PYPAF7, a Novel PYRIN-containing Apaf1-like Protein That Regulates Activation of NF-κB and Caspase-1-dependent Cytokine Processing*

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PYRIN-containing Apaf1-like proteins (PYPAFs) are members of the nucleotide-binding site/leucine-rich repeat (NBS/LRR) family of signal transduction proteins. We report here that PYPAF7 is a novel PYPAF protein that activates inflammatory signaling pathways. The expression of PYPAF7 is highly restricted to immune cells, and its gene maps to chromosome 19q13.4, a locus that contains a cluster of genes encoding numerous PYPAF family members. Co-expression of PYPAF7 with ASC results in the recruitment of PYPAF7 to distinct cytoplasmic loci and a potent synergistic activation of NF-κB. To identify other proteins involved in PYPAF7 and ASC signaling pathways, we performed a mammalian two-hybrid screen and identified pro-caspase-1 as a binding partner of ASC. Co-expression of PYPAF7 and ASC results in the synergistic activation of caspase-1 and a corresponding increase in secretion of interleukin-1β. In addition, PYPAF1 induces caspase-1-dependent cytokine processing when co-expressed with ASC. These findings indicate that PYPAF family members participate in inflammatory signaling by regulating the activation of NF-κB and cytokine processing.

The nucleotide-binding site (NBS) and leucine-rich repeat (NBS/LRR) family of proteins functions as a component of signaling pathways that regulate the activation of NF-κB, cytokine processing, and apoptosis. Each family member contains an N-terminal protein-protein interaction domain that mediates assembly with a downstream signaling partner, a central NBS domain that regulates activation of the signaling complex, and a C-terminal LRR domain that likely functions as a binding site for specific upstream regulators. CARD4 (Nod1), Nod2 (CARD15), and CARD12 (IapfClan) are NBS/LRR family members that contain an N-terminal caspase recruitment domain (CARD), a protein-protein interaction domain that belongs to the death domain-fold superfamily (1–6). CARD4 and Nod2 each activate NF-κB following assembly with RICK (RIP2/CARDIAK), a CARD-containing kinase that participates in both innate and adaptive immune responses (7, 8). Confirming the importance of NOD2 in inflammatory signaling, mutations within its gene confer susceptibility to Crohn’s disease and Blau syndrome, two chronic immune disorders (9–11). CARD12 also plays a role in inflammatory signaling by binding to and activating caspase-1, a CARD-containing caspase that processes pro-interleukin-1β and pro-interleukin-18 into biologically active cytokines. The upstream signals that regulate the assembly and activation of these NBS/LRR signaling complexes are not understood presently.

We recently identified the PYRIN domain as a novel protein-protein interaction domain that shares homology with pyrin, a protein that causes the rare inflammatory disorder called familial Mediterranean fever (12). Intriguingly, the PYRIN domain is found at the N terminus of numerous NBS/LRR family members instead of a CARD domain. Because of the structural similarity to the apoptosis protein Apa1, we have named these proteins PYPAFs for PYRIN-containing Apaf1-like proteins. Thus far, eight members of this NBS/LRR subfamily have been identified, including PYPAF1 (cryopyrin/NALP3), PYPAF2 (NBS1/NALP2), PYPAF3, PYPAF4 (PAN2/NALP4), PYPAF5, PYPAF6, MATER (PYPAF8), and CARD7 (NAC/DEFCAP/NALP1) (12, 14–20). At least six other genes predicted to encode novel PYPAFs are found in the High Throughout Genome (HTG) database of genomic sequences. The PYRIN domain is similar in structure to the CARD domain, death domain, and death effector domain, and it is therefore a new member of the death domain-fold superfamily (12–14, 21). This domain likely mediates homotypic interactions between PYRIN-containing proteins, resulting in the formation of a complex that participates in signal transduction. Indeed, PYPAF1 was shown recently to assemble with the PYRIN-containing protein ASC and activate NF-κB signaling (18). A role for PYPAF family members in inflammatory signaling is indicated by the recent finding that mutations within PYPAF1 are associated with Muckle-Wells syndrome and familial cold auto-inflammatory syndrome, two genetically determined immune disorders that are similar to familial Mediterranean fever (19). We report here that PYPAF7 is a novel PYRIN-containing NBS/LRR protein that is recruited by ASC to distinct cytoplasmic loci. Co-expression of PYPAF7 and ASC results in the synergistic activation of NF-κB and caspase-1-dependent cytokine processing. These findings identify
PYPAF7 as a novel regulator of inflammatory signal transduction pathways.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—Plasmids expressing either full-length PYPAF7 (PYPAF7-FL, residues 1–1061, accession number AY095146) or a PYPAF7 truncation mutant lacking the PYRIN domain (PYPAF7ΔPYRIN, residues 89–1061) with a C-terminal FLAG epitope were constructed using pCI (Promega). The panel of CARD domains used for the mammalian two-hybrid screen was described previously (22). Plasmid pCMV-CARD6-CARD/BD was constructed by inserting the CARD of CARD6 (residues 1–109, accession number AF356193) into pCMV/BD (Stratagene). Plasmid pCMV-ASC-PYRIN/AD was described previously (19). Plasmids expressing mouse pro-IL-1β with a C-terminal FLAG epitope or human pro-caspase-1 with a C-terminal Myc epitope were constructed using pCI. Plasmids expressing either dominant-negative IKKγ or inactive pro-caspase-1 (C285) were described previously (4, 18).

