Mapping of POP1-binding Site on Pyrin Domain of ASC*

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Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is an essential adaptor protein in the formation of a multiprotein complex that activates procaspase-1. ASC is also known as a modulator of NF-κB activation pathways. ASC has a bipartite domain structure, consisting of an N-terminal pyrin domain (PYD) and a C-terminal caspase-recruitment domain. The PYD of ASC (ASC_PYD) is known to interact with various PYD-containing intracellular danger signal sensors and PYD-only proteins. Using purified proteins, we characterized the in vitro interaction of ASC_PYD with PYD-only protein 1 (POP1). POP1 specifically interacts with ASC_PYD with a dissociation constant of 4.08 ± 0.52 μM but does not interact with Cryopyrin. NMR and mutagenesis experiments show that a negative electrostatic potential surface patch (EPSP) on ASC_PYD, consisting of the first (H1) and fourth (H4) helices, is essential in the interaction with POP1. A positive EPSP on POP1, consisting of the second (H2) and third (H3) helices, is a counterpart of this interaction. The interaction between ASC_PYD and POP1 is similar to the interaction between caspase recruitment domains of Apaf-1 and procaspase-9. In addition, we present evidence that conformational changes at the long loop of ASC_PYD between the H2 and H3 helices can affect its interaction with POP1. Based on our observations, we propose that the positive EPSP of ASC_PYD, including the H2 and H3 helices, may be the binding site for Cryopyrin, and the interaction with Cryopyrin may induce the dissociation of POP1 from ASC_PYD.

Accumulating evidence indicates that ASC_CARD is involved in the recruitment and activation of procaspase-1 (3, 4). ASC_PYD interacts with PYD-containing NALP proteins to initiate a multiprotein platform known as an inflammasome, where procaspase-1 is activated by the induced proximity mechanism (3, 4). The NALP proteins are characterized by a common structure, consisting of N-terminal PYD, an intermediate NATCH domain, and C-terminal leucine-rich repeats. The NALP proteins are expected to function as intracellular danger signal sensors (5, 6). Cryopyrin (also known as NALP3, PYPAF1, and CIAS1) is a relatively well studied member of the NALP proteins. Recent studies using Cryopyrin−/− mice indicate that this protein is pivotal to the interleukin-1β production by macrophages in response to bacterial RNA (7), certain Gram-positive bacteria (8), uric acid, calcium pyrophosphate crystals (9), cellular toxins (10), and some Toll-like receptor agonists (8). In addition, Cryopyrin and other NALP proteins, in particular NALP6 (also known as PYPAF5) and NALP12 (also known as Monarch and PYPAF7), induce NF-κB activation in conjunction with ASC (11–13). The physiological importance of Cryopyrin-induced signaling pathways has been well demonstrated by showing a strong association of mutations within the NALP3 gene with three autoinflammatory disorders: Muckle-Wells syndrome, familial cold autoinflammatory syndrome (14), and chronic infantile neurological cutaneous and articular syndrome (15). There are several human and viral PYD-only proteins (POPs) that reportedly interact with ASC_PYD to regulate the ASC-mediated immune response (16–20). Among POPs, human POP1 is 64% identical (88% similar) in amino acid sequence with the ASC_PYD and has been identified as a modulator of NF-κB activation and procaspase-1 activation through its interaction with ASC_PYD (16).

The PYD is a subfamily of the death domain (DD) superfamily, which is characterized by a common six-antiparallel α-helical structure and mediates homotypic interactions. The DD superfamily consists of the DD, the death effector domain, the CARD, and the PYD. Recent NMR structures of the PYDs of ASC (21), POP1 (22), and NALP1 (23) revealed that the structure of PYDs is different from the canonical DD fold. For example, ASC_PYD, POP1, and the PYD of NALP1 (NALP_PYD) have a long loop between the second (H2) and third (H3) helices (H2-H3 loop). In addition to the long loop, the region that normally corresponds to the H3 helix in the DD fold is disordered in NALP1_PYD. The NMR structure of NALP1_PYD raised the possibility that PYD interactions may involve a conformational change upon binding and that the folding/unfolding transition of the H3 helix may be an important determinant of the func-

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S6.

