PYPAF1, a PYRIN-containing Apaf1-like Protein That Assembles with ASC and Regulates Activation of NF-κB*

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The PYRIN domain is a recently identified protein-protein interaction domain that is found at the N terminus of several proteins thought to function in apoptotic and inflammatory signaling pathways. We report here that PYPAF1 (PYRIN-containing Apaf1-like protein 1) is a novel PYRIN-containing signaling protein that belongs to the nucleotide-binding site/leucine-rich-repeat (NBS/LRR) family of signaling proteins. The expression of PYPAF1 is highly restricted to immune cells, and its gene maps to chromosome 1q44, a locus that is associated with the rare inflammatory diseases Muckle-Wells syndrome and familial cold urticaria. To identify downstream signaling partners of PYPAF1, we performed a mammalian two-hybrid screen and identified ASC as a PYRIN-containing protein that interacts selectively with the PYRIN domain of PYPAF1. When expressed in cells, ASC recruits PYPAF1 to distinct cytoplasmic loci and induces the activation of NF-κB. Furthermore, co-expression of PYPAF1 with ASC results in a potent synergistic activation of NF-κB. These findings suggest that PYPAF1 and ASC function as upstream activators of NF-κB signaling.

The CED4/Apaf1 family of proteins coordinate the assembly of signaling complexes that regulate the activation of NF-κB, cytokine processing, and apoptosis. Members of this family include Apaf1, CARD4 (Nod1), Nod2 (CARD15), CARD7 (DEF-CAP/NAC/NALP1) and CARD12 (Ipaf/Clan) (1–11). Each family member contains a caspase recruitment domain (CARD) that mediates assembly with a downstream CARD-containing signaling partner and a nucleotide-binding site (NBS) that regulates activation of the signaling complex. In addition, each member contains a domain of either WD-40 repeats (Apaf1) or a Toll/interleukin-1 receptor homology region in place of a plant NBS/LRR proteins contain either a leucine zipper motif or a CARD domain (12). The regulation of NF-κB signaling by CARD4 and Nod2 and the activation of caspase-1 by CARD12 identifies these CED4/Apaf1 family members as important components of inflammatory signaling pathways. Consistent with this hypothesis, mutations within the Nod2 gene have been found to confer susceptibility to Crohn’s disease and Blau syndrome, two chronic inflammatory disorders (13–15).

We recently identified CARD7 and NBS1 as NBS/LRR proteins with N-terminal PYRIN domains (5). The PYRIN domain belongs to the death domain-fold superfamly that also includes the CARD domain, death domain, and death effector domain (5, 10, 16–18). Similar to other death domain-fold modules, the PYRIN domain likely mediates homotypic interactions between family members resulting in the formation of PYRIN-PYRIN signaling complexes. Proteins containing the PYRIN domain share homology with the N-terminal region of pyrin, a protein that functions to regulate inflammatory signaling in myeloid cells (19, 20). Mutations within the pyrin gene confer susceptibility to familial Mediterranean fever, a type of hereditary periodic inflammatory disease (21). In addition, the apopotosis proteins ASC and zebrafish caspase-13 each contain N-terminal PYRIN domains suggesting that PYRIN family members function in both inflammatory and apoptotic signaling (22, 23). Interestingly, analysis of human genomic sequences predicts a large family of PYRIN-containing proteins of unknown function, including at least 11 novel NBS/LRR proteins with N-terminal PYRIN domains.2 We report here that PYPAF1 (PYRIN-containing Apaf1-like protein 1) is a novel PYRIN-containing NBS/LRR family member that interacts selectively with the PYRIN domain of ASC. When expressed in cells, PYPAF1 and ASC colocalize to distinct cytoplasmic loci and signal the activation of NF-κB. These findings identify PYPAF1 and ASC as NF-κB activators of a novel inflammatory signaling pathway.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Plasmids expressing either full-length PYPAF1 (PYPAF1-FL, residues 1–1034, accession number AF420469) or a PYPAF1 truncation mutant lacking the PYRIN domain (PYPAF1ΔPYRIN, residues 90–1034) with a C-terminal FLAG epitope were constructed using pCMV-Tag 4a (Stratagene). Plasmids expressing either a PYPAF1 truncation mutant lacking the LRR domain (PYPAF1ΔLRR, residues 1–739) with a C-terminal FLAG epitope or ASC with a C-terminal HA epitope were constructed using pCI (Promega). For mammalian two-hybrid assays, pCMV-PYRIN/AD plasmids were constructed by inserting individual PYRIN domains into the mammalian two-hybrid screen and identified ASC as a PYRIN-containing protein that interacts selectively with the PYRIN domain of PYPAF1. When expressed in cells, ASC recruits PYPAF1 to distinct cytoplasmic loci and induces the activation of NF-κB. Furthermore, co-expression of PYPAF1 with ASC results in a potent synergistic activation of NF-κB. These findings suggest that PYPAF1 and ASC function as upstream activators of NF-κB signaling.

