PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and caspase-1

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Abstract

Recently, a large subfamily of nucleotide-binding and oligomerization domain-containing proteins that have an N-terminal pyrin-like domain and C-terminal leucine-rich repeats has been described. In this study, we identified PYNOD, a novel member of this family that lacks the leucine-rich repeats. We found that human PYNOD mRNA is expressed in various tissues and at high levels in heart, skeletal muscle and brain. It is also expressed in various cell lines, including haematopoietic cell lines. PYNOD oligomerizes and binds to ASC, an adaptor protein that plays a role in apoptotic and inflammatory signal transduction, and to caspase-1 and IL-1β. PYNOD inhibits apoptosis-associated speck-like protein containing a CARD (ASC)-mediated NF-κB activation and apoptosis, and caspase-1-mediated IL-1β maturation, and it does so in the presence and absence of constitutively active mutants of CARD12 and PYPAF1, which are enhancers of these processes. Thus, PYNOD is a novel regulator of apoptosis and inflammation.

Introduction

A large family of mammalian Apaf-1-like proteins has recently emerged (1–4). The first-reported members of this family, CIITA, Nod1, Nod2 and CARD12 (also called Ipaf, or CLAN), possess an N-terminal caspase activation and recruitment domain (CARD), a mid-sequence nucleotide-binding and oligomerization domain (NOD) and C-terminal leucine-rich repeats (LRRs). More recently reported members of this family (the PYPAF or NALP family) possess an N-terminal pyrin-like domain (PYD) instead of a CARD. The 3-dimensional structure of PYD determined by NMR spectroscopy (5,6) revealed that PYD is the fourth member of the death domain (DD) fold domains; the others are DD, the death effector domain, and CARD. The N-terminal domains of Apaf-1-like molecules recruit downstream signaling molecules. In addition, because the truncation of C-terminal LRRs enhances the activity of Apaf-1-like molecules, it has been postulated that the LRRs are negative regulatory domains, and that ligand binding to this region relieves the suppression (7–10).

Some members of the Apaf-1-like molecules have been show to play important roles in the immune system. For instance, CIITA, originally identified as product of the gene that restores class II MHC expression in the cells from patients with bare lymphocyte syndrome, is a transactivator of the class II MHC gene expression (11). On the other hand, Nod1 and Nod2 were recently demonstrated to be cytoplasmic pathogen receptors that play an important role in the innate immune system (12–14), in parallel with toll-like receptors that are cell-surface pathogen receptors. Nod1 and Nod2 recognize partial structures of bacterial peptidoglycans, γ-D-glutamyl-meso-diaminopimelic acid and muramyl dipeptides respectively, using their LRRs (12–14), and induce NF-κB activation through interaction with RICK/RIP2 with their N-terminal CARD domains (8,9). Several other members of Apaf-1-like molecules, including CARD12, NALP1 (also called DEFCAP, NAC or CARD7), PYPAF1 (Cryopyrin), PYPAF5 (NALP6) and PYPAF7 (NALP12, Monarch-1), induce NF-κB activation, caspase-1-mediated IL-1β processing, and/or apoptosis (1,10,15–18). Based on these functions, it is likely that these Apaf-1-like molecules also play an important role in innate immunity. However, our knowledge about their physiological roles are limited, because their upstream signaling molecules or ligands (possibly some pathogen-derived molecules in analogy with Nod1 and Nod2) are not known. Finally, PAN2 (PYPAF4, NALP4) interacts with IKKα and inhibits TNFα- and IL-1β-induced NF-κB activation (19). Thus, some Apaf-1-like molecule may play regulatory roles in immune/inflammatory responses. Many other Apaf-1-like molecules remain uncharacterized.
Some PYD-containing members of Apaf-1-like molecules (such as PYPAF1, PYPAF5, PYPAF7 and NALP1) bind apoptosis-associated speck-like protein containing a CARD (ASC) (also called as PYCARD or TMS1), an adaptor protein consisting of PYD and CARD. ASC links these Apaf-1-like molecules and caspase-1, and induces IL-1β maturation (1, 10, 16, 17). ASC also couples these Apaf-1-like molecules onto an NF-kB activating pathway, although the downstream molecular links to this direction is not known. In addition to the ASC linker, CARD12 requires ASC to induce NF-PYD-containing Apaf-1-like molecules described above, and constitute a molecular link to this direction (1, 10, 16, 17). Card12 also couples these Apaf-1-like molecules and caspase-1, and induces IL-1β processing through direct interaction with caspase-1 in the absence of ASC (20).

