, that fold into an anti-parallel $\alpha$-helical bundle. The $\alpha$-helical bundle tightly packed around a central hydrophobic core comprised of residues Leu$^6$, Leu$^{10}$, Leu$^{13}$, Leu$^{14}$, and Leu$^{17}$ from $\alpha_1$; Leu$^{22}$, Phe$^{25}$, and Leu$^{29}$ from $\alpha_2$; Leu$^{34}$ and Leu$^{38}$ from the $\alpha_3$ loop (residues 30–35); and Leu$^{60}$ and Leu$^{63}$ from $\alpha_4$, which position $\alpha_3$ with respect to $\alpha_2$ (Fig. 1D).

Thr$^{41}$ and Trp$^{43}$ from $\alpha_3$, which position $\alpha_3$ with $\alpha_2$ (Fig. 1D). Short linker regions, including residues 18–20, 30–35, 48–50, 61–64, and 79–82 connect the six helices (Fig. 1B). All of the loops are well defined by dense NOE networks that connect the loops with the six $\alpha$-helices (Fig. 1A).

NLRP7 PYD and the PYDs of NLRP1, ASC, and ASC2 Show Distinct Structural Features—The PYD domains of NLRP7, NLRP1 (24% sequence identity; Protein Data Bank accession code 1PN5) (34), ASC (27% sequence identity; Protein Data Bank accession code 1UCP) (31, 57), and ASC2 (25% sequence identity; Protein Data Bank accession code 2HM2) (32) only share modest primary sequence similarity (Fig. 2). ASC2 (also referred to as POP1 and ASCI) functions as a dominant negative inhibitor of ASC and other PYD-containing proteins
involved in NF-κB and caspase-1 activation. The structure of mouse NLRP10 has also been determined (Protein Data Bank accession code 2DO9), but because the structure determination of the mNLRP10 PYD has not been published and no dynamics data have been reported for this domain, we refer to our comparison with the mNLRP10 PYD in supplemental Figs. S1–S3.

Despite the low overall sequence similarity, multiple hydrophobic residues that are essential for the formation of the PYD hydrophobic core are highly conserved, not only among these PYD domains but also across the entire NLRP1–14 family. These residues include Leu14, Leu17, Phe25, and Leu58. Although the hydrophobic core of PYDs is conserved, the architecture of the helices, especially helix α3 and the loop connecting helices α2 and α3, differs significantly (Fig. 3A). The α2-α3 loop and helix α3 are well ordered in NLRP7 PYD. The well ordered α2-α3 loop in NLRP7 is strongly anchored by six hydrophobic residues (Trp30, Phe32, Leu35, Leu38, Thr41, and Trp43; Fig. 3B, left panel). An α-helical turn can be identified for residues Val17–Lys39 in NLRP7 PYD. Similarly, the α2-α3 loops of ASC and ASC2 are also well ordered and adopt a similar structure, although helix α3 in ASC2 is shorter by one turn. Four or three hydrophobic residues anchor the α2-α3 loop in ASC and ASC2, respectively (Leu38, Leu32, Tyr36, and Ile39 in ASC; Leu32, Phe36, and Ile39 in ASC2; Fig. 3B, middle panels). In addition, hydrophobic and polar interactions between helix α3 and α4 (Fig. 3, C and D) further stabilize helix α3 in the NLRP7, ASC, and ASC2 PYDs.

In contrast, no α2-α3 loop hydrophobic residues or hydrophobic contacts between helices α2, α3, and α4 can be identified in the NLRP1 PYD that could contribute toward any conformational stability of this loop (Fig. 3, B and D, right panels). As a result, helix α3 is missing in the NLRP1 PYD structure, and the α2-α3 loop is highly dynamic, as shown by dynamics NMR measurements (34), and extended toward helix α4.