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1 The abbreviations used are: CARD, caspase recruitment domain; POP1, peripheral blood leukocyte; POP1, PYRIN-only protein 1.

2 J. Bertin, unpublished data.

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pCMV/AD (Stratagene): ASC (residues 1–110), CARD7 (residues 22–112), PYPAF1 (residues 1–116), NBS1 (residues 1–115), and POP1 (residues 1–89, accession number AF454669). Similarly, pCMV-PYPAF1-PYRIN/BD and pCMV-ASC-CARD/AD plasmids were constructed by inserting the PYRIN domain of PYPAF1 (residues 1–116) and the CARD domain of ASC (residues 92–195) into pCMV-BD and pCMV-AD, respectively. The plasmid expressing dominant negative FLAG epitope-tagged IKKβ (residues 1–250) was constructed using pCMV2 (Stratagene). Myc-tagged CARD9 expression plasmid was described previously (24).

PYPAF1 Expression Analysis—Total RNA from cells in culture was extracted (Qiagen) and expression profiles were determined by real-time-PCR analysis (Taqman™). In brief, an oligonucleotide probe (5'-TCTCAGCAGCAGGATTCGATTCG-3') was designed to anneal to PYPAF1 between two PCR primers (forward: 5'-AGAACAGATTCGAGTACGCTG-3' and reverse: 5'-AGCGTTTGAACTGGAGTAAAGCT-3'). The probe for β2 microglobulin incorporated VIC as the reporter dye. PCR reactions were

**FIG. 1. Sequence and domain structure of PYPAF1.** A, amino acid sequence of PYPAF1. N-terminal PYRIN domain (residues 1–90, red shading), NBS domain (residues 219–534) with seven consensus motifs that are found in the NACHT subfamily of NTPases (blue shading), and C-terminal leucine-rich repeats (black shading). B, amino acid sequence alignment of the PYRIN domain of PYPAF1 with those of pyrin, CARD7, ASC, NBS1, and POP1. Black shading indicates identical residues. C, domain structure of PYPAF1 compared with NBS1, CARD7, pyrin, ASC, and POP1.

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performed on cDNAs from various cell types using primers and probes for both PYPAF1 and β2 microglobulin genes. Fluorescent emissions generated during PCR reaction was measured by Sequence Detector 7700. The expression level of PYPAF1 was normalized to the expression of β2 microglobulin for each sample.

**Reporter Gene Assays**—NF-κB and mammalian two-hybrid assays were described previously (25). For mammalian two-hybrid assays, 293T cells were transfected with pCMV/AD and pCMV/BD plasmids, pFR-Luc firefly reporter (Stratagene), and pRL-TK Renilla reporter (Promega). For NF-κB assays, 293T cells were transfected with pNF-κB luciferase reporter (Stratagene), pRL-TK Renilla reporter (Promega), and indicated expression plasmids. Cells were harvested, and firefly and Renilla luciferase activities were determined using the dual-luciferase reporter assay system (Promega).

**Immunostaining Assays**—293T cells were transfected with plasmids expressing epitope-tagged proteins and fixed 24 h after transfection. Cells were processed and images were acquired as described previously (26).

