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PAN1/ NALP2/PYPAF2, an Inducible Inflammatory Mediator That Regulates NF-κB and Caspase-1 Activation in Macrophages*

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Genes encoding proteins with PYRIN/PAAD/DAPIN domains, a nucleotide binding fold (NACHT), and leucine rich repeats have recently been recognized as important mediators in autoimmune inflammatory disorders. Here we characterize the expression and function of a member of the PYRIN and NACHT domain (PAN) family, PAN1 (also known as NALP2 and PYPAF2). PAN1 protein expression is regulated by lipopolysaccharide (LPS) and interferons (IFNβ and IFNγ) in THP-1 macrophage cells. In gene transfection studies PAN1 manifests an inhibitory influence on NF-κB activation induced by various pro-inflammatory stimuli, including tumor necrosis factor TNFα and interleukin-1β (IL-1β). Gene transfer-mediated elevations in PAN1 protein also suppressed activation of IκB kinases induced by inflammatory cytokines. Conversely, reducing endogenous levels of PAN1 using small interfering RNA enhanced LPS-induced production of ICAM-1 (intercellular adhesion molecule 1), an NF-κB-dependent gene. We also show here that PAN1 binds via its PYRIN domain to ASC, an adapter protein involved in caspase-1 activation. This binding is disrupted by mutation of the α1 helix of ASC. In gene transfer experiments PAN1 enhances caspase-1 activation and IL-1β secretion in collaboration with ASC. Conversely, reducing endogenous levels of PAN1 using small interfering RNA significantly reduced LPS-induced secretion of IL-1β in monocytes. We propose that PAN1 functions as a modulator of the activation of NF-κB and procaspase-1 in macrophages.

Genes encoding proteins with a combination of PYRIN (PAAD/DAPIN/PYD) domains, a nucleotide binding domain (NACHT), and leucine-rich repeats (LRRs)[1] have recently been recognized as important mediators in autoimmune and inflammatory disorders (1–6). Multidimensional NMR analysis and other biophysical techniques (2, 7) indicate that the PYRIN domain is a member of the death domain-fold superfamily that includes death domains, death effector domains, and caspase recruitment domains (CARDs). PYRIN domains serve as homotypic protein interaction modules, creating a network of protein associations mediated by PYRIN-PYRIN interactions. The human genome contains at least 19 genes encoding PYRIN domain proteins (8). The founding member of the family, Pyrin, is mutated in families with familial Mediterranean fever, a hereditary autoinflammatory disease. Mutant alleles of the *CIAS1* gene encoding another PYRIN-family protein, Cryopyrin/PYPAF1/NALP3, have been associated with familial cold autoinflammatory syndrome, chronic infantile neurological cutaneous and articular syndrome, and Muckle-Wells syndrome, providing further hints of a role for PYRIN domain proteins in control of inflammatory responses (6, 9–11).

NF-κB family transcription factors play critical roles in regulating expression of genes involved in inflammatory and immune responses, including certain cytokines, lymphokines, immunoglobulins, and leukocyte adhesion proteins in mammals. NF-κBs exist in the cytoplasm in inactive form sequestered by inhibitory proteins called inhibitor of NF-κB (IκB). Proteasome-dependent degradation of IκBs is linked to their phosphorylation by the IκB kinase (IKK) complex, which consists of two related kinases, IKKα and IKKβ, and a scaffold subunit IKKγ (NEMO). Phosphorylation of IκBs triggers their polyubiquitination and degradation, thus freeing NF-κB-family transcription factors to enter the nucleus and transactivate promoters of various target genes. Some PYRIN-family proteins have been shown to associate with the IKK complex, affecting activation of NF-κB (12–14). How PYRIN domains bind IKK complex proteins is unclear. Alternatively, ASC or NAC (NALP1) conceivably could connect to IKKγ via Cardinal/TUCAN/CARD8/NDPP/Dakar, which can bind ASC and NAC (NALP1)[2] and also reportedly binds IKKγ. However, other routes to IKK complex proteins must also exist since some PYRIN proteins, such as PAN2/NALP4/PYPAF4, associate with IKK proteins but apparently do not interact with ASC or NAC (NALP1) (14) or Cardinal. It has been shown recently that Cryopyrin/PYPAF1/NALP3/CIAS1 can suppress TNFα-induced nuclear translocation of endogenous p65 and that transcriptional activity of exogenous NF-κB p65 was also blocked by Cryopyrin (11).