The abbreviations used are: PYD, pyrin domain; CARD, caspase recruitment domain; EPSP, electrostatic potential surface patch; POP, PYD-only protein; DD, death domain; SPR, surface plasmon resonance; βME, β-mercaptoethanol; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; kT, kiloteslas.
tion and disease-related dysfunction of this domain (23). Through amino acid sequence and structural analysis, it has been shown that the amino acid composition of the H2-H3 loop is highly variable and that this loop is either reduced in length or not present in some human PYDs and is not present in viral PYDs (22). These variations of the H2-H3 loop were suggested to reflect differences in their cellular functions. Based on this observation, the region containing the H2-H3 loop and H3 helix was proposed as a hot spot for structural diversity in the DD superfamily (22, 23). NMR structures of ASC_PYD and POP1 (21, 23) showed that these proteins are highly bipolar molecules, suggesting that charge-charge interactions may play an important role in their interactions. It was postulated that PYD interactions might be analogous to those between the CARDs of Apaf-1 and procaspase-9 (21, 22), where the charged amino acid residues on the H2 and H3 helices of one protein interact with the charged amino acid residues on the first (H1) and fourth (H4) helices of the complementary protein. Currently, the molecular mechanisms of the interactions among PYDs remain largely unknown due to the lack of structural and mutational studies of PYD-mediated heterodimer formations.

To understand the molecular basis of PYD interactions, we have investigated the interactions among ASC_PYD, POP1, and the PYD of Cryopyrin (Cryo_PYD) using purified PYDs. NMR and mutagenesis experiments indicate that POP1 binds to the negative electrostatic potential surface patch (EPSP), containing Asp6, Glu13, Asp48, and Asp54 on ASC_PYD. A positive EPSP on POP1, consisting of amino acid residues on the H2 and H3 helices, is a counterpart in the interaction with ASC_PYD. We show that the structural changes of the region including the H2 and H3 helices can affect the interaction between ASC_PYD and POP1. We propose a potential molecular mechanism for the interaction of ASC_PYD with POP1 in the Cryopyrin-induced signaling pathway based on our observations.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification** — The coding region of ASC_PYD (Met1–Ala99) POP1 (Met1–Ala89) and Cryo_PYD (Met1–Ser100) were amplified by PCR using their respective human cDNAs as templates. ASC_PYD and Cryo_PYD were cloned into pET28a (Novagen), and POP1 was cloned into pET21d (Novagen). Mutated ASC_PYD and POP1 were generated using the QuikChange II kit (Stratagene). *Escherichia coli* BL21DE3 star cells (Novagen) were used to express the recombinant proteins. Cells were grown in LB medium. When the OD600 of the cultures reached 0.6, the protein expression was induced by adding 1 mM isopropyl-D-thiogalactopyranoside at 37 °C for 3 h. ASC_PYD and its mutants were purified from the soluble fractions of *E. coli* cell pellets that were lysed by sonication in a lysis buffer consisting of 30 mM potassium phosphate (pH 7.0), 3.0 M urea, 300 mM KCl, 10% (v/v) glycerol, 20 mM imidazole, and 3 mM β-mercaptoethanol (βME). Cleared cell extract was then incubated with Ni2+-nitrilotriacetic acid resin (Qiagen) that was pre-equilibrated with the lysis buffer. The resin containing bound recombinant proteins was extensively washed with the lysis buffer. The proteins were eluted from the Ni2+-nitrilotriacetic acid affinity column with an elution buffer consisting of 50 mM sodium acetate (pH 4.0), 2 mM urea, 500 mM KCl, 10% (v/v) glycerol, 100 mM EDTA, and 3 mM βME. The purified ASC_PYD was renatured by extensive dialysis in a buffer containing 30 mM sodium acetate (pH 4.5), 150 mM NaCl, 2 mM dithiothreitol (DTT), and 5% (v/v) glycerol. ASC_PYD and its mutants were further purified through size exclusion chromatography (Superdex 75; GE Healthcare) in 20 mM sodium acetate (pH 4.5), 100 mM NaCl, and 2 mM DTT. Cryo_PYD was purified using the same purification method as for ASC_PYD.

POP1 was purified using a lysis buffer consisting of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole, and 2 mM βME. Cleared cell extract was then incubated with Ni2+-nitrilotriacetic acid resin (Qiagen) that was pre-equilibrated with the lysis buffer. The resin containing bound recombinant proteins was extensively washed with a wash buffer consisting of 30 mM sodium phosphate (pH 6.0), 200 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, and 2 mM βME. The proteins were eluted from the Ni2+-nitrilotriacetic acid affinity column with an elution buffer consisting of the wash buffer with an additional 280 mM imidazole. POP1 was further purified through size exclusion chromatography (Superdex 75; GE Healthcare) in 20 mM MES (pH 6.0), 100 mM NaCl, and 2 mM DTT.

