The NLRP12 Pyrin Domain: Structure, Dynamics, and Functional Insights

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The initial line of defense against infection is sustained by the innate immune system. Together, membrane-bound Toll-like receptors and cytosolic nucleotide-binding domain and leucine-rich repeat-containing receptors (NLR) play key roles in the innate immune response by detecting bacterial and viral invaders as well as endogenous stress signals. NLRs are multi-domain proteins with varying N-terminal effector domains that are responsible for regulating downstream signaling events. Here, we report the structure and dynamics of the N-terminal pyrin domain of NLRP12 (NLRP12 PYD) determined using NMR spectroscopy. NLRP12 is a non-inflammasome NLR that has been implicated in the regulation of Toll-like receptor-dependent nuclear factor-κB activation. NLRP12 PYD adopts a typical six-helical bundle death domain fold. By direct comparison with other PYD structures, we identified hydrophobic residues that are essential for the stable fold of the NLRP PYD family. In addition, we report the first in vitro confirmed non-homotypic PYD interaction between NLRP12 PYD and the pro-apoptotic protein Fas-associated factor 1 (FAF-1), which links the innate immune system to apoptotic signaling. Interestingly, all residues that participate in this protein:protein interaction are confined to the α2–α3 surface, a region of NLRP12 PYD that differs most between currently reported NLRP PYD structures. Finally, we experimentally highlight a significant role for tryptophan 45 in the interaction between NLRP12 PYD and the FAF-1 UBA domain.

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Introduction

Vertebrates have evolved numerous strategies to defend against environmental pathogens, including the innate and adaptive immune responses. Innate immunity is the first line of host defense against pathogenic infection and relies on the detection of pathogen- and danger-associated molecular patterns by a set of germ-line-encoded receptors collectively called pattern-recognition receptors. Among pattern-recognition receptors, the membrane-anchored Toll-like receptors (TLRs) have emerged as essential components of innate immunity. They recognize diverse pathogen-derived molecules, causing the activation of intracellular signaling cascades that ultimately lead to an inflammatory response.1–4

During the last 10 years, it has become clear that TLRs are not the only pathogen- and danger-associated signals sensors in innate immunity. Nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) are expressed in the cytoplasm, and their importance in pathogen-associated molecular pattern and danger-associated molecular pattern recognition is rapidly growing.5,6 Additionally, numerous human auto-inflammatory disorders have been associated with mutations in NLRs, emphasizing their role as central regulators of disorders have been associated with mutations in NLRs, emphasizing their role as central regulators of immunity. They recognize diverse pathogen-derived molecules, causing the activation of intracellular signaling cascades that ultimately lead to an inflammatory response.1–4

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NLRs possess a tripartite architecture. They are composed of a C-terminal leucine-rich repeat domain, necessary for ligand binding; a central NACHT domain, capable of ATP binding and responsible for oligomerization; and an N-terminal effector domain, linking the NLR to downstream signaling cascades.8 The human genome encodes for 22 NLRs, which are further grouped into subfamilies according to their N-terminal effector domain (pyrin domain (PYD), caspase activation and recruitment domain (CARD), or baculovirus inhibitor repeat domain (BIR)). NLRs containing a pyrin domain (NLRP) constitute the largest subfamily of NLRs (NLRP1-14).9 Two members of the NLRP family, NLRP1 and NLRP3, activate the innate immune response via their interaction with the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) and, subsequently, procaspase-1. This multicomponent complex is called the inflammasome,10,11 a macromolecular machine that activates the cleavage of pro-inflammatory cytokines such as pro-IL-1β and pro-IL-18 into their mature, secreted forms.12–14

Other members of the NLRP family regulate key functions in the immune system without forming an inflammasome. For example, some NLRPs including NLRP12 (previously called Monarch-1 or PYPAF-7) regulate nuclear factor-κB (NF-κB) activation.12–14 NLRP12 was one of the earliest identified NLRPs and its expression is restricted to myeloid cells.15 Furthermore, mutations in NLRP12 are linked to hereditary periodic fevers and atopic dermatitis, stressing its role in immunity and inflammation.16,17

Through the use of gene silencing, it was recognized that NLRP12 fine-tunes downstream signaling via down-regulation of TLR-dependent NF-κB activation. Subsequently, NLRP12 suppresses the production of pro-inflammatory cytokines by inhibiting noncanonical NF-κB activation. This regulation is likely achieved via the inhibition of necessary processing kinases, especially the noncanonical MAP3 kinase NIK (NF-κB-inducing kinase).13,15 In addition, NLRP12 has been reported to interact with the chaperone Hsp90 and postulated to play a role in the proteasome pathway.18 Although the precise mechanism by which NLRP12 inhibits NIK-induced NF-κB activation is unknown, it is thought that NLRP12 drives the degradation of NIK via a proteasome-dependent pathway. Recent reports speculate that this modulation can be achieved via the interaction of effector domains with “adaptor-like” proteins, such as Fas-associated factor 1 (FAF-1),19–21 and thus links the innate immune system to apoptotic signaling.22

We23,24 and others25–29 have recently shown that NLRP PYDs can vary in structure and dynamics and, as a result, in surface charge, a critical parameter directing their protein:protein interactions. Thus, to understand the biological function of PYDs in general and NLRP12 PYD specifically, we determined the structure and auto-correlated fast timescale backbone dynamics of NLRP12 PYD. Interestingly, we identified differences among all currently published NLRP PYD structures in regions described to be central for homotypic PYD:PYD interactions. Finally, we tested the previously proposed heterotypic interaction of NLRP12 PYD with the pro-apoptotic protein FAF-1 in vitro. By testing several different FAF-1 single- and multi-domain fragments, we identified a direct interaction of NLRP12 PYD with the UBA domain of FAF-1.