Excessive production of IL-1β is implicated in various diseases, including septic shock, inflammatory bowl disease, autoimmune diabetes, and rheumatoid arthritis. PYRIN domain proteins have also been reported to induce activation of caspase-1, a cysteine protease responsible for proteolytic activation of pro-inflammatory cytokines. Pro-interleukin-1β (Pro-IL-1β) is the best known substrate of caspase-1 (15). Processing of pro-IL-1β is tightly controlled by various molecules that either inhibit or enhance activation of the zymogen (inactive)

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[1] The abbreviations used are: LRR, leucine-rich repeat; CARD, caspase recruitment domain; IKK, IκB kinase; TNF, tumor necrosis factor; Pro-IL-1β, pro-interleukin-1β; IFN, interferon; LPS, lipopolysaccharide; siRNA, small nuclear RNA; HA, hemagglutinin; GFP, green fluorescent protein; TK, thymidine kinase; ICAM-1, intercellular adhesion molecule 1; TLR, toll-like receptor.

[2] J. M. Bruey and J. C. Reed, unpublished observations.

form of caspase-1. Cardiak (CARD-containing IL-1β-converting enzyme-associated kinase) also known as Rip2 (Rick) and Ipaf (caspase-1-activating protein related to Apaf-1) (also known as CLAN), for example, are known activators of pro-caspase-1, which bind pro-caspase-1 via CARD-CARD interactions, activating this protease by an induced proximity mechanism involving protein oligomerization (16–18). The bipartite adapter protein ASC uses its CARD to interact with the CARD of pro-caspase-1 and certain caspase-1 activators, whereas its PYRIN domain binds the PYRIN domains of various other proteins including Pyrin, Cryopyrin, and NAC (NALP1) (13, 19). However, ASC can function as either a caspase-1 activator or inhibitor, depending on the levels of its expression and on whether certain other PYRIN-family proteins are coexpressed, and depending on whether other inflammatory pathways were activated (5, 20).

Here we characterized the PAN1 (NALP2/PYPAF2) protein and gene. PAN1 protein expression is regulated by specific inflammatory mediators in macrophage-lineage cells. We also show that the PAN1 protein is capable of regulating NF-κB and pro-caspase-1 activation. Furthermore, PAN1 binds ASC, collaborating with this adapter protein in the regulation of IL-1β production, consistent with a recent report (6). Thus, PAN1 shares functional similarity with selected members of the PYRIN family, including NAC (NALP1), Cryopyrin (NALP3/PYPAF1), and PYPAF5 (PAN3), Pyrin (13, 19, 21).

MATERIALS AND METHODS

Reagents—Human TNFα, human interferon IFNγ, and IFNβ were purchased from Invitrogen. Human IL-1β was purchased from Roche Applied Science. Lipopolysaccharide (LPS, purified from *Escherichia coli* O55:B5) was obtained from Sigma-Aldrich.

Expression Plasmids—The complete open reading frame and segments corresponding to full-length (amino acids 1–1062), the PYR (amino acids 1–112), NACHT (amino acids 112–591), ΔPYR (amino acids 110–1062), ΔLRR (amino acids 1–585), and LRRs (amino acids 590–1062) of PAN1 were amplified by high fidelity PCR (Stratagene, La Jolla, CA) from pCMV6-XL4 clone FB1924-A07 (Origene Technologie, Inc.) and subcloned into pcDNA3 vectors (Invitrogen) containing an N-terminal Myc epitope tag. Expression plasmids encoding pro-caspase-1, pro-IL-1β, Cardiak/Rip2/Rick, NIK, TRAF2, TRAF6, p65, Bcl-10 were described (13). Plasmids encoding IKKα, IKKβ, and IKKγ were gifts of Michael Karin (University of California, San Diego, CA), whereas IKKepsilon (IKKi) and TBK1 were gifts from Shizuo Akira (Osaka University, Osaka, Japan). Plasmids encoding full-length NAC (DEFCAP/CARD7/NALP1), ASC, and the PYRIN domain of ASC or NAC have been described (13, 22, 23). ASC (E13W) was generated using the QuikChange mutagenesis kit (Stratagene). Authenticity of all constructs was confirmed by DNA sequencing.