ASC also plays an important role in apoptosis, as it was originally identified as a protein that generates speck-like structure in apoptotic HL-60 cells treated with chemotherapeutic agents (21). The expression of ASC gene has been found ubiquitously in various tissues, while its highest expression was observed in peripheral blood lymphocytes. Interestingly, the expression of ASC is suppressed by hypermethylation in various tumor cell lines and tissues, suggesting that ASC is a tumor suppressor gene (22–26). Consistent with this notion, overexpression of ASC only or its moderate expression together with PYPAF1 or CARD12 induces apoptosis (27). Interestingly, apoptosis induced by the expression of CARD12 plus ASC was mediated by caspase 8. In contrast, it was recently reported that the expression of ASC was induced by p53, and ASC promotes translocation of BAX to mitochondria and caspase-9-dependent apoptosis, suggesting that ASC plays an essential role in the p53-mediated apoptosis (28). Thus, ASC may activate different apoptotic signaling pathways in different contexts.

Mutations of Apaf-1-like proteins have been connected to several genetic inflammatory diseases. For example, loss-of-function mutations in the Nod2 gene are associated with susceptibility to Crohn’s disease (29–31). On the other hand, gain-of-function mutations in Nod2 are responsible for Blau syndrome (32). Furthermore, mutations in the PYPAF1/cryopyrin genes cause familial cold autoinflammatory syndrome and Muckle–Wells syndrome (33). Thus, the dysregulation of Apaf-1-like molecules may be involved in immunological diseases such as inflammatory diseases, autoimmune diseases or allergy.

By basing a search on homology to PYPAF1, we have identified a gene encoding a novel Apaf-1-like protein, PYNOD, and have characterized its expression pattern and function. Unlike other Apaf-1-like molecules, PYNOD lacks the C-terminal LRRs. Our analyses demonstrate that PYNOD is a novel negative regulator of inflammatory and apoptotic signal transduction.

**Methods**

**Cell lines**

HepG2, PK-1, PK-8, PK-9, SW480, DLD-1 and SH-10-TC were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. HEK293 cells were obtained from Dr Hiroshi Sato (Cancer Research Institute, Kanazawa University). HEK293T cells were obtained from two sources, HEK293T-S from Dr Hiroshi Sato and HEK293T-Y from Dr Takashi Yokota (Graduate School of Medicine, Kanazawa University). Transfection with expression plasmids for Fas, Bid or ASC induces apoptosis in HEK293T-Y cells, whereas HEK293T-S cells are relatively resistant to the same treatment.

**Isolation of PYNOD cDNAs**

Human and mouse PYNOD cDNAs were amplified by nested RT-PCR from human heart total RNA (Clontech, Palo Alto, CA) and mouse brain poly(A) positive RNA, respectively, using the following primers: human PYNOD first round sense, AGCTTTGCAACATGTATCTCG; antisense, TCTTTCTCCAGAGTGTTGTC; second round sense, CCTTCCCCAGATGACCACT; antisense, TCCTCATAGATCCTGTAC; mouse PYNOD first round sense, TTCTCGTTCAGGTGCCAG; antisense, TGACATTGTGAAGTCTCG; second round sense, GCTGAACCTCTGACCATCAG; antisense, CTACCCA-TTCATCTTACT.