Results

Protein expression and purification

The N-terminal effector PYD of NLRP12 (residues 1–98, two N-terminal cloning artifacts; herein referred as NLRP12 PYD) showed high levels of soluble overexpression in Escherichia coli and was readily purified to homogeneity. NLRP12 PYD is a monomer in solution as verified by size-exclusion chromatography. Under low salt conditions (100 mM NaCl), NLRP12 PYD precipitates at a concentration higher than 0.2 mM. This is not surprising, as PYDs are known for their poor solubility and tendency to aggregate.26 To achieve
concentrations of NLRP12 PYD sufficient for accurate structural and dynamics studies, 500 mM NaCl was necessary. Under these conditions, NLRP12 PYD is readily concentrated to 0.6 mM without precipitation or aggregation and stable during the course of several weeks.

FAF-1 constructs (FAF-11–99, FAF-11–57, and FAF-199–180) were overexpressed in E. coli and purified to homogeneity. An maltose-binding protein (MBP) tag was used to enhance the solubility of FAF-11–57 during expression. All FAF-1 constructs are monomers in solution as verified by size-exclusion chromatography.

Three-dimensional structure of NLRP12 PYD

The solution structure of NLRP12 PYD was determined using heteronuclear NMR spectroscopy. Assignments were obtained for 95% of the backbone nuclei (N, HN, C’, Cα, Hα) and 95% of the side-chain 13CHn moieties. Of the 96 expected backbone amide NH pairs (3 prolines), 93 were identified; the missing assignments correspond to the N-terminal two-residue cloning artifact Gly2 and His1, as well as Thr4 (Supplemental Fig. S1). All aliphatic and aromatic side-chain resonances that are routinely observed were assigned, except those of residues Gly2, His1, Arg3, Ser13, Lys29, and Glu100, where confident assignments were uncertain. An ensemble of 100 structures was calculated from 1859 nuclear Overhauser enhancement spectroscopy (NOESY)–derived distance constraints [∼18 nuclear Overhauser enhancement (NOE) constraints/residue] using a simulated annealing protocol within the program CYANA30 and refined in explicit solvent using CNS.31 The 20 lowest-energy structures of NLRP12 PYD are shown in Fig. 1a. All structures have excellent geometry, with no violations of distance restraints greater than 0.5 Å and no dihedral angle violations greater than 5° (Table 1). In addition, all structures have excellent stereochemistry, with 98.8% of residues in the most favored and additionally allowed regions of the Ramachandran diagram, 0.8% of residues in the generously allowed region, and 0.5% of residues in the disallowed region (Table 1). The NLRP12 PYD structure is well defined, with the exception of the N- and C-termini, residues 2 to 9 and 92 to 100, respectively, which are flexible (Supplemental Fig. S2a and b).

As expected, NLRP12 PYD folds into a tightly packed helical bundle (residues 10–91) consisting of six helices (α1–α6) arranged in an antiparallel fashion (Fig. 1b), characteristic of PYDs. However, unlike other PYD structures, NLRP12 PYD helix α3 only forms a short 310 helix. The residues forming the six helices are 10–19 (α1), 22–34 (α2), 47–50 (α3–310 helix), 53–64 (α4), 66–80 (α5), and 83–91 (α6).

Table 1. Structural and CNS refinement statistics

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Structural quality

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<td>All heavy atoms (10–91)</td>
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Fig. 1. NMR structure of NLRP12 PYD. (a) Ensemble of the 20 lowest-energy structures calculated for NLRP12 PYD superimposed on the backbone atoms of residues 10–91 (PDB ID 2L6A). The six helices, characteristic of the DD fold, are highlighted in light pink, while loops are highlighted in gray. (b) Ribbon representation of the lowest-energy conformer of NLRP12 PYD in an orientation identical with that shown in (a). The N- and C-termini as well as the six helices are labeled. (c) Top view of NLRP12 PYD [rotated by 90° about the x-axis relative to (a) and (b)]. Residues forming the central hydrophobic core are shown as cyan sticks.
The NLRP12 PYD six-helical bundle is stabilized by a central hydrophobic core formed by residues Leu13, Tyr16, Leu17, and Leu20 from helix α1; Leu25, Phe28, and Leu32 from helix α2; Met56, Leu60, and Phe64 from helix α4; Ala69, Trp70, Ala73, Phe77, and Ile80 from helix α5; and Leu85 from helix α6 (Fig. 1c). All α-helices in the structure of NLRP12 PYD are connected by short well-defined loops, with the exception of the loop that connects helices α2 and α3 (310 helix), herein called the α2–α3 loop. The α2–α3 loop comprises residues 35–46. Despite the fact that it does not contain any regular secondary structure, the α2–α3 loop is quite ordered in the structure of NLRP12 PYD. This order in the α2–α3 loop is due to hydrophobic interactions of residues Leu38 and Ile43 both within the α2–α3 loop itself and with neighboring residues, especially residue Phe64 in helix α4 (Supplemental Fig. S3).