Cell Culture and Transfection—HEK293N and HEK293T cells were cultured in Dulbecco's modified Eagle's medium, whereas THP-1 and ZR-75-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Stably transfected ZR-75-1 cells (ZR-NEO or ZR-PAN1 cells) were generated by culture in 800 μg ml^{-1} G418 (Calbiochem). Cells were treated with or without 10–20 ng ml^{-1} TNFα, IL-1β, 1 μg ml^{-1} LPS, 1000 units of IFN-γ or INF-β for various times. Transfection of HEK293 cells was accomplished using Superfect (Qiagen), holding total DNA content constant. THP-1 cells were transfected with double-strand small interfering RNAs (siRNAs) using Oligofectamine (Invitrogen) in Opti-MEM media. The siRNAs were purchased from Dharmacon, Inc. and prepared by annealing complementary sequences corresponding to region, (5′-AGUUGAGCAAGUUCAAGUAdTdT-3′, 5′-UCAACUCGUUCAAGUUCAUdTdT-3′) or GFP used as control (5′-GCGCGCUUUGUAGGAUUCGdTdT-3′, 5′-CGCGCGAAACAUCCUAAGCdTdT-3′).

Antibodies—A polyclonal anti-PAN1 antiserum was generated by repeated immunization of rabbits with an 18-mer synthetic peptide NH_2-CLKSFNKRKPLSLGITRKER-(amide) conjugated to maleimide-activated carrier proteins keyhole limpet hemocyanin and ovalbumin (Pierce). Epitope-specific antibodies for FLAG, HA, or Myc tag were obtained from Sigma, Roche Applied Science, and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Polyclonal anti-ASC (AL177) was obtained from Apotech.

Co-immunoprecipitation Assay—Cells (5×10^5–1×10^6) were lysed in isotonic lysis buffer (150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 0.3% Nonidet P-40, 12.5 mM β-glycerophosphate, 2 mM NaF, 200 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, containing 1× protease inhibitor mix (Roche Applied Science)). Clarified lysates were subjected to immunoprecipitation using agarose-conjugated anti-c-Myc, anti-FLAG M2 (Sigma), and anti-HA antibodies. After incubation at 4 °C for 4–12 h, immune complexes were washed four times in lysis buffer, separated by SDS/PAGE, and analyzed by immunoblotting using anti-Myc, anti-FLAG, anti-HA, or anti-PAN1 antibodies as indicated in conjunction with ECL detection system (Amersham Biosciences). Where indicated, cell lysates (10% volume) were run alongside immune complexes. Alternatively, lysates were directly analyzed by immunoblotting after normalization for total protein content.

NF-κB Reporter Gene Assay—Typically, 10^5 HEK293 cells, cultured in 24-well plates in 5% serum, were transiently transfected with a total of 1 μg of plasmid DNA (normalized for total DNA) including 144 ng of pNF-κB -LUC (Clontech) and 6 ng of a *Renilla luciferase* gene driven by a constitutive thymidine kinase (TK) promoter (pRL-TK; Promega). Lysates were analyzed using the dual luciferase kit (Promega). Where indicated, cells were treated with 10 ng/ml TNFα for 8 h before analysis.

IL-1β Secretion Assay—IL-1β secreted into culture supernatants of HEK293N cells transiently transfected in 24-well plates was measured by enzyme-linked immunosorbent assay using a commercial kit (R&D Systems); data were normalized for cell numbers, and assays were performed in triplicate.

Caspase Activity Assay—HEK293N cells were transiently transfected in 6-well dishes, and at 24 h post-transfection cells were suspended in caspase lysis buffer (10 mM HEPES (pH 7.4), 25 mM NaCl, 0.25% Triton X-100, and 1 mM EDTA, 5 mM dithiothreitol) and normalized for protein content. Typically, 15–25 μg of lysates were analyzed for protease activity by continuously measuring the release of fluorigenic substrate Ac-YVAD-aminofluoromethylcoumarin product from (Bachem) at 37 °C as described (24).

Statistical Analysis—Data were analyzed using the PRISM Statistics software package; employing an unpaired *t* test method.