**RESULTS AND DISCUSSION**

To identify novel NBS/LRR proteins with N-terminal PYRIN domains, we searched Millennium Pharmaceuticals’ data base of expressed sequence tags. We found a 3.8-kilobase cDNA that encodes a PYRIN-containing protein of 1034 amino acids with a predicted molecular mass of 118 kDa (Fig. 1A). A BLAST search of the protein data base indicated that the protein was comprised of an N-terminal PYRIN domain linked to an NBS domain and multiple C-terminal LRRs. Because of its structural similarity to several CED4/Apaf1 family members, we designated this protein PYPAF1 for PYRIN-containing Apaf1-like protein 1. The PYRIN domain of PYPAF1 (residues 1–90) shares significant sequence similarity with the PYRIN domains of other PYRIN-containing proteins, including pyrin (33% identity), CARD7 (24% identity), ASC (25% identity), NBS1 (24% identity), and POP1 (PYRIN-only protein 1, 28% identity) (Fig. 1B). The NBS domain (residues 219–434) belongs to the NACHT subfamily of NTPases and contains all seven signature motifs, including the P-loop and the Mg²⁺-binding site (Fig. 1A) (27). The C-terminal domain (residues 740–991) contains at least nine LRRs predicted to function as protein-protein interaction motifs (28) (Fig. 1C).

Northern blot analysis showed PYPAF1 to be highly expressed in peripheral blood leukocytes (PBLs) as a single 3.8-kilobase transcript (Fig. 2A). Further analysis using a multiple tissue expression array (CLONTECH, Palo Alto, CA) also showed high expression in PBLs (data not shown). However, little or no PYPAF1 expression was detected in 75 other tissues and cancer cell lines indicating a role for this protein in inflammatory signaling. To determine the cells that express PYPAF1, PBLs were fractionated into distinct cell populations. Real-time PCR analysis showed PYPAF1 to be predominantly expressed in monocytes, with some expression in granulocytes and T-cells (Fig. 2B).

By analogy to other NBS/LRR family members such as CARD4 and Nod2, the N-terminal PYRIN domain of PYPAF1
likely interacts with a PYRIN-containing signaling partner. To identify PYRIN domains that interact with the PYRIN domain of PYPAF1, we performed a mammalian two-hybrid analysis and screened for binding to the PYRIN domains of other family members, including CARD7, ASC, NBS1, and POP1 (Fig. 3A). The PYRIN domain of PYPAF1 interacted with the PYRIN domain of ASC, resulting in a 16-fold increase in relative luciferase activity. However, PYPAF1-PYRIN failed to bind to the PYRIN domain of other family members (CARD7, NBS1, and POP1) indicating that the PYRIN domain of PYPAF1 interacts selectively with the PYRIN domain of ASC. Furthermore, the PYRIN domain of PYPAF1 failed to interact with the CARD domain of ASC (Fig. 3B). Taken together, these findings identify ASC as a putative signaling partner of PYPAF1 and demonstrate that PYRIN-PYRIN interactions between family members can be highly selective.

Our finding that the PYRIN domain of PYPAF1 binds to the PYRIN domain of ASC prompted us to further examine the interactions between these two proteins when overexpressed in 293T embryonic kidney cells. Immunoprecipitation of FLAG-tagged PYPAF1 failed to coprecipitate HA-tagged ASC (data not shown). We speculate that our inability to detect an interaction by immunoprecipitation analysis is due to the relative insolubility of PYPAF1-ASC complexes that form in the cell when both proteins are overexpressed (see below). A similar difficulty in immunoprecipitating ASC-containing complexes has been reported by others (20). We therefore performed cellular colocalization studies to further examine the interactions between these two PYRIN-containing proteins. We expressed FLAG-tagged PYPAF1 and HA-tagged ASC in cells and detected the proteins using a mixture of anti-HA and anti-FLAG antibodies. When expressed alone, the two proteins exhibited a distinct

![Fig. 4. PYPAF1 is recruited to ASC punctate structures. HA-tagged ASC (blue staining) and FLAG-tagged PYPAF1 proteins (red staining) were expressed in 293T cells. A, E and I show PYPAF1-FL, PYPAF1ΔPYRIN and PYPAF1ΔLRR when expressed alone. B-D, F-H and J-L show the immunostaining patterns observed when each protein was coexpressed with ASC. Note the colocalization of PYPAF1-FL (B-D) and PYPAF1ΔLRR (J-L), and the lack of colocalization of PYPAF1ΔPYRIN (F-H) to the ASC punctate structures (arrows).](image)