Backbone dynamics of NLRP12 PYD

The PYDs of NLRP1 and NLRP7 show distinct dynamics behaviors, especially in the α2–α3 loop, which exhibits substantially increased fast timescale dynamics in NLRP1 PYD (picosecond–nanosecond as detected by $^{15}$N$[^1]$H-NOE measurement) and slow timescale dynamics in NLRP7 PYD (microsecond as detected by $^{15}$N CSA, $^{15}$N$[^1]$H dipole–dipole cross-correlation relaxation rates, and on-resonance $R_1^\rho$ measurements).\(^\text{24,26}\) To investigate the dynamics in NLRP12 PYD, we measured auto-correlated $^{15}$N-relaxation data, including measurements of $^{15}$N$[^1]$H-NOE, as well as $^{15}$N longitudinal ($R_1$) and transverse ($R_2$) relaxation rates. Using a model-free analysis, we calculated a correlation time ($\tau_c$) of $\sim$7 ns for NLRP12 PYD at 298 K. Comparison of the $^{15}$N$[^1]$H-NOE data, which reports on fast timescale (picosecond–nanosecond) backbone motions, of the PYDs of NLRP1, NLRP7, and NLRP12 is shown in Fig. 2. The dynamics of the α2–α3 loop in NLRP12 PYD are much more similar to the dynamics of NLRP7 PYD than those of NLRP1 PYD. NLRP12 PYD shows a small increase in fast timescale dynamics in the α2–α3 loop, but no slow-intermediate timescale dynamics, when compared to NLRP7 PYD. Indeed, the dynamics detected for NLRP12 PYD seem to be most similar to the dynamics reported for the PYD of the inhibitor protein ASC\(^\text{27}\).

A $\sim$10% cis/trans proline isomerization for Pro33 and Pro42 in NLRP7 PYD and Pro42 in NLRP1 PYD was reported. These proline residues flank the α2–α3 loop. In contrast, no significant cis/trans proline isomerization was detected in NLRP12 PYD.

Structural and dynamics comparison of NLRP12 PYD with members of the NLRP family

Despite a relatively low sequence identity between NLRP12 PYD and the PYDs of NLRP7 [29% sequence identity; Protein Data Bank (PDB) ID 2KM6] and NLRP1 (36% sequence identity; PDB ID 1PN5), their structures superimpose well with RMSD values of 2.4 Å for the backbone atoms between NLRP12 and NLRP7 PYDs and 3.1 Å between NLRP12 and NLRP1 PYDs. Interestingly, a structure-based sequence alignment identified that the conserved residues between all three PYD structures form the hydrophobic core of the PYD fold. This hydrophobic core is highly conserved among the entire NLRP family (NLRP1–14) and, therefore, defines the overall fold of NLRP PYDs (Fig. 3).

Superposition of the structure of NLRP12 PYD with those of NLRP1 and NLRP7 PYDs revealed small differences in the length and orientation of the helices. In particular, helices α1 and α6 differ in overall length between the three PYD structures. Furthermore, the relative angle of helix α2 with respect to the overall helical bundle shows the largest difference between the structures of NLRP12 and NLRP7 PYDs.

Clearly, the most significant differences among the three PYD structures are helix α3 and the preceding well-formed, stable secondary structural elements. While the α2–α3 loop and helix α3 in NLRP1 PYD show a substantial increase in fast timescale backbone dynamics, these motions are missing in NLRP7 PYD. The α2–α3 loop and helix α3 in NLRP12 PYD show increased flexibility when compared to NLRP7 PYD.
The NLRP12 Pyrin Domain

Fig. 3. Sequence alignment of human NLRP PYDs. Residues contributing to the hydrophobic core of NLRP12 PYD are conserved among the entire NLRP family and are highlighted by gray boxes. Experimentally derived secondary structure elements of NLRP12 PYD are depicted by gray cylinders on top of the figure.

α2–α3 loop. The overall length of NLRP12 PYD is shorter than that of NLRP7 and NLRP1 PYDs, and the structure-based sequence comparison (Fig. 3) identified a two-amino-acid deletion in the α2–α3 loop region of NLRP12 PYD. This deletion has multiple consequences. First, helix α3 (two turns) in NLRP7 PYD is replaced by a 3_10 helix in NLRP12 PYD. Furthermore, this secondary structure element is entirely missing in NLRP1 PYD, where α3 is replaced by a flexible disordered loop that connects helix α2 to helix α4. Second, the dynamics of the α2–α3 loop in all three NLRP PYDs differ. While the α2–α3 loop in NLRP1 PYD shows significantly increased fast timescale dynamics, the α2–α3 loop
is rigid in NLRP7 PYD. In NLRP12 PYD, the α2–α3 loop is slightly more flexible than in NLRP7 PYD but much more rigid than in NLRP1 PYD. Previously, we identified a hydrophobic cluster (consisting of six hydrophobic residues) that stabilizes both the α2–α3 loop and helix α3 in NLRP7 PYD. Interestingly, this hydrophobic cluster only contains three hydrophobic residues in NLRP12 PYD. The other three hydrophobic residues in NLRP12 PYD are replaced by two glycines and one alanine, smaller nonpolar residues, that likely account for the reduced rigidity of the α2–α3 loop. Strikingly, NLRP1 PYD lacks all six hydrophobic residues entirely (Fig. 4a and b). Thus, the dynamics of the α2–α3 loop in NLRP12 PYD are more similar to those of NLRP7 PYD than to those of NLRP1 PYD, and these differences are directly correlated with the size of the hydrophobic cluster.