**Surface Plasmon Resonance** — Surface plasmon resonance (SPR) experiments were performed on a Biacore 1000 instrument (Biacore Life Sciences) at 25.0 °C. Purified ASC_PYD and its mutants were immobilized on CM5 chips by standard amine coupling through injecting 20 μl of the purified proteins (100 μg/ml) following the instructions provided by the manufacturer. The amount of immobilized protein was about 3000 resonance units. A reference surface was generated by immobilizing either bovine serum albumin or Cryo_PYD in flow cell 4. Purified POP1 (3 mg/ml) was extensively dialyzed in a running buffer consisting of 30 mM MES (pH 6.0), 100 mM NaCl, 2 mM DTT, 5% (v/v) glycerol, and 0.1% CHAPS. Then POP1 was diluted to various concentrations with the running buffer. POP1 was injected over the immobilized ASC_PYD, ASC_PYD mutants, and Cryo_PYD or bovine serum albumin at a flow rate of 5 μl/min for 15 min, followed by a dissociation for 15 min. At each cycle, the sensor chip was regenerated by passing a buffer consisting of 20 mM MES (pH 6.0) and 1 mM NaCl. The recorded sensograms were analyzed using BIAevaluation software (Biacore Life Sciences). Prior to the calculations, the binding data were corrected for nonspecific interactions by subtracting the reference surface data (bovine serum albumin or Cryo_PYD) from the reaction surface data. The data thus obtained were globally fit using a 1:1 binding model to calculate the dissociation constants (KD).

**NMR Spectroscopy** — To produce uniformly 15N-labeled ASC_PYD, ASC_PYD_L25A, in which Leu25 of ASC_PYD was replaced with Ala, transformed *E. coli* cells were grown in M9 minimal medium supplemented with Invitrogen vitamin solution, 1 g/liter L-[15N]NH4Cl, and 5 g/liter glucose. The expression and purification procedures were the same as described for the unlabeled ASC_PYD. NMR data were collected on a Bruker DMX-600 spectrometer equipped with a pulsed field gradient triple reso-
nance probe (Bruker Biospin GmbH). NMR data for the ASC_PYD_L25A were acquired at a protein concentration of 0.9 mM in 20 mM MES (in 90% H$_2$O and 10% D$_2$O (pH 5.5)) containing 100 mM NaCl and 2 mM DTT. In order to map the interaction site of POP1 on ASC_PYD, 0.5 mM unlabeled POP1 was mixed with 0.55 mM 15N-labeled ASC_PYD, and 1H-15N HSQC spectra were recorded. Chemical shift values for 1H and 15N were assigned on the basis of 15N HSQC, 15N HSQC-TOCSY, 15N HSQC-NOESY, HNHA, and HNBA experiments collected at 25 °C and also using the information available from BioMagResBank entry 15313. The spectra were processed and analyzed using FELIX (Felix NMR, Inc.) and Sparky (University of California). The total chemical shift change for a given peak was determined from the equation,

$$\Delta \delta = \left(\frac{\delta_H}{2} + \left(\frac{\delta_N}{10}\right)^2\right)^{1/2}$$

(parts per million), where $\delta_H$ and $\delta_N$ represent the changes in 15N and 1H chemical shift, respectively, upon POP1 binding to ASC_PYD.

Spectroscopic Experiments—Far-UV CD spectra of ASC_PYD, ASC_PYD mutants, POP1, POP1 mutant, and Cryo_PYD were recorded using an AVIV 62DS spectropolarimeter (AVIV Associates) equipped with a thermodrlectric sample holder. The protein concentration was 10 $\mu$M in a buffer consisting of 20 mM sodium acetate (pH 4.5), 100 mM NaCl, and 2 mM DTT. Data were collected from 200 and 250 nm at 25 °C. Fluorescence measurements were performed on a PTI fluorimeter (Photon Technology International), using an excitation wavelength of 295 nm to record intrinsic Trp fluorescence changes of Cryo_PYD (5 $\mu$M) upon the addition of various concentrations of ASC_PYD, ASC_PYD mutants, and POP1. The emission spectra were collected from 300 to 400 nm.