To further quantify these differences in hydrophobic interactions, the hydrophobic surface buried by helices α2 and α3 and the α2-α3 loop was calculated using GetArea1.1 (58): NLRP1 PYD, 1763 Å²; ASC2 PYD, 2026 Å²; ASC PYD, 2199 Å²; and NLRP7 PYD, 2266 Å². These calculations show a large 25% increase in hydrophobic surface burial between the NLRP1 and NLRP7 PYDs (this increase in hydrophobicity is highlighted by a black arrow in Fig. 3B).

The significant increase of the number of hydrophobic residues in the α2-α3 loop and helix α3 from NLRP1 to NLRP7 allows for the formation of an exceedingly stable second hydrophobic cluster in the NLRP7 PYD (Fig. 1D). These hydrophobic residues are also responsible for the differential flexibility of the α2-α3 loop (described in the next section), as well as the formation of helix α3. We used the program AGADIR (59) to predict the helical behavior of the residues between the C termini of helices α2 and α3. The calculations show very low propensities for helix formation (<1%), confirming that the presence of helix α3 in the structures of NLRP7, ASC, and ASC2 PYDs is due primarily to tertiary contacts within the proteins.

Thus, although the conserved central hydrophobic cores of PYDs are expected to lead to a similar fold for helices α1, α2, and α4–α6 (Figs. 1C and 2) for all PYDs, further differences in the conformations of the α2-α3 loop and helix α3 are expected.
The NLRP7, NLRP1, ASC, and ASC2 PYDs Have Distinct Electrostatic Surfaces—Electrostatic surface charge plays a major role in the formation and regulation of death domain protein-protein interactions (60). Initial work on the RAIDD CARD, as well as caspase-9 and Apaf-1 CARD domains, showed the importance of highly charged surface patches for the specific interaction of CARD domains (35, 61). A similar mode of electrostatic interaction was also identified for the PYD-PYD interaction of ASC PYD and ASC2 PYD (37). Critically, the PYD-PYD interaction between NLRP1 PYD and ASC PYD and, in turn, the CARD-CARD domain interaction between the ASC CARD and caspase-1 CARD domains are essential for the formation of the inflammasome. Only NLRP1, NLRP3, and NLRC4 have been identified so far to form inflammasomes with clear physiological roles (14).

The electrostatic surface of ASC PYD is distinctively two-faced. A highly positively charged surface is formed near helices \(a2\) and \(a3\), and a negatively charged surface is observed near helices \(a1\) and \(a4\) (Fig. 5, A and D, respectively). ASC PYD binds via its positively charged helices \(a2\) and \(a3\) surface to the negative charged surface formed by residues from helices \(a1\) and \(a4\) of ASC PYD (comprised of residues Lys\(^{23}\), Lys\(^{22}\), Arg\(^{36}\), and Arg\(^{41}\) and residues Asp\(^{6}\), Asp\(^{10}\), Glu\(^{13}\), Asp\(^{48}\), Asp\(^{51}\), and Asp\(^{54}\) from ASC2 and ASC, respectively). Thus oppositely charged electrostatic surfaces are important for mediating these PYD-PYD interactions (37).

Fig. 5 compares the electrostatic surface of NLRP7 PYD with those of ASC, ASC2, and NLRP1. We used the Protein Interaction Property Similarity Analysis server (62) to quantify the similarities between the electrostatic surfaces of the PYDs from NLRP1, NLRP7, ASC, and ASC2. The Protein Interaction Property Similarity Analysis server uses the University of Houston Brownian Dynamics program to calculate the electrostatic potential of a protein. Pairwise calculations comparing the NLRP7 PYD to the PYDs of ASC, ASC2, and NLRP1 were used to assess overall electrostatic similarity, where 0 indicates identical electrostatic surfaces, and 2 indicates completely different electrostatic surfaces. Based on an overall electrostatic distance, NLRP7 PYD is
most similar to ASC PYD (electrostatic distance, 1.115) followed by NLRP1 PYD (electrostatic distance, 1.145) and ASC2 PYD (electrostatic distance, 1.293), although none of the other PYDs are identical/highly similar to the NLRP7 PYD (i.e. an electrostatic distance between 0 and 0.75).