The hydrophobic cluster in NLRP7 PYD that stabilizes the α2–α3 loop is anchored by two stacking Trp side chains (residue Trp30 and Trp43). However, Trp30 of NLRP7 PYD is replaced

![Fig. 4. Structural comparison of NLRP PYDs. (a) NLRP12 PYD (light pink) overlaid with the PYDs of NLRP7 (light blue; RMSD, 2.4 Å) and NLRP1 (light green; RMSD, 3.1 Å). The pairwise RMSD between NLRP12 PYD and all other PYDs was calculated by superposition of helices α1–α6. The largest structural difference is localized to the α2–α3 loop as well as helix α3, which is illustrated in the current orientation. (b) Hydrophobic residues in the α2–α3 loop as well as helix α3 are highlighted as dark-blue sticks and labeled. A six-residue hydrophobic cluster stabilizes the α2–α3 loop as well as helix α3 in NLRP7 PYD. The corresponding cluster in NLRP12 PYD consists only of two hydrophobic residues. NLRP1 PYD lacks all six hydrophobic residues. (c) Residues Gly33 and Trp45 in NLRP12 PYD, as well as Trp30 and Trp43 in NLRP7 PYD, are highlighted as dark-red sticks and labeled. While Trp43 in NLRP7 PYD is buried and forms stacking interactions with Trp30, the corresponding Trp45 in NLRP12 PYD is surface exposed.](image-url)
in NLRP12 PYD by a Gly residue (Gly33). This substitution has two effects. First, it plays a key role in the increased flexibility of the α2–α3 loop displayed by NLRP12 PYD. Second, it also changes the role of NLRP12 PYD Trp45 (Trp43 is the corresponding residue in NLRP7 PYD). Instead of being highly buried, as it is in NLRP7 PYD, it becomes surface exposed in NLRP12 PYD (Fig. 4c).

**Structural comparison of NLRP12 PYD among the death domain superfamily**

The most similar structures to NLRP12 PYD, based on Dali z-scores, are the structures of ASC2 PYD (z-score, 10.5) and ASC PYD (z-score, 10.4). The overall length of NLRP12 PYD is shorter than that of the ASC2 PYD, and a structure-based sequence comparison also identified a two-amino-acid deletion in the α2–α3 loop. Similar assessments are possible for the ASC PYD. Nevertheless, this has very little influence in the overall structure, as well as dynamics of the α2–α3 loop.

The electrostatic surface of PYDs has two distinct faces. One surface is formed by helices α2 and α3, and a second surface is composed of helices α1 and α4. In both the ASC and ASC2 PYDs, these surfaces are highly charged and complementary with one another. The α2–α3 electrostatic surface of NLRP12 PYD is also charged but also has a significant hydrophobic patch clustered around Trp45 on helix α3.

**Figure 5** compares the electrostatic surface of NLRP12 PYD to those of ASC, ASC2, NLRP7, and NLRP1. We used the PIPSA (Protein Interaction Property Similarity Analysis) server to quantify the similarities between the electrostatic surfaces of the PYDs from NLRP1, NLRP7, ASC2, ASC, and ASC2. The PIPSA server uses the UHBD program to calculate the electrostatic potential of a protein. Pairwise calculations comparing the NLRP12 PYD to the PYDs of ASC, ASC2, NLRP7, and NLRP1 were used to assess overall electrostatic similarity, where 0 indicates identical and 2 indicates completely different electrostatic surfaces. Based on an overall electrostatic distance, NLRP12 PYD is most similar to ASC PYD (electrostatic distance, 0.634) followed by NLRP7 PYD (electrostatic distance, 0.994), ASC2 PYD (electrostatic distance, 1.324), and NLRP1 PYD (electrostatic distance, 1.339). Therefore, based on this analysis, ASC PYD can be classified as identical/highly similar to the NLRP12 PYD (i.e., an electrostatic distance between 0 and 0.75).

Numerous reports have argued that differences in surface charge/hydrophobicity are the key drivers for the interaction specificity of NLRP PYDs. We have tested the direct interaction of NLRP12 PYD with ASC PYD at various ratios using NMR spectroscopy (data not shown), but no chemical shift perturbations (CSPs) were identified in the titration experiments, showing that there is, as expected, no interaction between NLRP12 PYD and ASC PYD.