**PYPAF7 Expression Analysis**—Total RNA from cells in culture was extracted (Qiagen), and expression profiles were determined by real time quantitative PCR analysis (Taqman™). In brief, an oligonucleotide probe (5′-TCCCGAGAAAATCCGGTCACT-3′) was designed to anneal to PYPAF7 between two PCR primers (forward, 5′-CCCCAGAAACCTACAGGGACTA-3′ and reverse, 5′-CGCGCATGGGGTCTT-3′). The probe was then fluorescently labeled with FAM (reporter dye) on the 5′-end and TAMRA (quencher dye) on the 3′-end. Probe and PCR primers designed for β2 microglobulin were described previously (18). PCR reactions were performed on cDNAs from specific cell types using primers and probes for both PYPAF7 and β2 microglobulin genes. Fluorescent emissions generated during the PCR reaction were measured using a Sequence Detector 7700 (PerkinElmer Life Sciences). The expression level of PYPAF7 was normalized to the expression of β2 microglobulin for each sample.

**Reporter Gene Assays**—NF-κB and mammalian two-hybrid assays were described previously (22). For mammalian two-hybrid assays, 293T cells were transfected with pCMV/AD and pCMV/BD plasmids, pFRLuc-firefly reporter (Stratagene), and pRL-TK Renilla reporter system (Promega). For NF-κB assays, 293T cells were transfected with pNF-κB luciferase reporter (Stratagene), pRL-TK Renilla reporter, 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 in poly(t-lysine)-coated glass chamber slides (BioCoat, BD Labware) with plasmids expressing HA-tagged ASC and either FLAG-tagged PYPAF7 or PYPAF7ΔPYRIN using SuperFect transfection reagent (Qiagen). Cells were fixed 24 h after transfection in 4% paraformaldehyde, permeabilized and blocked in a buffer containing 0.3% Triton X-100, and incubated with the following primary and secondary antibodies: rabbit anti-HA polyclonal (Santa Cruz Biotechnology), mouse anti-FLAG monoclonal antibody M2 (Sigma), Alexa-350 goat anti-rabbit IgG (Molecular Probes), and Alexa-598 goat anti-mouse IgG (Molecular Probes). Images were generated as described previously (23).

**IL-1β Secretion Assays**—COS-7L cells (Invitrogen) were co-transfected in 12-well (22-mm) plates using LipofectAMINE 2000 transfection reagent (Invitrogen) with plasmids encoding mouse pro-IL-1β and indicated expression plasmids (total DNA, 1.04 μg). Supernatants were collected 24 h after transfection and subjected to ELISA for mouse IL-1β according to the manufacturer’s protocol (R&D Systems). The caspase-1 inhibitor z-WEHD-FMK was added to cells following transfection at a final concentration of 100 μM (R&D Systems).

**RESULTS AND DISCUSSION**

We searched the High Throughput Genome (HTG) data base of genomic sequences using the TBLASTN and GENESCAN programs and identified a novel PYPAF family member, which was designated PYPAF7 (Fig. 1A). PYPAF7 maps to chromosome 19q13.4, a locus that contains a cluster of genes that encode PYPAF family members including PYPAF2, PYPAF3, PYPAF4, PYPAF6, and MATER.2 A 3.8-kilobase cDNA corre-
responding to the predicted gene was isolated and found to encode a protein of 1061 amino acids with a predicted molecular mass of 120 kDa. A BLAST search of the protein data base revealed a tripartite domain structure consisting of an N-terminal PYRIN domain (residues 13–87), a central NBS domain (residues 212–528), and a C-terminal domain (residues 712–1061) comprised of at least 12 LRR motifs (Fig. 1B). The NBS domain belongs to the NACHT subfamily of NTPases and contains seven signature motifs including the P-loop (residues 217–224, Fig. 1B) (24). The PYRIN domain of PYPAF7 shares significant sequence similarity with the PYRIN domains of other PYRIN-containing proteins, including PYPAF1 (45% identity) and pyrin (33% identity) (Fig. 1C). Northern blot analysis using a multiple tissue expression array (CLONTECH) showed PYPAF7 expression in peripheral blood leukocytes (data not shown). Little or no expression was detected in 75 other tissue and cancer cell lines, indicating a role for this protein in inflammatory signaling. To determine the cells that express PYPAF7, peripheral blood leukocytes were fractionated into distinct cell populations. Real-time quantitative PCR analysis showed PYPAF7 to be expressed predominantly in eosinophils and granulocytes, with some expression in monocytes (Fig. 2).