RESULTS

Characterization of PAN1 mRNA and Protein Expression—The predicted PAN1 protein contains a PYRIN, NACHT, and eight LRR domains (Fig. 1*A*). Using reverse transcription-PCR, we surveyed adult and fetal human tissues for the presence of *PAN1* transcripts (Fig. 1*B*). First-strand cDNAs generated from equivalent amounts of RNA from a variety of normal human tissues were used as templates to amplify a region of the *PAN1* cDNA corresponding to the NACHT domain. This analysis revealed that *PAN1* is expressed at highest levels in lungs, placenta, and thymus, with detectable expression also observed in ovary, intestine, and brain.

To assess expression at the protein level, an antiserum was raised in rabbits using a synthetic peptide of the human PAN1 as an immunogen. This antibody detected the 110-kDa PAN1 protein, which was expressed in transfected HEK293 cells (Fig. 1*C*). This antibody did not cross-react with other PAN-family proteins tested, PAN2 or NAC (data not shown).

Using the anti-PAN1 antiserum, we assessed expression of the endogenous PAN1 protein in various cancer cell lines by immunoblotting. Lysates were normalized for total protein content before analysis. The relative levels of PAN1 protein varied widely among the tumor lines tested, with highest levels found in MCF7 and MDA-MB-435 breast cancer, Caco2 colon cancer, and UACC62 melanoma cells (Fig. 1*D*).

Regulation of PAN1 Protein Expression by Inflammatory Mediators—We examined the effect of pro-inflammatory stimuli on PAN1 protein expression in THP-1 macrophages by immunoblotting (Fig. 2). Culturing THP-1 macrophages with either type I or type II interferons resulted in transient elevations in PAN1 protein expression, which peaked at 4–8 h after treatment. Comparisons with IFI16, another PYRIN domain-containing protein known to be induced by interferons (25), served as a positive control (Fig. 2, *A* and *B*). Although culturing THP-1 macrophages with TLR4 ligand LPS induced increases