**NLRP12 PYD interacts directly with the UBA domain of FAF-1**

FAF-1 is a 74-kDa multi-domain protein that has been shown to function in diverse biological processes, such as the regulation of apoptosis and NF-κB activity, as well as ubiquitination and proteasomal degradation. FAF-1 consists of multiple protein-interaction domains, including a Fas-interacting domain (FID), a death effector domain-interacting domain, and multiple ubiquitin-related domains. Recently, Kinoshiba et al. used yeast two-hybrid screening to search for novel FAF-1-interacting proteins and identified the PYDs of NLRP3, NLRP7, and NLRP12 as potential FAF-1 targets. In their work, they used the entire FAF-1 FID (residues 1–180) as bait. The FID of FAF-1 consists of a UBA (ubiquitin associated) domain (residues 1–57) and an UB1 (ubiquitin related) domain (residues 99–180) connected by a flexible linker sequence (residues 58–98). We recently tested the interaction of NLRP7 PYD with the FAF-1 FID (residues 1–180) using NMR titrations but were unable to detect a direct interaction. This was expected, as no heterotypic interactions had been previously reported for a PYD domain.

Nevertheless, we performed the identical titration study with NLRP12 PYD and FAF-1 FID. Surprisingly, we detected small CSPs, indicating a direct interaction between the two proteins. In order to investigate this interaction in more detail, we subcloned the FAF-1 FID into multiple shorter, structurally and biologically meaningful sub-domains: (1) FAF-11–57 (UBA domain only), (2) FAF-11–99 (UBA domain and flexible linker), and (3) FAF-11–180 (UB1 domain only). All FAF-1 constructs expressed solubly and were purified to homogeneity, and one-dimensional 1H NMR spectra were recorded to confirm their folded state (Supplemental Fig. S5). Because it has been reported that death domain (DD) interactions are more robustly identified in higher pH solution, all titrations were performed at pH 7 or 7.5. NMR CSPs (detected using 15N-labeled NLRP12 PYD) showed that NLRP12 PYD interacts with the N-terminal UBA domain of FAF-1 (residues 1–57) (Supplemental Fig. S6). Interestingly, when the NMR titration experiment was carried out with FAF-11–99 (UBA domain and linker region), the resulting spectrum was identical with that of the FAF-11–57 titration (Supplemental Fig. S7). This shows that the UBA domain of FAF-1 is both necessary and sufficient for the interaction with NLRP12 PYD and that the residues comprising the flexible linker region (58–98) do not play a
role in this interaction. We also show that this interaction is specific, as no shifts were observed when NLRP12 PYD was titrated with FAF-199–180 (UB1 domain) (Supplemental Fig. S8).

Using the results from the NMR titrations, we identified the residues of NLRP12 PYD that mediate binding with FAF1 UBA. The majority of the perturbed residues (greater than 2 SD from the mean) were located exclusively to the α2–α3 surface, composed of helix α2, the α2–α3 loop, and helix α3 of NLRP12 PYD. It is interesting to note that this surface has been previously implicated in mediating homotypic interactions of PYDs. Specifically, the interaction between NLRP12 PYD and FAF1 UBA is mediated by residues Lys27, Thr34, Thr36, Glu40, Lys42, Ile43, Trp45, Gly46, and Lys50 (Fig. 6). The largest chemical shift changes were detected for residues Glu40, Lys42,
and Trp45. Based on the poor solubility of both proteins and the limitations of sample concentrations necessary for reliable NMR measurements, the highest feasible titration ratio used was 1:10 (NLRP12 PYD:FAF-11–57). This ratio does not identify a plateau in the titration curve that is characteristic of saturation of the interaction and necessary for a defined calculation of a $K_d$ value. Nevertheless, based on these chemical shift changes, we estimated a dissociation constant ($K_d$) $\geq 150$ μM between NLRP12 PYD and FAF-11–57 as well as FAF-11–99 (Supplemental Fig. S9).

To elucidate the importance of hydrophobic contacts in the interaction between NLRP12 PYD and FAF-11–57, we generated numerous point mutants of NLRP12 PYD residue Trp45 (W45A, W45E, W45R, W45F, and W45I). The ability of the NLRP12 PYD mutants to bind FAF-11–57 was then assayed using NMR titration experiments. Substitution of NLRP12 PYD W45 by an A, E, or R residue completely abolished binding with FAF-11–57, as no chemical shift changes were observed in the titration experiments (Supplemental Figs. S10–S12). In contrast, chemical shift changes were identified for W45F and W45I mutations (Supplemental Figs. S13 and S14). This highlights a role for the surface-exposed hydrophobic Trp45 residue in the binding of NLRP12 PYD to FAF-11–57.

Recently, a crystal structure of FAF-1–57 was reported (PDB ID 3E21), as were the chemical shift assignments for FAF-11–81.21 While the domain boundaries for these constructs differ from those used in our studies, we were nevertheless able to rapidly transfer the reported sequence-specific backbone assignments to FAF-11–57, allowing the detection of NLRP12-PYD-caused CSPs on 15N-labeled FAF-11–57 in a two-dimensional (2D) [1H,15N] heteronuclear single quantum coherence (HSQC) spectrum. However, these reverse titration experiments (unlabeled NLRP12 PYD into 15N-labeled FAF-11–57) did not result in any significant