In fact, a closer look at specific surfaces, such as the α2 and α3 and α1 and α4 interaction surfaces (hereafter referred to as interaction patches) illustrates distinct differences in the electrostatic surfaces of these PYDs. ASC2 PYD has the most similar interaction patch electrostatic surface to ASC PYD. Conversely, the interaction patch electrostatic surfaces of NLRP1 PYD and NLRP7 PYD are highly dissimilar. NLRP1 PYD has only a small positively charged interaction patch, similar to ASC PYD and ASC2 PYD, formed mainly by Lys22 on helix α2. This positively charged patch is completely absent on NLRP7 PYD. Indeed, this surface of the NLRP7 PYD is nearly completely hydrophobic with small negatively charged patches. Conversely, the negative charges on opposing surfaces, especially Glu15 and Glu56 from helices α1 and α4, respectively, are much more similar between the PYDs of NLRP7, ASC, and ASC2. Indeed, the NLRP1 PYD has the least negatively charged electrostatic surface at this interaction patch.

The PYDs of ASC and ASC2 have structurally opposed positively and negatively charged electrostatic interaction patches. Critically, these oppositely charged patches have also been shown to be essential for mediating homotypic protein-protein interactions between these PYDs. These electrostatic surfaces of these interaction patches are significantly different for the PYDs of NLRP1 and NLRP7, with the positively charged surface patch necessary for a potential ASC PYD interaction completely missing in NLRP7 PYD. This observation predicts that a protein-protein interaction between the PYDs of ASC and NLRP7 will not occur. To confirm this hypothesis, we experimentally tested the interaction of the NLRP7 PYD with ASC PYD using NMR spectroscopy. Unlabeled ASC PYD was titrated into 15N-labeled NLRP7 PYD. No chemical shift changes in the NLRP7 PYD two-dimensional 1H,15N HSQC spectrum were identified upon titration with ASC PYD (1:1 molar ratio), confirming our hypothesis that the NLRP7 PYD does not bind directly to the ASC PYD (supplemental Fig. S6).

NLRP7 PYD Does Not Interact Directly with FAF-1—FAF-1 is a multidomain protein and a conserved pro-apoptotic factor that also has a central role in the regulation of NF-κB. Recently, it was reported that NLRP3, NLRP7, and NLRP12 interact with FAF-1 via its Fas-interacting domain (FID) (19). The reported interaction studies showed that the interaction must be mediated by residues 1–180 of the FAF-1 FID domain. The FAF-1 FID domain consists of an N-terminal UBA domain, known to interact with polyubiquitinated proteins, as well as a C-terminal adjacent UB1, which has a typical ubiquitin fold and is implicated in the inhibition of NF-κB signaling.

We used NMR spectroscopy to experimentally test the potential direct interaction of NLRP7 PYD with the FAF-1 FID domain. This is of interest because it would be the first nonhomotypic PYD interaction ever confirmed in vitro. The unlabeled FAF-1 FID domain was titrated into 15N-labeled NLRP7 PYD. No chemical shift changes in the NLRP7 PYD two-dimensional 1H,15N HSQC spectrum were identified upon titration.

**FIGURE 5.** Helices α2 and α3 form a predominantly hydrophobic surface on NLRP7 PYD. A, electrostatic surface representation of PYD of NLRP7, ASC, ASC2, and NLRP1. Blue, positive charge; red, negative charge. B, residues responsible for these charges are highlighted on the lowest energy conformer of each structure in pink (NLRP7 (light blue), ASC (salmon), ASC2 (gold), and NLRP1 (green)). C, identification of the second PYD interaction site (rotation Y = -130°; X = 30°) formed by residues on helices α1 and α4. Residues responsible for these charges are highlighted on the lowest energy conformer of each structure in pink. D, corresponding electrostatic surface representation of the PYDs of NLRP7, ASC, ASC2, and NLRP1. The arrows indicate experimentally confirmed direct homotypic PYD-PYD interactions.
with FAF-1 FID (1:1 and 1:10 molar ratio; supplemental Fig. S7), demonstrating that the NLRP7 PYD does not interact directly with the FAF-1 FID domain.