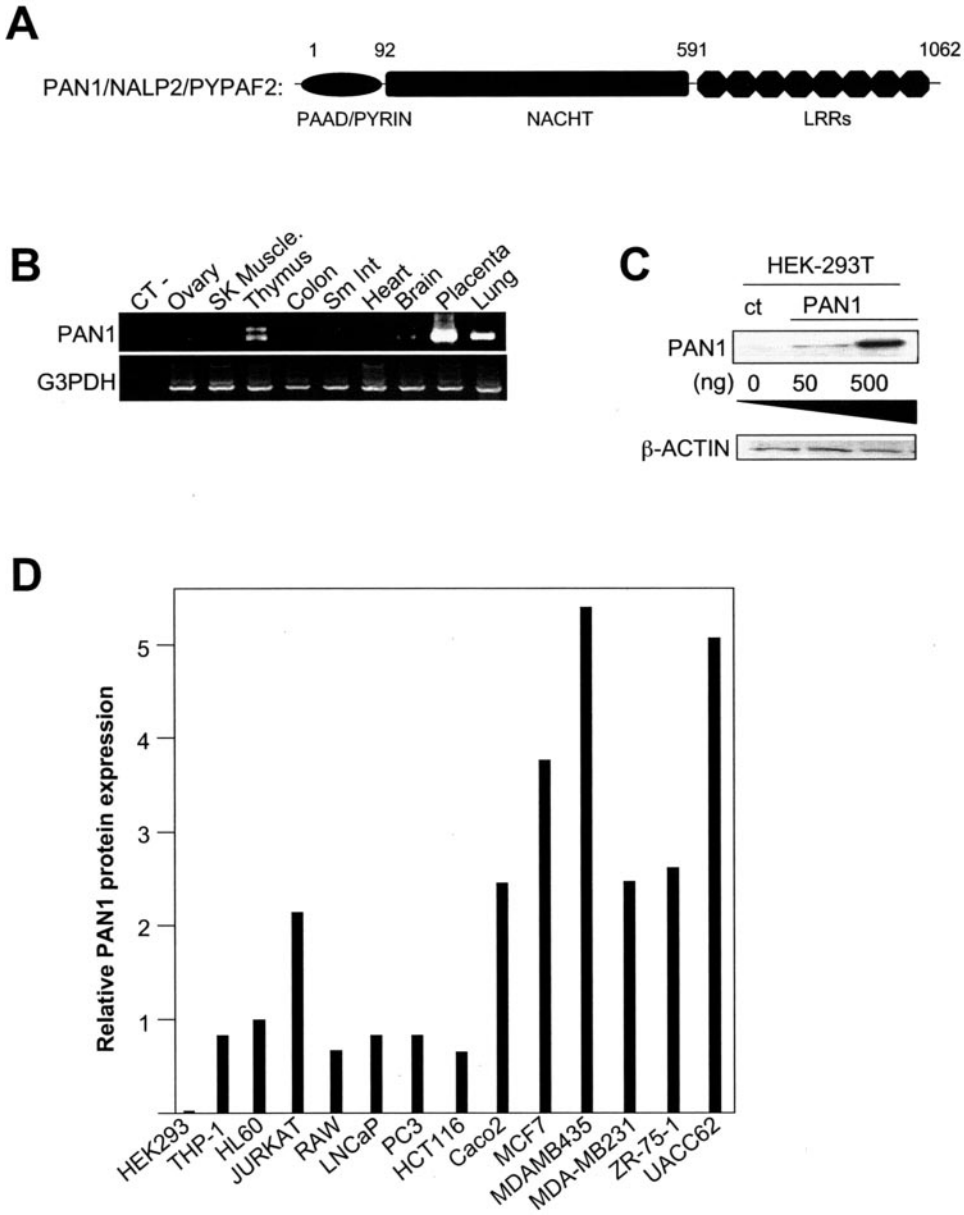

FIG. 1. **PAN1 expression in human tissues and tumor cell lines.** *A*, the structure of the PAN1/NALP2/PYPAF2 protein is depicted, showing the location of the PYRIN, NACHT, and LRR domains. The amino acid positions defining the borders of these domains are indicated. *B*, first-strand cDNAs prepared from 1 μg of total RNA from various human tissues were used as templates for amplifying either a region of PAN1 corresponding to the NACHT domain or glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) as a control. PCR products were analyzed by agarose gel electrophoresis and visualized by UV illumination of ethidium bromide-stained gels. *CT* refers to control RNA where the reverse transcription step was omitted, which was included to exclude signals from contaminating genomic DNA. *SK*, skeletal. *Sm Int*, small intestine. *C*, lysates were prepared from HEK293T cells that had been transiently transfected with various amounts of plasmid DNA encoding PAN1. Lysates were directly analyzed by SDS-PAGE/immunoblotting using anti-PAN1 or anti-β-actin antibodies. *D*, lysates (100 μg) prepared from the indicated human cell lines were analyzed by SDS-PAGE/immunoblotting using anti-PAN1 or anti-β-actin antibodies. Antibody detection was accomplished by ECL, with exposure to x-ray film. The resulting films were analyzed by densitometric scanning of the bands corresponding to PAN1 and normalized relative to β-actin.

in PAN1 protein levels, a TLR9 ligand consisting of a CpG oligonucleotide did not (26–31). Thus, some but not all TLR-family receptors also induce PAN1 protein expression in these cells. Probing blots with anti-β-actin antibody confirmed loading of equivalent amounts of protein for each sample. By confocal microscopy, the PAN1 was localized predominantly to the cytosol, although occasional cells also contained nuclear immunostaining (data not shown).

PAN1 Inhibits NF-κB Activation by Diverse Stimuli—The rapid induction of PAN1 by immunostimulatory molecules led us to hypothesize that PAN1/NALP2 may play a role in mediating inflammatory responses. Because NF-κB is intimately linked to inflammation (for review, see Ref. 32), we analyzed NF-κB activity in the presence of transfected PAN1. Transfection of various amounts of PAN1-encoding plasmid DNA into HEK293 cells caused dose-dependent suppression of TNFα and CD40-induced NF-κB activity, as measured by reporter gene assays (Fig. 3, *A* and *B*). This suppression of NF-κB activity was specific in as much as PAN1 did not suppress activity of other transcription factors, such as β-catenin/T cell factor and p53 (Fig. 3, *C* and *D*; and data not shown).