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**Fig. 6.** Mapping the interaction of NLRP12 PYD with FAF-11–57. (a) CSPs calculated for NLRP12 PYD upon titration of FAF-11–57 at a molar ratio of 1:10 [10 mM Na-phosphate buffer (pH 7.0), 100 mM NaCl, and 0.5 mM TCEP]. The color scheme denotes the intensity of the shifts observed in the titration experiment. CSP values higher than 2 SD from the mean are colored light blue; 3 SD, marine; and 4 SD, purple. Experimentally derived secondary structure elements of NLRP12 PYD are depicted by gray cylinders on top of the figure. (b) NLRP12 PYD structure displaying the residues that show the highest CSP values upon titration with FAF-11–57. Side chains are depicted in stick model, labeled and colored according to (a). (c) Surface representation of the NLRP12 PYD structure in the same orientation as (b), displaying the residues that show the highest CSP values. These residues are clustered on the α2–α3 surface, which has been previously implicated in homotypic interactions of PYDs.
CSPs, up to the protein ratios technically possible (Supplemental Fig. S15). The most likely explanation for the lack of detectable CSPs is that this interaction occurs in an intermediate exchange regime when detected by FAF-11–57. Therefore, it does not lead to progressive chemical shift changes, which are the hallmarks of CSP mapping experiments in a fast or slow exchange regime.

In order to test this hypothesis, we measured the peak intensities of free FAF-11–57 and FAF-11–57 bound to NLRP12 PYD (at the highest titration ratio of 1:10) under identical buffer, concentration, and NMR conditions. If NLRP12 PYD binds to FAF-11–57, the intensities of the peaks corresponding to those residues that interact directly with NLRP12 PYD will change. As shown in Fig. 7a, I/I₀ intensity changes that are 2× or 3× higher than the standard deviation were detected. These changes, which define the NLRP12 PYD interaction surface on FAF-1, occur in three α-helices that form the core structure of FAF-11–57. FAF-1 also interacts with ubiquitin and ubiquitin-like proteins, and the residues involved in these interactions differ from those that interact with NLRP12 PYD,21 showing that these proteins bind to distinct surfaces on FAF-1.

**Discussion**

PYDs are the most common members of the DD superfamily.35 PYDs play a key role in the control of signaling pathways in the immune system, as well as in many apoptotic pathways.36 While numerous structures of PYDs have been reported during the last few years, the differences, in both structure and dynamics, between them are larger than the similarities. Moreover, the currently available structures have neither revealed the differential specificities for their homotypic interactions, nor have heterotypic interactions of these domains been reported in vitro. Here, we report the three-dimensional (3D) structure of human NLRP12 PYD, one of the first NLRPs discovered that functions to downregulate TLR-dependent NF-κB activation via direct interaction with processing kinases. Furthermore, NLRP12 plays also a role in the proteasome pathway.13,15 Thus, NLRP12 is considered as an essential regulatory bridge between innate immunity and apoptotic signaling pathways.

The structure of NLRP12 PYD folds into an expected DD fold. Through the use of a structure-based sequence alignment of the currently three available NLRP PYD structures (NLRP1, NLRP7, NLRP12), the NLRP12 Pyrin Domain...
and NLRP12), it was possible to identify conserved residues that form the hydrophobic core of these PYDs. Critically, this hydrophobic core is nearly perfectly conserved among the NLRP family (NLRP1–14) and, thus, enabled us to define the overall hydrophobic core of the NLRP PYD family. However, because of a two-amino-acid deletion in the α2–α3 loop, helix α3 is replaced by a 310 helix in NLRP12 PYD. Furthermore, analysis of the dynamics in the α2–α3 loop of NLRP12 PYD shows slightly increased fast timescale dynamics when compared to NLRP7 PYD. Interestingly, the PYD domain that is structurally most similar to that of NLRP12 is that of ASC2. However, the ASC2 PYD has highly charged electrostatic surfaces formed by helices α2/α3 (positive) and α1/α4 (negative), which are indicated to be essential for the interaction with ASC PYD.25,27,37 These oppositely charged surfaces are mostly missing in NLRP PYDs. NLRP12 PYD has a higher surface charge than NLRP7 PYD (which explains why it was necessary to add 500 mM NaCl to stabilize the NLRP12 PYD sample for NMR measurements). However, NLRP12 PYD has fewer hydrophobic patches than NLRP7 PYD. Based on the differential electrostatic surfaces, it seems unlikely that the charge-driven NLRP1 PYD:ASC PYD interaction can be formed between NLRP12 PYD:ASC PYD, which is typically required for the formation of an inflammasome.38

While numerous homotypic and heterotypic interactions have been reported for NLRP PYDs, very few have been confirmed in vitro. Here, we tested the previously reported interaction with FAF-1, a vital protein involved in ubiquitination and proteasomal degradation regulation.19 We were able to use NMR chemical shift mapping to detect a very weak interaction (estimated a Kd of ≥150 μM based on chemical shift analysis) with the N-terminal UBA (ubiquitin associated) domain (residues 1–57) of FAF-1. This is the first in vitro confirmed heterotypic interaction of a PYD. The inflammatory response to danger signals is a difficult balancing act for the host and therefore must be tightly regulated. Whereas the most prominent NLRPs, including NLRP1 and NLRP3, are positive regulators of inflammatory responses, NLRP2, NLRP4, and NLRP12 were shown to be negative regulators of pro-inflammatory signaling by inhibiting NF-κB activation.12,13,18,39,40

As FAF-1 itself is indicated in inhibiting NF-κB signaling, it is likely that this interaction can lead to a synergistic effect between NLRP12 PYD and FAF-1 UBA. Thus, NLRP12 together with FAF-1 modulates the pro-inflammatory response, where NF-κB possesses a key role. Therefore, to understand the molecular basis of this interaction, we performed a number of mutagenesis experiments and showed that the hydrophobic nature of a surface-exposed Trp (Trp45) is critical for the interaction. Interestingly, the identical Trp residue in NLRP7 PYD is completely buried in the hydrophobic core of this protein. This likely explains the differential interaction with the FAF-1 UBA domain.