Recent studies indicate that ASC binds to several PYRIN-containing proteins including PYPAF1, CARD7, and pyrin (13, 18, 25). We therefore performed cellular co-localization studies to examine interactions between PYPAF7 and ASC. FLAG-tagged PYPAF7 and HA-tagged ASC were co-expressed in cells and detected using a mixture of anti-HA and anti-FLAG antibodies. When expressed alone, PYPAF7 (PYPAF7-FL) showed a broad cytoplasmic distribution that excluded the nucleus (Fig. 3A). As observed previously, ASC localized to cytoplasmic punctate structures when expressed alone (data not shown) (26). However, when the two proteins were co-expressed, PYPAF7-FL was found to co-localize with ASC (Fig. 3, B–D). We also examined the ability of a PYPAF7 mutant lacking the N-terminal PYRIN domain (PYPAF7ΔPYRIN) to be recruited to the ASC punctate structures. When expressed alone, PYPAF7ΔPYRIN showed a broad cytoplasmic distribution similar to that of PYPAF7-FL (Fig. 3E). However, PYPAF7ΔPYRIN failed to associate with the ASC punctate structures, demonstrating that the PYRIN domain of PYPAF7 is necessary for recruitment (Fig. 3, F–H). We have been unable to detect an interaction between PYPAF7 and ASC by performing co-immunoprecipitation experiments when both proteins are overexpressed in cells. Our inability to detect an interaction may be because of the relative insolubility of PYPAF7/ASC complexes that form in the cell when these proteins are co-expressed. Alternatively, the interaction between PYPAF7 and ASC may be transient, thereby precluding its detection by immunoprecipitation analysis. Nonetheless, the co-localization of PYPAF7 with ASC to cytoplasmic loci is required for the synergistic activation of NF-κB and caspase-1-dependent cytokine processing (see below).

We next examined whether PYPAF7 and ASC participate in NF-κB signaling pathways using a luciferase reporter plasmid. Expression of PYPAF7 alone in cells failed to activate NF-κB at all protein levels examined (Fig. 4B, lane 3 and data not shown). In contrast, NF-κB was activated when high levels of ASC were expressed in cells (data not shown) (18). However, when ASC was expressed at low protein levels that did not activate NF-κB, co-expression with increasing amounts of PYPAF7 resulted in a concentration-dependent increase in NF-κB activity (Fig. 4A). The N-terminal PYRIN domain of PYPAF7 was essential for NF-κB signaling because deletion of this domain (PYPAF7ΔPYRIN) eliminated the synergistic induction of NF-κB activity (Fig. 4B, compare lanes 4 and 6). Immunohistology analysis revealed that ASC protein levels were not increased when co-expressed with PYPAF7, demonstrating that the activation of NF-κB was not caused by increased levels of ASC (Fig. 4B, upper panels). NF-κB signaling occurred through the IKK complex because dominant-negative versions of IKKα and IKKβ blocked the ability of PYPAF7 to synergistically activate NF-κB (Fig. 4C, lanes 3 and 4). These data

**FIG. 2. Expression of PYPAF7 mRNA.** PYPAF7 expression in various immune cell types was normalized to the expression of β2 microglobulin.