**Fig. 4. PYPAF1 is recruited to ASC punctate structures.** HA-tagged ASC (blue staining) and FLAG-tagged PYPAF1 proteins (red staining) were expressed in 293T cells. A, E and I show PYPAF1-FL, PYPAF1ΔPYRIN and PYPAF1ΔLRR when expressed alone. B-D, F-H and J-L show the immunostaining patterns observed when each protein was coexpressed with ASC. Note the colocalization of PYPAF1-FL (B-D) and PYPAF1ΔLRR (J-L), and the lack of colocalization of PYPAF1ΔPYRIN (F-H) to the ASC punctate structures (arrows).

**Fig. 5. PYPAF1 augments ASC-induced NF-κB activity.** A, activation of NF-κB activity by ASC. Plasmids expressing ASC were transfected into 293T cells, and relative luciferase activities were determined to measure induction of NF-κB activity. B, PYPAF1 synergizes with ASC to induce NF-κB. 293T cells were transfected with plasmids expressing PYPAF1-FL, PYPAF1ΔPYRIN, PYPAF1ΔLRR, or CARD9 (500 ng) with or without ASC (32 ng). The amount of DNAs in each transfection was kept constant by addition of empty vectors. Relative luciferase activities were then determined as a measure of NF-κB activity (lower panel). Immunoblot analysis reveals expression of PYPAF1 or CARD9 (upper panel) and ASC (middle panel). C, PYPAF1 and ASC activate NF-κB through the IKK complex. 293T cells were transfected with plasmids expressing 32 ng of ASC and 500 ng of PYPAF1 with either 500 ng of empty vector or dominant negative mutants of IKKγ (IKKγ-DN) or IKK2 (IKK2-DN). Immunoblot analysis reveals expression of PYPAF1 (upper panel) and ASC (middle panel). Relative luciferase activities were measured as described before.
pattern of cellular localization. Whereas ASC localizes to cytoplasmic punctate structures (Ref. 22 and data not shown), full-length PYPAF1 (PYPAF1-FL) showed a broad cytoplasmic distribution that excluded the nucleus (Fig. 4A). However, when the two proteins were coexpressed, PYPAF1-FL was found to colocalize with ASC (Fig. 4, B, C, and D). To determine the regions within PYPAF1 necessary for colocalization, we examined the ability of PYPAF1 mutants lacking either the N-terminal PYRIN domain (PYPAF1ΔPYRIN) or C-terminal LRRs (PYPAF1ΔLRR) to be recruited to the ASC punctate structures. When expressed alone, PYPAF1ΔPYRIN showed a broad cytoplasmic distribution similar to PYPAF1-FL (Fig. 4E). However, PYPAF1ΔPYRIN failed to associate with the ASC punctate structures, demonstrating that the PYRIN domain of PYPAF1 is necessary for recruitment (Fig. 4, F, G, and H). In contrast, PYPAF1ΔLRR colocalized with ASC indicating that the C-terminal LRRs of PYPAF1 are dispensable for recruitment (Fig. 4, I, J, K, and L). Taken together, these findings provide additional evidence that the N-terminal PYRIN domain of PYPAF1 mediates the assembly of a PYPAF1-ASC complex.