To map where the PAN1 affects the NF-κB activation pathway, NF-κB activity was induced by transient transfection of plasmids encoding various intracellular signal transducers that operate within cytokine receptor pathways, leading to phosphorylation of IκB, a key event required for NF-κB release.

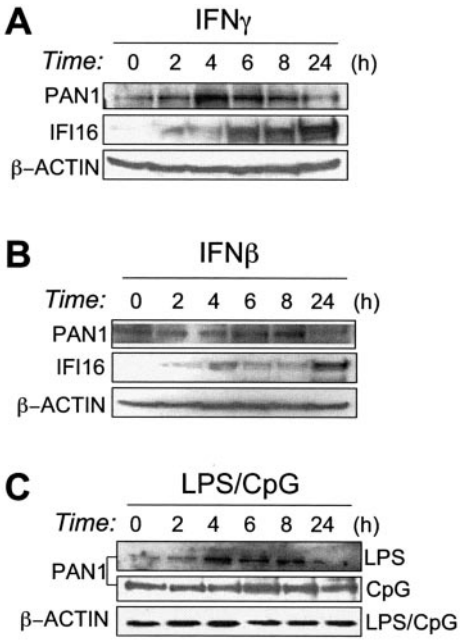

FIG. 2. **PAN1 expression is regulated by inflammatory stimuli.** Lysates (100 μg) derived from 12-*O*-tetradecanoylphorbol-13-acetate-differentiated THP-1 cells were prepared at various times after treatment with IFNγ (A), IFNβ (*B*), or LPS and CpG (*C*) and analyzed by SDS-PAGE/immunoblotting using anti-PAN1, anti-β-actin, or anti-IFI-116 antibodies.

We performed similar reporter gene assays, stimulating cells with the cytokine IL-1β or transfecting cells with NF-κB-inducing proteins such as RIP, TRAF6, TRAF2, Bcl-10, TLR4, IKKα, IKKβ (Fig. 3, *C* and *D*), and NIK, IKKepsilon (IKKi), and TBK1 panel (Fig. 3*E*). In every case examined transient overexpression of PAN1 suppressed NF-κB activity. In contrast, co-expression of PAN1 did not suppress reporter gene activation induced by NF-κB-p65. Thus, PAN1 blocks upstream of NF-κB, apparently at the level of the IKK complex.

To preliminarily explore the physiological significance of these findings, we compared the levels of endogenous PAN1 protein produced in differentiated THP-1 monocytes before and after LPS treatment, with the levels of PAN1 produced by transfection in HEK293T cells (Fig. 3*F*). Plasmid-derived expression of PAN1 was also compared with endogenous levels of PAN1 found in tumor cell lines such as MCF7, which was determined to express high levels of endogenous PAN1. All samples were normalized for total protein content before analysis by immunoblotting. These studies showed that the levels of PAN1 produced by transfection of 0.5 μg of plasmid DNA were comparable with the levels of endogenous PAN1 found in LPS-stimulated THP-1 monocytic cells. Probing the blots with anti-actin confirmed loading of comparable amounts of protein for all samples (Fig. 3*F*). Thus, the levels of PAN1 proteins produced by gene transfection are within the physiologically relevant range.

PAN1 Associates with the IKK Complex—Because the functional analysis of PAN1 suggested that it suppresses NF-κB activity induced by overexpression of IKKα or IKKβ (Fig. 3), we explored whether PAN1 might associate with components of the IKK complex. Using lysates from HEK293 cells in which PAN1 was co-expressed with HA epitope-tagged IKKα, IKKβ, or IKKγ, co-immunoprecipitation assays were performed, revealing association of IKKα, IKKβ, and IKKγ with PAN1 (Fig. 4*A*). Immunoblot analysis of the lysates confirmed production of IKKα, IKKβ, and IKKγ at comparable levels. In contrast to IKK components, PAN1 did not co-immunoprecipitate with other proteins kinases such as AKT or JNK1, demonstrating specificity.