**FIG. 3. PYPAF7 is recruited to ASC punctate structures.** HA-tagged ASC (blue staining) and FLAG-tagged PYPAF7 proteins (red staining) were expressed in 293T cells. A and E show PYPAF7-FL and PYPAF7ΔPYRIN, respectively, when expressed alone. B–D and F–H show the immunostaining patterns observed when each protein was co-expressed with ASC. Note the co-localization of PYPAF7-FL (B–D) and the lack of co-localization of PYPAF7ΔPYRIN (F–H) to the ASC punctate structures (arrowheads).
demonstrate that PYPAF7 is an activator of NF-κB signaling when co-expressed with ASC. ASC is comprised of an N-terminal PYRIN domain and a C-terminal CARD domain (26). The presence of a CARD domain in ASC suggests that a CARD-containing protein may be involved in PYPAF7 and ASC signaling. To identify CARD domains that interact with the CARD of ASC, we performed a mammalian two-hybrid analysis and screened for binding to the CARD domains of 22 family members (Fig. 5A). The CARD domain of ASC was found to interact selectively with the CARD domain of pro-caspase-1, resulting in a 24-fold activation of luciferase activity (Fig. 5A). The CARD domain of CARD12 also interacted with the CARD domain of ASC as observed previously (Fig. 5A) (4). The PYRIN domain of ASC failed to interact expressing PYPAF7 and ASC (30 ng) were transfected into 293T cells, and relative luciferase activities were determined to measure induction of NF-κB activity. B, the N-terminal PYRIN domain of PYPAF7 is required for synergy. 293T cells were transfected with plasmids expressing PYPAF7-FL and PYPAF7 ΔPYRIN (500 ng) with or without ASC (30 ng). The amount of DNA 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 shows expression of PYPAF7 and ASC (upper panels). C, PYPAF7 and ASC activate NF-κB through the IKK complex. 293T cells were transfected with plasmids expressing 30 ng of ASC and 500 ng of PYPAF7 with either 500 ng of empty vector or dominant negative mutants of IKKγ (IKKγ-DN) or IKK2 (IKK2-DN). Relative luciferase activities were then measured. Immunoblot analysis shows expression of PYPAF7 and ASC (upper panels).
FIG. 6. PYPAP7 and ASC activate pro-caspase-1. COS-7L cells were co-transfected with plasmids encoding mouse pro-IL-1β in combination with various amounts of indicated plasmids encoding pro-caspase-1, ASC, and PYPAP7. After 24 h, supernatants were collected and subjected to ELISA for IL-1β. A, activation of pro-caspase-1 by ASC. B, activation of pro-caspase-1 by PYPAP7. C, PYPAP7 and ASC induction of IL-1β secretion is dependent on active caspase-1.
with the CARD domain of pro-caspase-1, indicating that the CARD domain of ASC is both necessary and sufficient for binding (Fig. 5A). In addition, immunoprecipitation of HA-tagged ASC coprecipitated T7-tagged pro-caspase-1 (Fig. 5B). The amount of pro-caspase-1 that associates with ASC is low considering the high level of ASC that is expressed in the cell lysate. A difficulty in quantitatively coprecipitating proteins that associate with ASC has been reported in several studies (18, 25). When overexpressed in cells, ASC self-associates via CARD-CARD and PYRIN-PYRIN interactions and forms insoluble aggregates. We speculate that the low levels of pro-caspase-1 found to associate with ASC are caused by the relative insolubility of ASC/pro-caspase-1 complexes that form in the cell when both proteins are overexpressed. Alternatively, the interaction between ASC and pro-caspase-1 may be transient and/or weak. Taken together, the mammalian two-hybrid and immunoprecipitation interaction studies suggest that ASC binds to pro-caspase-1 through its C-terminal CARD domain.

Our finding that ASC interacts with pro-caspase-1 prompted us to examine whether PYPF7 and ASC also play a role in caspase activation. Active caspase-1 cleaves pro-IL-1β, resulting in the release of IL-1β from cells. To measure the activation of pro-caspase-1, a transient transfection assay was used in which plasmids expressing pro-caspase-1 and mouse pro-IL-1β were transfected into COS-7L cells with plasmids encoding ASC and PYPF7. In this assay, the amount of murine IL-1β released into the culture medium 1 day after transfection correlated with the amount of intracellular caspase-1 activity (27). We first examined whether ASC functions as an activator of pro-caspase-1. Co-expression of ASC and pro-caspase-1 resulted in a concentration-dependent increase in the levels of secreted IL-1β (Fig. 6A). The induction of cytokine production by ASC was caspase-1-dependent because little or no IL-1β was produced in the absence of plasmid encoding pro-caspase-1 (Fig. 6A, lane 9). Moreover, ASC was unable to induce cytokine production when co-expressed with a caspase-1 active site mu-