**Plasmids**

Human cDNAs for ASC, CARD12, PYPAF1, IL-1β, caspase-1, RIP and Bid were amplified by RT-PCR. The resultant cDNAs and human and mouse PYNOD cDNAs were cloned into the mammalian expression vector pEF-BOS-EX (34), and the plasmids were named pEF-PYNOD, pEF-mPYPAF1, pEF-IL-1β, pEF-ASC, pEF-CARD12 and pEF-caspase-1, respectively. To generate a plasmid (pEF-CARD12ALRR) expressing LRR-truncated CARD12 (residues 1–457), pEF-CARD12 was digested with BstXI and EcoRV, filled in, and ligated. To generate a similar mutant of PYPAF1 (residues 1–739), a stop codon and an Xbal site were introduced next to the codon for residue 739 using PCR. The HindIII–Xbal fragment of the PYPAF1 cDNA in pEF-PYPAF1 was replaced with the corresponding fragment of the PCR product to generate pEF-PYPYPAF1ALRR.

Plasmids expressing FLAG-tagged proteins were generated as described below. The full-length cDNAs for human PYNOD and IL-1β were cloned into p3XFLAG-CMV-7.1 (Sigma, Saint Louis, MO), and the plasmids were named p3XFLAG-PYNOD and p3XFLAG-IL-1β, respectively. The cDNAs encoding caspase-1 (whose initial methionine was replaced with leucine), RIP-DD (residues 558–671), L297A, E299A), and an IKKβ-ΔN (residues 135–419) were generated by PCR and cloned into pCMV-Tag2B (Stratagene, La Jolla, CA). The resultant FLAG-tagged cDNAs were subcloned into pEF-BOS-EX to generate pEF-FLAG-caspase-1, pEF-FLAG-RIPDD, pEF-FLAG-TRADDN, and pEF-FLAG-IKKΔN, respectively. The cDNAs encoding full-length Bid and the Bid C-terminal domain (BidΔC, residues 61–195) were amplified by PCR and cloned into pBluescript II (Stratagene). The glycine at position 94 of Bid-CT was mutated to glutamic acid using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega, WI). The full-length Bid and G94E-BidΔC cDNAs were cloned into pCMV-Tag2A. The resultant cDNAs were subcloned into pEF-BOS-EX to generate pEF-FLAG-Bid and pEF-FLAG-G94E-BidΔC. G94E-BidΔC showed somewhat weaker apoptosis-inducing activity than
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Analyses of PYNOD mRNA expression

Northern blot analyses were performed using a Human 12-Lane MTN Blot (Clontech) according to the manufacturer’s protocol. RT–PCR analyses were performed as described previously (36). The primers used to detect human PYNOD mRNA were as follows: sense, CCTTCCCCCAGATCACCATG; antisense, CCTCAGAATCTCTGTGACAG. The amounts of template cDNAs were adjusted so that a similar amount of a PCR fragment of β-actin was generated within the linear range of the PCR.

Assay for the transcriptional activity of NF-κB

HEK293 cells in a 24-well plate were transfected with plasmid DNA including 50 ng of pNF-κB-Luc (carrying a firefly luciferase cDNA driven by 5xNF-κB-binding sites, Stratagene, La Jolla, CA) and 50 ng pRL-TK (carrying renilla luciferase cDNA driven by HSV-TK promoter, Promega, Madison, WI) using the TransIT-LT1 reagent (TAKARA, Otsu, Japan) according to the manufacturer’s recommendation. The total amount of DNA in each transfection was kept constant by the addition of empty vector (pEF-BOS). Twenty-four hours after the transfection, the firefly and renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity (RLA) was calculated as follows: RLA = firefly luciferase activity / renilla luciferase activity. Fold induction of NF-κB activity was calculated as follows: fold induction of RLA = experimental RLA / RLA of vector control.