Molecular Modeling—For the structural analysis and model structure of ASC_PYD-POP1 complex, we used the NMR structures ASC_PYD (21) (Protein Data Bank entry 1UCP) and POP1 (22) (Protein Data Bank entry 2HM2). The electrostatic potential of the proteins was mapped on their individual solvent-accessible surfaces using APBS (24) and visualized by Pymol (available on the World Wide Web). To model the ASC_PYD-POP1 complex, we aligned the NMR structures of ASC_PYD and POP1 to the available Apaf1/procaspase-9 complex structure (25) (Protein Data Bank entry 3YGS) using the secondary structure matching function in Coot (26). Then modeling of the complexes was accomplished using RosettaDock (27). The model structure of Cryo_PYD was generated by Swiss-Model (28) using the NMR structure of NALP1_PYD (23) (Protein Data Bank entry 1PN5) as a reference.

RESULTS AND DISCUSSION

Protein Expression and Purification—Because recombinant ASC_PYD was not soluble, a purification protocol involving partial denaturation and renaturation at pH 4.5 was used. Purified ASC_PYD has a tendency to form a gel-like precipitate at a pH higher than 4.5 and is not suitable for studying in vitro interactions with other PYDs. To improve the solubility and stability of ASC_PYD, we carefully examined the structure of ASC_PYD and previously reported point mutational sites that could prevent ASC_PYD from self-association in vivo and in vitro (29). Among the reported mutational sites (Lys21, Leu25, Arg41, Asp48, and Asp51), only Leu25 on the H2 helix appeared to be free from potential interactions with other proteins (Fig. 1). In addition, Leu25 of ASC_PYD is not conserved among human and viral PYDs (Fig. 2). The other mutational sites could affect the interactions mediated by ASC_PYD, because charge-charge interactions are expected to play an important role in its interactions with other proteins (21, 22). We generated and purified a mutant ASC_PYD, ASC_PYD_L25A, in which Leu25 was replaced with Ala. ASC_PYD_L25A was partially soluble when expressed in E. coli cells and purified using the same method as for ASC_PYD purification. However, purified ASC_PYD_L25A is more stable than ASC_PYD and can be concentrated to 1 mM at pH 5.5. Recombinant POP1 was purified as a soluble and monomeric protein, as previously reported (22). We also purified Cryo_PYD using the same method as
ASC_PYD—CD spectroscopy confirmed that the purified PYDs consist of mainly α-helical structure (Fig. 3A). All purified PYDs showed elution peaks consistent with a monomeric conformation at pH 4.5 in size exclusion chromatography (Fig. 3B).

Quantitative Affinity Measurements for the Interactions of ASC_PYD and ASC_PYD_L25A with POP1 — The low stability of ASC_PYD hampered in vitro studies of its interactions with other proteins. We noticed that the purified PYDs are stable and can be immobilized on CM5 SPR sensor chip surfaces at pH 4.5. The immobilized PYDs are stabilized through covalent bonding with the sensor chip. We immobilized purified ASC_PYD and then injected various concentrations of POP1 into the fluid phase to measure the interaction affinity of ASC_PYD to POP1 at pH 6.0 (Fig. 4). The results show that the POP1 binds to ASC_PYD with a $K_D$ of 4.08 ± 0.52 μM. Using the same strategy, we also measured the interaction affinity of ASC_PYD_L25A to POP1 to test whether this mutation, which prevents aggregation, can affect the interaction with POP1. The measured $K_D$ value for the interaction between ASC_PYD_L25A and POP1 is 3.81 ± 0.8 μM, indicating that L25A mutation does not interfere with the ASC/POP1 interaction. Most of the amino acid residues (Lys21, Leu25, Lys26, Pro40, and Arg41), whose mutations were reported to prevent ASC_PYD from in vivo self-association without disturbing its hydrophobic core (29), are on the H2 and H3 helices (Fig. 1). The results therefore imply that the POP1-binding site on the ASC_PYD may be separate from the site for self-association, which includes Leu25.