**Fig. 7.** PYPF1 and ASC activate pro-caspase-1. COS-7L cells were co-transfected with plasmids encoding mouse pro-IL-1β in combination with various amounts of indicated plasmids encoding pro-caspase-1, ASC, and PYPF7. After 24 h, supernatants were collected and subjected to ELISA for IL-1β. A, activation of pro-caspase-1 by PYPF7. B, PYPF1 and ASC induction of IL-1β secretion is dependent on active caspase-1.
tant (C285A) that is unable to be processed and activated (Fig. 6A, lane 8) (5). We next examined whether PYPAF7 plays a role in pro-caspase-1 activation. When ASC was expressed at protein levels that resulted in low levels of caspase activity, co-expression with PYPAF7 resulted in a synergistic activation of pro-caspase-1 and a corresponding increase in IL-1β production (Fig. 6B, compare lanes 5 and 6). The N-terminal PYRIN domain of PYPAF7 was essential for pro-caspase-1 activation because deletion of this domain (PYPAF7ΔPYRIN) eliminated the synergistic production of IL-1β (Fig. 6B, compare lanes 6 and 7). Immunoblot analysis revealed that PYPAF7ΔPYRIN was expressed at levels similar to that of PYPAF7, indicating that loss of function was not caused by reduced protein levels (data not shown). PYPAF7 and ASC were unable to synergistically induce cytokine expression when co-expressed with a caspase-1 active site mutant (C285A) (Fig. 6C, lane 4). Moreover, addition of a caspase-1 inhibitor (z-WEHD) blocked the ability of PYPAF7 and ASC to induce the secretion of IL-1β (Fig. 6C, lane 3). Taken together, these data demonstrate that PYPAF7 and ASC synergistically activate pro-caspase-1.

We also examined whether another member of the PYPAF family, PYPAF1, functions as an activator of pro-caspase-1. Co-expression of PYPAF1 with ASC resulted in the activation of pro-caspase-1 and a corresponding increase in IL-1β production (Fig. 7A, compare lanes 5 and 6). The N-terminal PYRIN domain of PYPAF1 was essential for pro-caspase-1 activation because deletion of this domain (PYPAF1ΔPYRIN) eliminated the synergistic production of IL-1β (Fig. 7A, compare lanes 6 and 7). Immunoblot analysis revealed that PYPAF1ΔPYRIN was expressed at levels similar to that of PYPAF1, indicating that loss of function was not caused by reduced protein levels (data not shown). Thus, PYPAF1 is also an activator of pro-caspase-1 when co-expressed with ASC.

In conclusion, we have identified PYPAF7 as a novel PYPAF family member. Our finding that PYPAF7 is recruited to ASC punctate structures in the cytoplasm of cells suggests that these two proteins assemble together into a complex that mediates signal transduction. The PYRIN domain of PYPAF7 is required for its recruitment, suggesting that this domain mediates the assembly of a signaling complex comprised of PYPAF7 and ASC. The restricted expression of PYPAF7 to cytotoxic T cells and macrophages suggests a role for this PYPAF family member in inflammatory signaling. Our data suggest that PYPAF7 and ASC participate in the activation of NF-κB and pro-caspase-1. Activation of ASC and pro-caspase-1 by PYPAF7 may occur through an induced-proximity mechanism analogous to the activation of caspase-9 by Apaf1 (28). We also examined whether another member of the PYPAF family, PYPAF1, functions as an activator of pro-caspase-1. Co-expression of PYPAF1 with ASC resulted in the activation of pro-caspase-1 and a corresponding increase in IL-1β production (Fig. 6B, compare lanes 5 and 6). The N-terminal PYRIN domain of PYPAF1 was essential for pro-caspase-1 activation because deletion of this domain (PYPAF1ΔPYRIN) eliminated the synergistic production of IL-1β (Fig. 6B, compare lanes 6 and 7). Immunoblot analysis revealed that PYPAF1ΔPYRIN was expressed at levels similar to that of PYPAF1, indicating that loss of function was not caused by reduced protein levels (data not shown). PYPAF7 and ASC were unable to synergistically induce cytokine expression when co-expressed with a caspase-1 active site mutant (C285A) (Fig. 6C, lane 4). Moreover, addition of a caspase-1 inhibitor (z-WEHD) blocked the ability of PYPAF7 and ASC to induce the secretion of IL-1β (Fig. 6C, lane 3). Taken together, these data demonstrate that PYPAF7 and ASC synergistically activate pro-caspase-1.

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Pyrin-like proteins (PYPAF family) associate with ASC and promote the assembly of the inflammasome, a multiprotein complex that mediates the conversion of pro-caspase-1 to its active form and the secretion of mature IL-1β. This process is critical for the activation of the immune system in response to pathogens, but it can also contribute to the development of inflammatory diseases. The PYPAF7 protein, a member of the PYPAF family, has been shown to activate caspase-1 and induce IL-1β production in immune cells. Our study reveals that PYPAF7 cooperates with ASC to promote caspase-1 activation, highlighting its role in the innate immune response and its potential relevance in inflammatory diseases.