NF-κB electrophoretic mobility shift assay (EMSA)

An EMSA was carried out with extracts of HEK293 cells (10 μg protein) as described previously (37). Oligonucleotides containing the following NF-κB-binding sequence were used (sequence overhangs are in lowercase): forward, gatcTG-GGGACTTTCCGC; reverse, gatcGCGGAAAGTCCCCA. An EMSA was carried out with extracts of HEK293 cells (10 μg protein) as described previously (37). Oligonucleotides containing the following NF-κB-binding sequence were used (sequence overhangs are in lowercase): forward, gatcTG-GGGACTTTCCGC; reverse, gatcGCGGAAAGTCCCCA.

Assay for the caspase-1-mediated IL-1β secretion

HEK293T-S cells in a 24-well plate were transfected with plasmid DNA including 300 ng of pEFx-hIL-1β and 5 or 50 ng of pEF-hCaspase-1 using the Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA). The total amount of DNA in each transfection was kept constant by the addition of empty vector (pEF-BOS). Culture supernatants were harvested 24 h after the transfection, and the concentration of IL-1β was determined using the Human IL-1β OptEIA ELISA Set (PharMingen, San Diego, CA) according to the manufacturer’s protocol.

Apoptosis assay

HEK293T-Y cells in a 96-well plate were transfected with plasmid DNA including 1 ng of pEGFP-C1 (Clontech). Twenty-four hours after the transfection, the cells were stained with propidium iodide and Cy5-annexin V (BioVision, Palo Alto, CA) in binding buffer (10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2). The cells were then analyzed using a FACSCalibur equipped with a 488 nm argon laser and a 635 nm diode laser (Becton Dickinson, San Jose, CA).

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting were performed as described previously (38) with some modifications. In brief, HEK293T-S cells in a 24-well plate were transfected with various combinations of plasmids, and then harvested 24 h after the transfection. Cells were lysed in 100 μl of lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.2% NP-40, 1 mM APMSF, 1 μg/ml peptstatins, 1 μg/ml leupeptins). After centrifugation, the cleared lysates were incubated with mouse anti-ASC (a kind gift of Dr Junji Sagara, Graduate School of Medicine, Shinshu University), anti-FLAG (Sigma), or anti-Myc monoclonal antibody (MBL, Nagoya, Japan) for 1 h, and the immune complex was precipitated using Protein G Sepharose 4 Fast Flow beads (Amersham, Uppsala, Sweden). Precipitates were then subjected to western blotting using a peroxidase-conjugated anti-FLAG antibody (Sigma) or mouse anti-Myc antibody, followed by a peroxidase-conjugated anti-mouse IgG antibody (Chemicon, Temecula, CA).

Results

Identification of human, mouse and rat PYNOD genes

We performed a tblastn search using the amino acid sequence of human PYPAF1 as a query sequence against the nucleotide databases of GenBank. As a result, we identified a novel rat gene encoding a member of the PYPAF family proteins in the genomic clone RP32-100G3 (accession number AC118667) derived from the chromosome 1q32. We named it PYNOD because it consists of N-terminal PYD and C-terminal NOD, but it lacks LRRs (Fig. 1A–C). Using the rat PYNOD sequence as a query sequence, we found the genomic sequence of mouse PYNOD in the genomic clone RP24-449N4 (accession number AC122046) derived from mouse chromosome 7E3, and of human PYNOD in the genomic clone RP11-21N2 (accession number AC124259) derived from chromosome 11p15, where the genes for PYPAF5/NALP6 and NALP14/NOD5 are also located. We also found EST sequences derived from mouse olfactory brain (accession numbers BB622805 and AV344932) that contain a partial mouse PYNOD cDNA sequence. Human, mouse and rat PYNOD genes consist of two exons corresponding to PYD and NOD, and span about 4.5 kb, 3 kb and 4 kb, respectively.