Specific Interaction of POP1 with ASC—POP1 and ASC_PYD are 64% identical (88% similar) in amino acid sequence (Fig. 2) and have a very similar three-dimensional structure with root mean square deviation of 1.5 Å for Ca atoms. In addition, the distributions of charged amino acid residues on their surfaces are also similar (21, 22). POP1 was reported to enhance interleukin-1β production when coexpressed with ASC alone as well as when coexpressed with ASC and either Cryopyrin or Pyrin (16). These reported observations raise the possibility of direct interaction between POP1 and Cryopyrin. To test this possibility, we measured the interaction of Cryo_PYD with POP1 using SPR, in which purified monomeric Cryo_PYD was immobilized on the sensor chip and various concentrations of POP1 (from 2.5 to 200 μM) were injected. The results show that the affinity of Cryo_PYD to POP1 is negligible, when compared with the affinity of ASC_PYD or ASC_L25A to POP1 (data not shown). Since our Cryo_PYD construct has three Trp residues (Trp66, Trp71, and Trp92), whereas POP1 does not contain any Trp residues (supplemental Fig. S1), we were able to measure the intrinsic Trp fluorescence change of Cryo_PYD during the addition of POP1. We monitored the intrinsic Trp fluorescence of Cryo_PYD (5 μM) at pH 6 and pH 7 by adding various concentrations of POP1 (0.1–10 μM). The results show that the addition of POP1 does not affect the intrinsic Trp fluorescence of Cryo_PYD (data not shown). Thus, SPR and fluorescence experiments demonstrate that POP1 specifically interacts with ASC_PYD, but not with Cryo_PYD. This implies that the observed interleukin-1β production enhancement, when POP1 was coexpressed with ASC and Cryopyrin (16), may not be due to the interaction between Cryopyrin and POP1. The absence of specific interaction between POP1 and Cryo_PYD is consistent with our analysis that used the available NMR structure of POP1 and the model structure of Cryo_PYD (see below).
trated ASC_PYD_L25A formed gel-like precipitation at pH values higher than 5.5. About 90% of the main-chain resonances of ASC_PYD_L25A were assigned using standard 1H-15N-HSQC and with analysis of the sequential NOE connectivities in the 15N NOESY-HSQC and 15N TOCSY-HSQC. We also used the chemical shift information of BioMagResBank entry 15313 for full-length ASC at pH 3.8 as a reference. The chemical shifts for most amino acid residues of ASC_PYD_L25A upon the addition of POP1 generally agree with ASC_PYD_L25A alone (Fig. 5A). However, the addition of POP1 does induce notable chemical shift changes of three charged amino acid residues, Asp6, Glu13, and Asp48 of ASC_PYD_L25A (Fig. 5B). In addition, a hydrophobic residue, Phe23, shows significant chemical shift change by the addition of POP1 (Fig. 5B). In contrast, the chemical shifts of positively charged amino acid residues are not affected by the addition of POP1. The NMR structure of ASC_PYD_L25A shows that Phe23 is on the H2 helix, which contains Lys21, Lys22, Lys24, and Lys26 and is a major contributor of positive EPSP formation. This residue is completely buried in the hydrophobic core and is located very close to Leu12 on the H1 helix and Leu52 on the H4 helix. As a result, structural changes on the H1 and H4 helices of ASC_PYD, including an interaction with POP1, can affect the conformation of Phe23. This implies that there may be communication between the negative EPSP and the positive EPSP of ASC_PYD through a tightly packed hydrophobic core.

Importance of Negatively Charged Amino Acid Residues of ASC_PYD in the Interaction with POP1—We tested the role of the amino acid residues in the negative EPSP on ASC_PYD_L25A using site-directed mutagenesis. We first mutated Glu13 and Asp48 of ASC_PYD_L25A to Ala and purified the respective mutant proteins, ASC_PYD_L25/E13A and ASC_PYD_L25/D48A. The interactions of these mutant proteins with POP1 were tested using SPR. ASC_PYD_L25/E13A and ASC_PYD_L25/D48A were immobilized on the sensor chip and various concentrations of POP1 (2.5–15 μM) were injected. The results show that POP1 binds to the negative EPSP on ASC_PYD, which is composed of the H1 and H4 helices. Interestingly, Phe23 is on the H2 helix, which contains Lys21, Lys22, Lys24, and Lys26 and is a major contributor of positive EPSP formation. This residue is completely buried in the hydrophobic core and is located very close to Leu12 on the H1 helix and Leu52 on the H4 helix. As a result, structural changes on the H1 and H4 helices of ASC_PYD, including an interaction with POP1, can affect the conformation of Phe23. This implies that there may be communication between the negative EPSP and the positive EPSP of ASC_PYD through a tightly packed hydrophobic core.
ligible value, comparable with Cryo_PYD (Table 1 and supplemental Fig. S2), indicating that these mutational sites are critical for the interaction with POP1. The negative EPSP on ASC_PYD contains Asp\(^6\), Asp\(^{10}\), Asp\(^{51}\), and Asp\(^{54}\) as well as Glu\(^{13}\) and Asp\(^{48}\). In addition, the resonance peak of Asp\(^6\) is shifted upon the addition of POP1 in our NMR experiments (Fig. 5B). To confirm the importance of residues in the negative EPSP on ASC_PYD in the interaction with POP1, we mutated the two Asp residues (Asp\(^6\) and Asp\(^{54}\)), located on the outer rim of the negative EPSP of ASC_PYD_L25A, to Ala. We purified ASC_PYD_L25A/D6A and ASC_PYD_L25A/D54A and tested the interactions with POP1. In the SPR experiments, these two mutants generated similar signal levels as ASC_PYD_L25A/E13A and Cryo_PYD when 5 and 10 \(\mu\)M POP1 were injected (Table 1). These results demonstrate that the negative EPSP is indeed the POP1-binding site on ASC_PYD. The amino acid sequence alignment suggests that Asp\(^6\), Asp\(^{10}\), Asp\(^{51}\), and Asp\(^{54}\) of ASC_PYD may play a role as a specificity determinant in the ASC/POP1 interaction rather than in maintaining common structural and functional role of PYDs, because these amino acid residues are not conserved among human PYDs (Fig. 2).