To extend these co-immunoprecipitation studies to analysis of the endogenous proteins, we used lysates from hematopoietic cell lines in which elevations of PAN1 protein were induced by pro-inflammatory agents, such as LPS-stimulated THP-1 monocytic cells and INFγ-stimulated Jurkat T-cells. Lysates were subjected to immunoprecipitation using anti-IKKγ monoclonal antibody or control IgG, and the resulting immune-complexes were immunoblotted using anti-PAN1 antiserum. As shown in Fig. 4*B*, PAN1 was readily detected in association with IKKγ but not control immune-complexes. As expected, IKKγ immune-complexes also contained associated endogenous IKKα and IKKβ, as revealed by immunoblotting. Similar results were obtained using anti-IKKα instead of anti-IKKγ to pull down PAN1 (not shown). We conclude, therefore, that endogenous PAN1 associates with the endogenous components of the IKK complex, at least in hematopoietic cells stimulated with pro-inflammatory agents.

Overexpression of PAN1 Inhibits IκBα Degradation Induced by TNFα—Active IKK induces phosphorylation and subsequent degradation of IκB-family proteins (33). We, therefore, evaluated the effects of PAN1 on levels of endogenous IκBα in cells after treatment with IKK activator, TNFα. For these experiments breast cancer cell line ZR-75-1 was stably transfected with PAN1-encoding (ZR-PAN1) or control (ZR-NEO) plasmids, then stimulated for various times with TNFα followed by analysis of lysates by immunoblot using anti-IκBα antibody (Fig. 5*A*). In ZR-NEO cells TNFα stimulation caused the disappearance of IκBα protein after 15 min. In contrast, IκBα protein levels were sustained at detectable levels in ZR-PAN1 cells even after TNFα treatment. Clones of ZR-75-1 cells stably expressing PAN1 demonstrated ~70% reduced activation of a transfected NF-κB reporter gene in response to TNFα, consistent with reduced degradation of IκBα (data not shown).

SiRNA-mediated Knock-down of Endogenous PAN1 Expression Increases LPS-mediated Induction of NF-κB Target Gene—Several target genes of NF-κB-family transcription factors have been identified, including the cell adhesion protein ICAM-1. We used the technique of siRNA to reduce PAN1 expression in THP-1 cells, assessing the impact on LPS-mediated induction of endogenous ICAM-1 by immunoblotting. For these experiments, a 19-bp double-strand RNA corresponding to PAN1 nucleotides 5′-AGUUGAGCAAGUUCAAGUAdTdT-3′ was introduced into 12-*O*-tetradecanoylphorbol-13-acetate-differentiated THP-1 macrophages using a GFP siRNA as a negative control. Immunoblot analysis demonstrated that siRNA directed against PAN1 mRNA substantially reduced the levels of PAN1 protein in THP-1 cells before and after treatment with LPS, whereas GFP control siRNA did not (Fig. 5*B*). When normalized for β-actin levels, the reductions in PAN1 protein levels were determined to be ~60–70%. LPS-induced ICAM-1 expression was also reduced in PAN1 siRNA-treated THP-1 macrophages by approximately half compared with GFP siRNA-treated THP-1 cells stimulated with LPS (Fig. 5*B*). These data indicate that endogenous PAN1 is capable of suppressing LPS-induced expression of endogenous NF-κB target genes.

PAN1 Inhibits NF-κB Induction via Its PYRIN Domain—To map the domain in PAN1 required for suppression of NF-κB, a series of Myc-tagged deletion constructs of PAN1 were generated (Fig. 6*A*) and tested for their ability to suppress TNFα-mediated induction of NF-κB activity using reporter gene assays. Specifically, we compared the effects of full-length PAN1 with truncation mutants containing only the PYRIN, NACHT,