Taken together, our analysis revealed initial insights into a non-homotypic PYD interaction between NLRP12 PYD and the FAF-1 UBA domain. In contrast to homotypic PYD:PYD interactions, which are mainly driven by electrostatic contacts and anchored by hydrophobic residues, this interaction is mainly driven by a weak hydrophobic contact. The interaction between NLRP12 PYD and FAF-1 indicates a possible mechanism for inhibition of TLR-dependent NF-κB activation.

Materials and Methods

Protein expression and purification

NLRP12 PYD (residues 1–98) was subcloned into pHigparsembleSTOP,41 which encodes an N-terminal His6 purification tag and a tobacco etch virus (TEV) protease cleavage site. FAF-1 constructs FAF-1199 (UBA domain and linker region; residues 1–99) and FAF-199–180 (UB1 domain; residues 99–180) were subcloned into pET1B, which encodes a Hist6His6 expression/purification tag and a TEV protease cleavage site. FAF-199–180 (UB1 domain; residues 1–57) was cloned into pETM30-MBP, which encodes an N-terminal Hist6-MBP purification/solubility tag and a TEV cleavage site. The plasmids were transformed into E. coli BL21-Codon-Plus (DE3)-RIL (Stratagene) cells. The expression of uniformly 13C/15N-labeled and 15N-labeled protein was carried out by growing freshly transformed cells in M9 minimum medium containing 4 g/L [13C]glucose and/or 1 g/L 15NH4Cl (Cambridge Isotope Laboratory) as the sole source of carbon and nitrogen, respectively. Cell cultures were grown at 37 °C under vigorous shaking (250 rpm) in the presence of 34 μg/mL chloramphenicol and 50 μg/mL kanamycin in the case of all FAF-1 constructs or in the presence of 34 μg/mL chloramphenicol and 50 μg/mL ampicillin in the case of NLRP12 PYD until they reached an OD600 of 0.6. Expression of all proteins was induced by addition of 1 mM IPTG to the culture medium, and cultures were allowed to grow overnight (18 h) at 18 °C under vigorous shaking (250 rpm). Cells were harvested by centrifugation and stored at −80 °C.

Purification of NLRP12 PYD and all FAF-1 constructs was performed as follows. Cells were resuspended in lysis buffer [50 mM Tris (pH 8.0), 500 mM NaCl, 5 mM imidazole, and 0.1% Triton-X 100, supplemented with ethylenediaminetetraacetic-acid-free protease inhibitor tablets (Roche)] and lysed by high-pressure homogenization (Avestin C-3 Emulsiflex). Cell debris was removed by centrifugation at 35,000 g for 40 min at 4 °C, and the supernatant containing soluble proteins was loaded onto a HisTrap HP column (GE Healthcare) equilibrated with 50 mM Tris (pH 8.0), 500 mM NaCl, and 5 mM imidazole. Hist6-tagged proteins were eluted with a 5–500 mM imidazole gradient. Fractions containing the protein of interest, as identified by SDS-PAGE, were pooled, incubated with Hist6-TEV Nla (S219V) protease, and dialyzed at 4 °C against 50 mM Tris (pH 7.5) and 200 mM NaCl until...
cleavage was complete. The untagged proteins were separated from the enzymatically cleaved His6- or His6-MBP tag, as well as from His6-TEV by Ni²⁺-affinity subtraction purification. Proteins were subsequently purified by size-exclusion chromatography using a Superdex 75 26/60 column (GE Healthcare). Fractions containing the pure proteins, as identified by SDS-PAGE, were pooled and concentrated. All purifications were performed at 4 °C.

NMR spectroscopy

NMR experiments were acquired at 298 K on a Bruker Avance 500-MHz spectrometer. In addition, a 3D 13C-resolved [1H,1H] NOESY spectrum was recorded on a Bruker Avance 800-MHz spectrometer. Both spectrometers are equipped with a TCI HCN z-gradient cryoprobe. Proton chemical shifts were referenced directly to internal 2,2-dimethyl-2-silapentane-5-sulfonate. 13C and 15N chemical shifts were referenced indirectly to 2,2-dimethyl-2-silapentane-5-sulfonate using the absolute frequency ratios.