DISCUSSION

PYDs are the most recently identified members of the DD superfamily (60). Indeed, PYDs are now considered the most common DD. However, the currently available structures have not yet revealed their differential modes of interaction. Rather they have raised additional questions, especially because the region that corresponds to helix α3 in the DD fold was completely disordered in the structure of NLRP1 PYD (34), which in turn has led to the proposal that conformational change may play a role in PYD-PYD binding. The interest in PYDs stems from their central role in the control of signaling pathways in the immune system, as well as in many apoptotic pathways. Thus it has been suggested that cross-talk among effectors is modulating signaling between the immune system and apoptosis. How this is mediated at a molecular level is still poorly understood.

Here we report the three-dimensional structure of the human NLRP7 PYD. Conserved hydrophobic residues are essential for the formation of the DD fold of helices α1, α2, and α4–α6. The NLRP7 PYD has a significant second hydrophobic cluster that allows for the formation of helix α3 and anchors it to helix α2. Furthermore, this hydrophobic cluster also stabilizes the only extended loop between secondary structural elements, the α2-α3 loop. This is important, because the three-dimensional structure of the NLRP7 PYD is significantly different from the NLRP1 PYD and much more similar to the PYDs of ASC and ASC2, two essential proteins in innate immune system signaling. This second hydrophobic cluster is entirely absent in the NLRP1 PYD, resulting in a highly disordered α2-α3 loop that prohibits the formation of helix α3. Notably, primary sequence comparison of the NLRP7 PYD with other PYDs (Fig. 2B) demonstrates that this second hydrophobic cluster is generally conserved, and we therefore predict that the NLRP7 PYD is the prevalent fold for most of the 14 identified NLRP PYDs.

Both electrostatic and, to a lesser extent, hydrophobic interactions are important for DD protein-protein interactions. For example, oppositely charged surfaces have been identified as a critical selector of homotypic CARD-CARD and PYD-PYD interactions (35, 37), which likely become subsequently stabilized by additional hydrophobic interactions. In ASC PYD, lysine and arginine residues on helices α2 and α3 form a highly positively charged surface. Negatively charged residues (glutamic and aspartic acids) on helices α1 and α4 of ASC2 are complementary and allow for a strong and specific interaction between the ASC and ASC2 PYDs.

Interestingly, the NLRP7 PYD surface is unique compared with that of ASC, ASC2, and NLRP1 PYD. In the NLRP7 PYD, the positively charged residues on helices α2 and α3 are mostly missing. The α2 and α3 surface is predominantly hydrophobic, and consequently the NLRP7 PYD does not have the typical PYD positively/negatively charged surfaces on opposing sides of the protein. Indeed, for NLRP1 and ASC PYDs, either a highly charged fusion protein (GB1) or a low pH of 3.7, respectively, was required to produce a 1 mM sample for the subsequent successful structure determination. In contrast, for NLRP7 PYD, it was possible to use low salt conditions, a much more commonly used pH of 6.0, and no highly charged GB1 fusion protein to concentrate the sample to 1 mM, without the occurrence of aggregation or multimer formation. This behavior is similar to that seen for ASC2 PYD. Clearly, additional PYD structures must be determined to understand this behavior.

These significantly different electrostatic surfaces are likely one of the reasons for differential downstream signaling by the NLRP family. The NLRP1 PYD interacts with the ASC PYD to form the NLRP1 inflammasome (63). Conversely, no inflammasome formation has been reported so far for NLRP7. Based on the differential electrostatic surfaces, it seems unlikely that a charge-driven interaction such as NLRP1 PYD-ASC PYD can also be formed between NLRP7 PYD and ASC PYD. We confirmed the absence of such a direct interaction via an NMR titration experiment. Thus the inability of NLRP7 PYD to bind ASC PYD most likely prevents the formation of the NLRP7 inflammasome. However, the differential electrostatic surface of NLRP7 PYD likely allows for differential downstream signaling, leading to a negative regulation of pro-IL-1β and procaspase-1 processing.

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