The authenticity of the predicted mRNA sequences for human and mouse PYNOD was confirmed by isolating their cDNAs from human heart and BALB/c mouse brain mRNA using the RT–PCR technique. The nucleotide sequences of human and mouse PYNOD cDNAs determined in this paper have been submitted to GenBank with accession numbers AY489192 and AY489193. The homology of the PYNOD amino acid sequences between different species are as follows: human and mouse, 55.5%; human and rat, 55.9%; and mouse and rat, 91.5% (Fig. 1D). Recently, the human PYNOD gene...
Expression of PYNOD

The tissue distribution of PYNOD mRNA was examined using northern blot analyses (Fig. 2A). A PYNOD mRNA of ~2.4 kb was detected at high levels in heart, brain and skeletal muscle, while weak expression was observed in all other tissues. The ~4.4 kb transcript was also observed in brain, heart and skeletal muscle. Other variations in PYNOD mRNA size may occur in the small intestine and placenta. We also detected PYNOD mRNA by RT-PCR analysis in various cell lines, including those derived from the haematopoietic lineage, endothelium, liver, pancreas and digestive tract (Fig. 2B). In addition, the PYNOD mRNA expression was enhanced after the treatment with PMA plus ionomycin in K562 and Jurkat but not U937 and THP-1 cells. Its expression in Jurkat cells was also slightly increased by LPS stimulation. Thus, PYNOD expression in some haematopoietic cells may enhanced upon inflammatory stimulation.

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has been reported as NALP10 and Nod8 (3,4). These reviews mentioned the presence of this gene in the chromosome 11p15.

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PYNOD specifically inhibited ASC-mediated NF-κB activation.

PYPF1, 5, 7, and CARD12 activate NF-κB in the presence of ASC (1,10,16,27). Deletion of the LRRs in these proteins
enhances this activity. Thus, we expected that PYNOD might act as a constitutive NF-κB activator. To investigate this possibility, we transiently transfected HEK293 cells with a human PYNOD expression vector together with an NF-κB-luciferase reporter plasmid in the presence or absence of expression vectors carrying ASC or CARD12/δLRR. The expression of PYNOD alone did not induce significant NF-κB activation (Fig. 3A and B). Instead, and unexpectedly, PYNOD inhibited the NF-κB activation induced by ASC or CARD12/δLRR plus ASC in a dose-dependent manner (Fig. 3A–C). However, PYNOD did not inhibit the NF-κB activity induced by the transfection of MEKK1 or MyD88 cDNA or by stimulation with TNFα (Fig. 3A and B). Consistent with NF-κB transcriptional activity, nuclear NF-κB DNA-binding activity was increased by the expression of CARD12/δLRR plus ASC and inhibited by PYNOD expression (Fig. 3D).

PYNOD inhibits maturation of IL-1β mediated by caspase-1.

The proinflammatory cytokine IL-1β is produced in its proform. Caspase-1 cleaves Pro-IL-1β into its active form, and this process is also essential for the secretion of IL-1β. As reported
previously, the expression of CARD12ΔLRR (independent of ASC) or a high dose of ASC stimulates the caspase-1-mediated secretion of IL-1β (20). In contrast, PYNOD did not induce the caspase-1-mediated IL-1β maturation by itself; rather, it inhibited the IL-1β release induced by high-dose caspase-1, or by a combination of caspase-1 and ASC or CARD12ΔLRR (Fig. 4A). PYPAF1ΔLRR but not PYNOD enhanced caspase-1-mediated IL-1β release in the presence of a limited amount of ASC (Fig. 4B). Instead, PYNOD inhibited the IL-1β release induced by the combination of PYPAF1ΔLRR and ASC in a dose-dependent manner.