**Comparison of the ASC/POP1 Interaction with Apaf-1/Pro-caspase-9 Interaction**—The interaction of ASC_PYD with POP1 is reminiscent of the interaction of procaspase-9 with Apaf-1, since it uses the negative EPSP of ASC_PYD, consisting of the H1 and H4 helices. The crystal structure of procaspase-9 and Apaf-1 complex showed that the H1 and H4 helices of the CARD of procaspase-9 (Csp9_CARD), containing Arg\(^{14}\), Arg\(^{53}\), and Arg\(^{57}\), interacted with the H2 and H3 helices of the CARD of Apaf-1 (Apaf_CARD), containing Asp\(^{27}\) and Glu\(^{40}\) (25). To identify the potential ASC interaction site on POP1, we calculated the surface electrostatic potential of the protein. Electrostatic complementarity is defined using the correlation of surface electrostatic potential at the protein-protein interface and provides a better explanation of protein interaction than charge complementarity, which is defined using the correlation of charges on the nearest neighbor atoms at the interface (30). Our surface electrostatic potential analysis of the available POP1 NMR structure (22) shows that Lys\(^{21}\), Lys\(^{22}\), and Lys\(^{26}\) on the H2 helix and Arg\(^{41}\) on the H3 helix form a positive EPSP (Fig. 6). However, the negative EPSP of POP1 is less well defined than the positive EPSP and the corresponding negative EPSP of ASC_PYD (Fig. 1). This is also evident in the comparison of the electrostatic isosurfaces of ASC_PYD and POP1 (supplemental Fig. S3). This analysis indicates that the negative EPSP of ASC_PYD may interact with the positive EPSP of POP1 in a similar way as Csp9_CARD interacts with Apaf_CARD. To gain insight into the ASC_PYD interaction with POP1, we generated a model structure of ASC/POP1 complex using our chemical shift perturbation and mutagenesis data in combination with available NMR structures of ASC_PYD (21) and POP1 (22). The structure of Apaf_CARD/Csp9_CARD complex (25) was used as a reference. The model structure shows that the positive EPSP of POP1 has a complementary surface in both electrostatic potential and three-dimensional structure to the negative EPSP of ASC_PYD (Fig. 7). The result predicts that about 1300 \(\AA^2\) of solvent-accessible surface area from the two proteins (about 660 \(\AA^2\) from each protein) would be buried at the ASC_PYD/POP1 interface. Hydrogen bond and salt bridges would be formed between negatively charged residues of ASC_PYD and positively charged residues of POP1 in the interface (supplemental Table S1). The model structure also shows that the EPSPs on ASC_PYD and POP1 are divided into two subpatches by intermediate hydrophobic amino acid residues.

![Figure 5. Chemical shift changes of ASC_PYD_L25A upon the addition of POP1. A, \(^{1}H-{^{15}N}\) HSQC of \(^{15}N\)-labeled ASC_PYD alone (red) and with POP1 (blue) are overlaid. Resonance peaks of amino acid residues in the negative and positive EPSPs on ASC_PYD are labeled. B, a graph of the chemical shift change (\(\Delta\delta\)) versus residue number.](image)

**TABLE 1**

| ASC_PYD Mutation position | Affinity to POP1 (K\(_D\)) |
|---------------------------|---------------------------|
| Wild type                 | 4.08 ± 0.69               |
| L25A                      | 3.81 ± 0.8                |
| D6A/L25A                  | H2 helix/H2 helix         |
| E13A/L25A                 | H1 helix/H2 helix         |
| K21A                      | H2 helix                  |
| Y36A                      | H2-H3 loop                |
| D48A/L25A                 | H3-H4 loop/H2 helix       |
| D54A/L25A                 | H4 helix/H2 helix         |