Chemical shift assignments and structure calculation

All NMR experiments for chemical shift assignments and structure determination were performed with either a 15N- or a 15N/13C-labeled NLRP12 PYD sample at a final concentration of 0.6 mM in 20 mM sodium phosphate (pH 6.5), 500 mM NaCl, and 0.5 mM tris(2-carboxyethylphosphine) (TCEP). The following spectra were used to achieve the sequence-specific backbone and side-chain resonance assignments of NLRP12 PYD: 2D [1H,15N] HSQC, 3D HNCA, 3D CBCA(CO)NH, 3D HNCO, 3D HN(CA)CO, 3D CC(CO)NH [CC-total correlated spectroscopy (TOCSY) mixing time of 12 ms], 3D HBHA (CO)NH, and 3D HC(H)H-TOCSY (CC-TOCSY mixing time of 12 ms). TopSpin 2.1 (Bruker) was used for data acquisition and processing. NMR spectra were analyzed using the program CARA‡.

The following spectra were used for the structure calculation of NLRP12 PYD: 3D 15N-resolved [1H,1H] NOESY (mixing time of 80 ms), 3D 13C-resolved [1H,1H] NOESY (mixing time 80 ms), and 2D [1H,1H] NOESY (mixing time of 80 ms; acquired in 100% D2O solution). NOESY peak picking, NOESY peak assignment, and 3D structure calculation were performed automatically using the ATNOS/CANDID module in the CNS software. The input for the structure calculations of NLRP12 PYD was the amino acid sequence, the complete chemical shift lists, and the 2D and 3D NOE spectra. Default program parameters were used for all calculations. Constraints for backbone dihedral angles derived from 13C chemical shifts were only used in the initial structure calculation. The 20 conformers from the final CYANA cycle with the lowest residual CYANA target function values were energy minimized in a water shell using CNS and the RECOORD script package (Table 1). The structure quality was assessed by PSVS (Protein Structure Validation Suite†). All structural comparisons throughout the study were performed using the lowest-energy conformer of the ensemble of structures.

Relaxation measurements and analysis

15N relaxation experiments were performed under the exact same conditions as described for the structure determination of NLRP12 PYD. 15N longitudinal (R₁) and transverse (R₂) relaxation rates and 15N[1H]-NOE (hetNOE) measurements were acquired using sensitivity-enhanced pulse sequences. T₁ experiments were acquired with relaxation delays (T) of 20, 100, 200, 400, 550, 700, 850, and 1000 ms. T₂ experiments were acquired with relaxation delays (T) of 20, 80, 110, 140, 180, 220, 250, and 350 ms. A recycle delay of 3 s between scans was used for all T₁ and T₂ experiments. 15N[1H]-NOEs were measured from a pair of spectra acquired with and without presaturation recorded in an interleaved manner. A recycle delay of 5 s between scans was used for the heteronuclear NOE experiments.

All spectra were processed with NMRPipe and analyzed with NMRView. R₁ and R₂ relaxation rates were determined by fitting the peak intensities as a function of the relaxation delays using an exponential decay function, I(T)=Ae⁻(r/τ)², where I(T) is the peak intensity after a time delay T, A is the intensity at time zero, and r=R₁ or R₂. 15N[1H]-NOEs were calculated by dividing the intensity of the peaks in the spectra recorded without presaturation by the intensity of the peaks in the presaturated spectra.

NLRP12 PYD: FAF-1 interactions

The interaction of NLRP12 PYD with FAF-1 was investigated by NMR titration experiments using a 15N-labeled NLRP12 PYD or FAF-11–57 sample at a final concentration of 35 μM in 10 mM Na-phosphate buffer (pH 7.0 or 7.5), 100 mM NaCl, and 0.5 mM TCEP (pH varied depending on the pl of the FAF-1 constructs used). Titration experiments were performed with three different FAF-1 constructs (FAF-11–99, FAF-199–180, and FAF-11–57) at 1:2, 1:5, 1:10, and 1:15 molar ratios, with the highest concentration ratio dependent on the solubility of the FAF-1 construct in the case of detection by 15N-labeled NLRP12 PYD. 15N[1H]-NOEs were measured from a pair of spectra acquired with and without presaturation recorded in an interleaved manner. CSPs in the 2D [1H,15N] HSQC spectrum of NLRP12 PYD upon titration with FAF-1 constructs were used to monitor binding. CSPs were calculated using the following equation: \[ \text{CSP} = \left( \frac{\Delta\text{NH}}{\Delta\text{15N}} \right)^{2} + \left( \frac{\Delta\text{15N}/10}{\Delta\text{15N}} \right)^{2} \] where ΔNH and Δ15N represent the difference between free and bound 1H and 15N chemical shifts, respectively. A dissociation constant (Kd) for the interaction between NLRP12 PYD and FAF-11–57 was calculated using the chemical shift changes in the NMR titration experiments. Only the chemical shift resonances of NLRP12 PYD that exhibited the most significant change upon titration with FAF-11–57 were used for this calculation. CSPs were fitted to a one-site saturation binding equation using Sigmamap 11.0 (Systat Software Inc.).

Site-directed mutagenesis

NLRPP12 PYD mutants W45A, W45E, W45R, W45F, and W45I were generated using the QuickChange Mutagenesis

†http://www.nmr.ch
‡http://psvs-l4-dev.nesg.org
kit (Agilent). All mutated DNA constructs were sequence verified (Beckman Coulter).

Accession numbers

Chemical shift assignments of NLRP12 PYD were deposited in the Biological Magnetic Resonance Data Bank under accession number 17305, and the atomic coordinates were submitted to the PDB under accession code 2L6A.

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Supplementary Data

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References