**PYNOD inhibits apoptosis induced by ASC**

The apoptosis protein Ced-4, the *Caenorhabditis elegans* ortholog of mammalian Apaf-1, lacks the C-terminal regulatory domain, just like PYNOD. In addition, ASC causes speck-like aggregation in apoptotic HL-60 cells (21), and the overexpression of ASC induces apoptosis in HEK293T-Y cells (27). Therefore, we investigated the effect of PYNOD on apoptosis. As reported previously, a high dose of ASC or the combination of CARD12ΔLRR and ASC induced apoptosis (Fig. 5A). Unlike the Fas death receptor or Bid, the proapoptotic BH3-only member of the Bcl-2 family, PYNOD did not induce apoptosis by itself in HEK293T-Y cells (Fig. 5B). Interestingly, PYNOD selectively inhibited ASC-mediated apoptosis but not that induced by Fas or Bid.

**PYNOD interacts with multiple proteins**

Since PYNOD inhibits various activities of ASC, we investigated the interaction of PYNOD with ASC in HEK293T-S cells. As shown in Fig. 6A, in the presence of ASC, anti-ASC antibody co-precipitated FLAG-tagged PYNOD but not the RIP-DD. These results suggest that PYNOD interacts with ASC in cells. It has been hypothesized that ligand-induced oligomerization of Apaf-1-like proteins is essential for their downstream signaling. Since PYNOD interacts with ASC and inhibits its function, we speculated PYNOD might not be able to self-oligomerize. However, when Myc-tagged and FLAG-tagged PYNOD were co-expressed in cells, Myc-PYNOD was co-precipitated with FLAG-PYNOD and vice versa (Fig. 6B and data not shown). Thus, although PYNOD oligomerizes and interacts with ASC, these interactions did not result in the activation of ASC.

Since PYNOD inhibits the IL-1β maturation induced by high-dose caspase-1 in the absence of ASC or CARD12ΔLRR, we investigated the interaction between PYNOD and caspase-1 or IL-1β. We found that human PYNOD interacted with both IL-1β and caspase-1 (Fig. 6B). When we expressed mouse proteins in a similar experiment, mouse PYNOD also interacted with mouse IL-1β and caspase-1 (data not shown). In contrast, FLAG-tagged RIP-DD, TRADD-DN, IKKγ-DN and full-length Bid did not coprecipitate with Myc-PYNOD (Fig. 6B).

**Discussion**

Here we identified a novel Apaf-1 like molecule, PYNOD, that consists of a PYD and a NOD, but lacks LRRs. This is probably the major form of PYNOD, because 3’RACE analyses for mouse PYNOD mRNA failed to detect a cDNA containing LRRs. The ~4.4 kb transcript may represent premature PYNOD mRNA, because its length is similar to that of the human PYNOD gene. However, we cannot exclude a possibility that some longer transcripts represent splicing variants carrying LRRs.

We demonstrated that PYNOD interacts with ASC and inhibits multiple functions of ASC, including activation of NF-κB and caspase-1, and induction of apoptosis. PYNOD also interacts with caspase-1 and IL-1β and inhibits caspase-1-mediated IL-1β maturation. Thus, PYNOD is likely to be a multifunctional negative regulator of inflammation and apoptosis. On the other hand, PYNOD does not inhibit MEKK1-, MyD88-, or TNFα-induced NF-κB activation, or Fas- or Bid-
induced apoptosis. We also failed to show that PYNOD inhibited apoptosis of HEK293T-Y cells induced by staurosporine, etoposide (VP-16), Fas ligand, or TNF-α (see Supplementary figure S2, available at International Immunology Online). Thus, the inhibitory activity of PYNOD in NF-κB activation and apoptosis occurs only when they are mediated by ASC, suggesting that ASC is a direct target of PYNOD. In this context, PYNOD is different from PAN2/PYPAF4, which has been reported to interact directly with IKKα and inhibits the NF-κB activation induced by multiple...
intracellular molecules that are involved in the TNF receptor and IL-1/toll-like receptor signal transduction pathways (19). On the other hand, PYNOD inhibited the caspase-1-mediated IL-1β secretion in the absence of exogenous ASC (Fig. 4A). Because ASC expression was not detected in HEK293 cells, it is unlikely that this IL-1β secretion was mediated by endogenous ASC. Therefore, it is likely that PYNOD has the ability to inhibit this response by directly binding to caspase-1 and/or IL-1β. However, we have not succeeded in showing that PYNOD disrupted the interaction between caspase-1 and IL-1β. Further experiments are necessary to clarify this point.