\(*\text{ND, not detected.}\)
On ASC_PYD, one subpatch consists of Asp⁶, Asp¹⁰, and Glu¹³ on the H1 helix, and the other subpatch consists of Asp⁴⁸ on the H3-H4 loop and Asp⁵¹ and Asp⁵⁴ on the H4 helix. These subpatches are separated by Leu⁹ on the H1 helix and Leu⁵⁰ on the H4 helix. On POP1, Met²⁵ on the H2 helix and Leu⁴⁴ on the H4 helix divide one subpatch (Lys²¹, Lys²², and Lys²⁶ on the H2 helix) from the other subpatch (Arg⁴⁰ on the H2-H3 loop and Arg⁴¹ on the H3 helix). It appears that the subdivision of each EPSP by hydrophobic residues is critical for structural complementarity (Fig. 7). Our structural analysis in combination with the amino acid sequence alignment indicates that the positive EPSP on ASC_PYD is unlikely to interact with POP1. The surface of POP1, which corresponds to the negative EPSP of ASC_PYD, has a greatly reduced electrostatic potential (supplemental Fig. S3) due to the presence of two basic residues (Lys¹⁰ and Arg⁵). Lys¹⁰ of POP1 is replaced with Asp in ASC_PYD. Arg⁸ of POP1, which is located between Asp⁶ on the H1 helix and Asp⁵⁴ on the H4 helix, partly neutralizes the negative EPSP, whereas the Arg⁸ of ASC_PYD does not.

Role of Positively Charged Amino Acid Residues of POP1 in the Interaction with ASC_PYD—To test the results from our model structure analysis, we generated and purified two mutant POP1 proteins, POP_K21A and POP_R41A, containing Ala substitutions at Lys²¹ and Arg⁴¹, respectively. Our structural analysis suggests that these two amino acid residues are major contributors in the positive EPSP formation of POP1 (Fig. 6). We tested the interaction of these two mutants with ASC_PYD and ASC_PYD_L25A by SPR, using the same strategy previously used with ASC_PYD and POP1. The results show that POP1 mutants do not interact with ASC_PYD or with ASC_PYD_L25A (data not shown). Therefore, it is clear that the negative EPSP of ASC_PYD is the binding site for the positive EPSP on POP1.

Role of the H2-H3 Loop in ASC/POP1 Interaction—It has been proposed that POP1 might interfere in the recruitment of ASC by activated Cryopyrin and the subsequent NF-κB activation (11, 19). Similarly, human POP2 was reported to interact with ASC_PYD to block the interaction between Cryopyrin and ASC to inhibit the NF-κB activation (18, 19). Based on the observation that TNF treatment induces phosphorylation of ASC_PYD as well as POP1, the post-translational modification of PYDs has been proposed as a regulatory mechanism in NF-κB activation pathways (16). We hypothesized that the TNF-induced phosphorylation of ASC_PYD might cause conformational changes, which could interfere in the interaction with other proteins, including POP1. To test this hypothesis, we first predicted potential phosphorylation sites on ASC_PYD.
and POP using NetPhos 2.0 (available on the World Wide Web) (31) with a threshold of 0.5 and PPSP (available on the World Wide Web) (32) with the option of high specificity. The two programs commonly predicted an amino acid residue, Tyr^{36} of ASC. Our structural analysis shows that Tyr^{36} of ASC_PYD, which is on the H2-H3 loop, forms a hydrophobic patch with Tyr^{60} and Tyr^{64}, anchoring the loop on the protein (supplemental Fig. S4). Interestingly, the predicted phosphorylation site appears to play a role in the stabilization of the H2-H3 loop structure in ASC_PYD. Although the H2-H3 loop has been proposed as a hot spot for PYD interactions (22, 23), our NMR results show that this region of ASC_PYD is not directly involved in the interaction with POP1 (Fig. 5). To test the importance of the H2-H3 loop in ASC_PYD-mediated interactions, we generated and purified ASC_PYD_Y36A, where Tyr^{36} is replaced with Ala. This mutant was expected to have a disrupted surface hydrophobic patch around the mutational site. However, CD and size exclusion chromatography profiles of purified ASC_PYD_Y36A are very similar to those of ASC_PYD and ASC_PYD_L25A (supplemental Fig. S5), indicating the mutation does not affect the global fold of ASC_PYD.