The NBS/LRR proteins CARD4 and Nod2 function as upstream regulators of NF-κB signaling (2, 4). We therefore determined whether PYPAF1 and ASC also function to regulate the activation of NF-κB using a luciferase reporter plasmid. When expressed at high protein levels in 293T cells, ASC induced NF-κB activity 40–50-fold (Fig. 5A). In contrast, PYPAF1 failed to induce the activation of NF-κB at all protein levels examined (Fig. 5B, lane 3 and data not shown). Because PYPAF1 interacts with ASC, we next examined whether PYPAF1 synergizes with ASC to activate NF-κB. When ASC was expressed at low protein levels that did not activate NF-κB, coexpression with PYPAF1 resulted in a 30-fold increase in NF-κB activity (Fig. 5B, lane 4). Immunoblot analysis revealed that ASC protein levels were not increased when coexpressed with PYPAF1, demonstrating that the activation of NF-κB was not due to increased levels of ASC (Fig. 5B, compare lanes 2 and 4). NF-κB signaling occurred through the IKK complex because dominant negative versions of IKK-γ and IKK-2 blocked the ability of PYPAF1 to synergistically activate NF-κB (Fig. 5C, lanes 3 and 4). The N-terminal PYRIN domain of PYPAF1 was essential for NF-κB signaling, since deletion of this domain (PYPAF1ΔPYRIN) eliminated the synergistic induction of NF-κB activity (Fig. 5B, lane 6). Immunoblot analysis revealed that PYPAF1ΔPYRIN was expressed at levels similar to that of PYPAF1 (Fig. 5B, upper panel), indicating that loss of function was not due to reduced protein levels. In contrast, deletion of the C-terminal domain resulted in a 2-fold increase in the synergistic activation of NF-κB relative to full-length protein suggesting that the LRRs may function as a negative regulator of PYPAF1 activity (Fig. 5B, lane 8). To confirm that the synergistic effect was specific for PYPAF1, we also coexpressed ASC with CARD9, a CARD-containing NF-κB activator (24). When expressed alone, CARD9 induced NF-κB activity 15–20-fold compared with empty vector (Fig. 5B, lane 9). However, when CARD9 and ASC were coexpressed, CARD9 failed to synergistically activate ASC-induced NF-κB activity (Fig. 5B, lane 10). Taken together, these data demonstrate that PYPAF1 functions as an activator of ASC activity and is an upstream regulator of NF-κB signaling.

In conclusion, we have identified PYPAF1 as a novel NBS/LRR family member with an N-terminal PYRIN domain. Our finding that PYPAF1 interacts with ASC through a PYRIN-PYRIN interaction suggests that these two proteins assemble together into a complex that mediates signal transduction. PYPAF1 may function in a manner analogous to other NBS/LRR family members and transmit upstream signals to the activation of NF-κB and other molecules recruited to the signaling complex. Activation of ASC by PYPAF1 may occur through an induced-proximity mechanism analogous to the activation of caspase-9 by Apaf1 (29). The restricted expression of PYPAF1 to monocytes indicates a role for this protein and its binding partners in inflammatory signaling. Indeed, our findings suggest that ASC links PYPAF1 to the activation of NF-κB. The presence of a C-terminal CARD domain in ASC suggests that a CARD-containing protein is also recruited to the PYPAF1-ASC signaling complex. Preliminary studies indicate that ASC also interacts with the CARD-containing protein caspase-1, suggesting that PYPAF1 might also coordinate signaling pathways that regulate the processing of pro-inflammatory cytokines (data not shown). ASC has been recently identified as a signaling partner of pyrin, a protein involved in the rare inflammatory disorder known as familial Mediterranean fever (20). Our findings on PYPAF1-ASC signaling suggest that pyrin may also engage NF-κB through its binding to ASC. In a search of the HTG genomic data base, we identified a single BAC clone, RP11–433K2, containing the partial genomic sequence of PYPAF1. This BAC clone maps to chromosome 1 at q44. Intriguingly, 1q44 is a locus that is associated with Muckle-Wells syndrome and familial cold urticaria, two genetically determined auto-inflammatory disorders that are similar to Mediterranean fever (31–33). Taken together, these data demonstrate that the gene encoding cryopyrin, a protein identical in sequence to PYPAF1, causes Muckle-Wells syndrome and familial cold urticaria. In addition, 1q44 has also been associated with rheumatoid arthritis (30) suggesting that PYPAF1 variants might be associated with more prevalent auto-inflammatory diseases. At least 11 other NBS/LRR proteins with N-terminal PYRIN domains are found in the HTG data base of genomic sequences. The findings presented here suggest that certain members of the emerging PYPAF family function to regulate NF-κB activity and inflammatory signaling.

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