It has been hypothesized that Apaf-1-like proteins are oligomerized via their C-terminal region (WD40 repeats or LRRs) upon ligand binding, and this oligomerization then induces the proximity of downstream adapter molecules that interact with the N-terminal region (CARD or PYD) of the Apaf-1-like molecules (3). Here we found that, like the C-terminal truncation mutants of PYPAF1 and CARD12, PYNOD self-oligomerizes and interacts with ASC. However, PYNOD behaves as an inhibitor rather than an activator of ASC. The precise mechanism of this difference between PYNOD and other Apaf-1-like molecules is unclear at present. It is possible that not only the proximity of ASC proteins induced by an oligomer of an Apaf-1-like protein but also a conformational change of ASC is essential for its activation, and that PYNOD may not be able to induce the conformational change. Alternatively, PYNOD may sterically inhibit the interaction between ASC and its downstream interacting molecules.

Pyrin, the gene mutated in familial Mediterranean fever, has been shown to inhibit apoptosis and NF-κB activation induced by ASC (39, 40). Furthermore, targeted disruption of the pyrin gene in macrophages leads to the enhancement of caspase-1 activation and IL-1β production upon LPS stimulation, and overexpression of pyrin suppresses IL-1β production (41). Thus, PYNOD has a similar activity to pyrin. The expression plasmids for PYNOD and pyrin showed similar efficacy in the inhibition of NF-κB activation and apoptosis based on the dose–response curves (data not shown), suggesting that these functions of PYNOD are physiologically relevant. In addition, we found here that PYNOD inhibits the PYPAF1 pathway of IL-1β secretion. It is noteworthy that mutations in the PYPAF1 gene cause cold-induced auto-inflammatory syndrome (33). In this context, an interesting possibility is that mutation of the PYNOD gene may cause a disease similar to familial Mediterranean fever or cold-induced auto-inflammatory syndrome. On the other hand, the augmentation of PYNOD expression might be a remedy for these genetic diseases.

We found significant expression of PYNOD mRNA in all the organs we examined. High levels of PYNOD expression were found in brain, heart, and skeletal muscle. In contrast, consistent with a previous report (21), the expression of ASC in these organs is relatively low (Supplementary figure S1). Considering the inflammatory and apoptotic activity of ASC, a high expression of ASC could have deleterious effects in these organs. Therefore, PYNOD may play a protective role in ASC-mediated inflammation or apoptosis in these organs. In addition, PYNOD mRNA was detected by RT–PCR analysis in all cell lines tested, including those of the lymphoid and myeloid lineages. Stimulation of K562 or Jurkat cells with PMA plus ionomycin and/or LPS enhanced their expression of PYNOD mRNA. Thus, given that Apaf-1-like proteins are intracellular pathogen receptors that activate innate immune responses, PYNOD may play a regulatory role in the innate immune system. Future studies using PYNOD-deficient mice or transgenic mice should elucidate the physiological role of PYNOD in animals.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.
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Abbreviations

ASC  apoptosis-associated speck-like protein containing a CARD
BidCT  BID C-terminal
CARD  caspase activation and recruitment domain
DD  death domain
DN  dominant-negative mutant
EMSA  electrophoretic mobility shift assay
LRR  leucine-rich repeat
PYD  pyrin-like domain
NOD  nucleotide-binding and oligomerization domain
RLA  relative luciferase activity

References


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