The effect of the Y36A mutation on the interaction of ASC_PYD with POP1 was tested using SPR. Surprisingly, we could not detect the interaction between ASC_PYD_Y36A and POP1 when 5 and 10 μM of POP1 were injected (Table 1 and Supplemental Data Fig. S6). The result shows that structural changes in the H2-H3 loop can affect the interaction of ASC_PYD with other proteins, as shown in the interaction with POP1. Our experiment suggests that the TNF-induced phosphorylation of ASC_PYD, most possibly at Tyr^{36}, can dissociate POP1 from ASC_PYD. Thus, TNF can induce ASC-mediated NF-κB activation pathways, bypassing the interference of POP1 inhibition. However, further work is necessary to identify the interaction of Cryo_PYD with ASC_PYD may take place through one of two possible mechanisms. One possibility is that this interaction occurs on the negative EPSP of ASC_PYD through competition with POP1. The other possibility is that the Cryo_PYD binds to the positive EPSP of ASC_PYD. To gain insight into the interaction of ASC_PYD with Cryo_PYD, we generated a model structure of Cryo_PYD using the NMR structure of NALP1_PYD as a template (Fig. 8). The model structure shows that Cryo_PYD has a different surface charge distribution when compared with ASC_PYD and POP1. Cryo_PYD has a well developed negative EPSP that consists of Glu^{13}, Asp^{14}, Glu^{16}, Asp^{17}, and Glu^{19} on the H1-H2 loop and Asp^{48} on the H3-H4 loop. However, Cryo_PYD does not have a clear positive EPSP, since the electrostatic potential of the region corresponding to the positive EPSP of ASC_PYD and POP1 is markedly reduced by negatively charged amino acid residues located nearby (Fig. 8). The obvious difference in the polarity of the surface electrostatic potential between Cryo_PYD and POP1 suggests that Cryo_PYD may not compete with POP1 for the same binding site on ASC_PYD. Rather, Cryo_PYD may bind to the positive EPSP of ASC_PYD, which consists of the H2 and H3 helices and contains Lys^{21}, Lys^{22}, Lys^{26}, and Arg^{41}. However, we were unable to detect an in vitro interaction between ASC_PYD and Cryo_PYD using purified PYDs using SPR and intrinsic Trp fluorescence of Cryo_PYD. This might be because Cryopyrin requires NACHT domain-mediated self-oligomerization to interact with ASC (35, 36).

Our results of NMR experiments show that the binding of POP1 induces the chemical shift change of Phe^{23} on the H2 helix of ASC_PYD. This result indicates that the negative EPSP of ASC_PYD may communicate with the positive EPSP through a tightly packed hydrophobic core. As an alternative way to test the interaction between ASC_PYD and Cryo_PYD,
we tested whether conformational changes in the positive EPSP of ASC_PYD could affect its interaction with POP1. To explore the possible communication between the positive and negative EPSPs of ASC_PYD, we first replaced the Arg18 of ASC_PYD with Trp (ASC_PYD_R41W) to mimic the result of a charge-charge interaction at the protein interface. The affinity of purified ASC_PYD_R41W to POP1 was measured with SPR using the same method described for measuring the affinity of ASC_PYD to POP1. The result shows that the R41W mutation reduces the affinity of ASC_PYD to POP1, resulting in a $K_D$ of $29.7 \pm 0.8 \mu M$ (Table 1 and supplemental Fig. S6). Then we mutated the Lys11 of ASC_PYD to Ala to confirm the communication between the two EPSPs. This mutation blocks the interaction of ASC_PYD with POP1 (Table 1). These results, in combination with our NMR results (Fig. 5A), suggest that conformational changes in the positive EPSP of ASC_PYD can affect its interaction on the negative EPSP, although residues in the positive EPSP are not directly involved in that interaction. Based on our observations and analysis of the Cryo_PYD model structure, we propose that the interaction of Cryo_PYD with ASC_PYD, which is probably mediated by the positive EPSP of ASC_PYD, may induce conformational changes in the positive EPSP resulting in the dissociation of POP1. In turn, this dissociation may induce the oligomerization of ASC or ASC_CARD, whose local concentration is considerably increased by interactions with activated, thereby oligomeric, Cryopyrin.

In this report, we present the in vitro evidence for the interaction of ASC_PYD with POP1 and the first quantitative characterization of the PYD interaction. In addition, we propose the potential importance of the H2-H3 loop region in the function of ASC, based on our experimental results and structural analysis. Determination of the molecular mechanisms underlying the PYD-mediated inflammatory signaling pathways relies on detailed biochemical and structural analysis of their interactions; thus, this study represents a first step toward understanding the molecular basis for these signaling mechanisms.

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