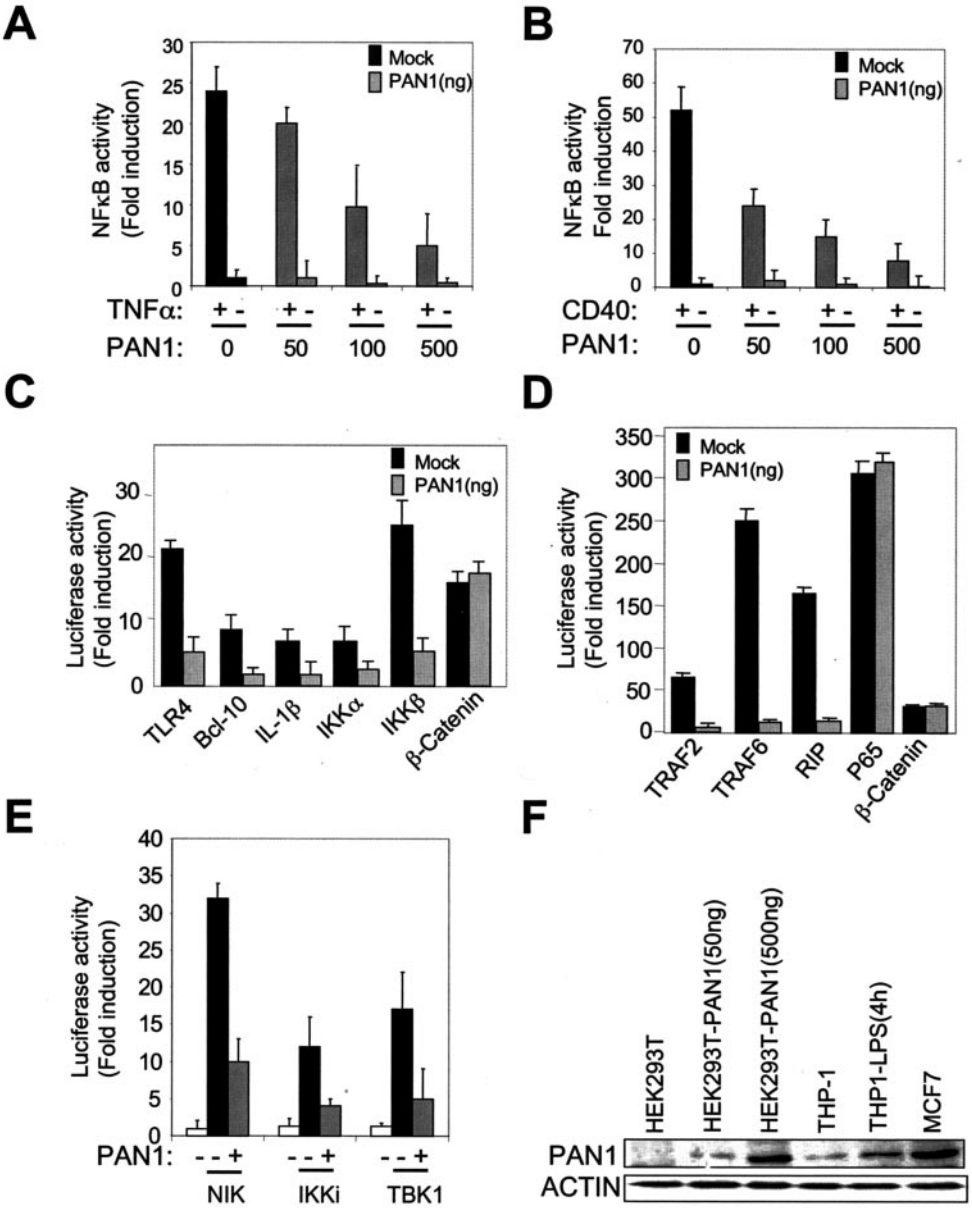

FIG. 3. **PAN1 inhibits NF-κB induction at the level of the IKK complex.** *A*, HEK293T cells were transfected with either 500 ng of control plasmid pcDNA3 (*black bars*) or various amounts of pcDNA3-PAN1 plasmid encoding PAN1 (*gray bars*), as indicated (50, 100, 500 ng). Transfections also included 100 ng of pNF-κB and 6 ng of pRL-TK, maintaining the total DNA of transfections at 1 μg. At 36 h after transfection cells were either cultured with or without 20 ng/ml TNFα for 8 h, and NF-κB activity was measured by a dual luciferase assays, expressing normalized data as a fold induction above basal activity measured in unstimulated, control-transfected cells (mean ± S.D.; $n = 3$). *B*, HEK293N cells transfected as above were co-transfected with 100 ng of CD40 plasmid. *C*, *D*, and *E*, NF-κB activity as measured by reporter gene assays in HEK-293N cells transiently transfected with 200 ng of plasmids encoding various NF-κB-inducing proteins as indicated and 150 ng of PAN1-encoding plasmid. NF-κB transcriptional activity was measured 2 days later by reporter gene assay, and results are presented as -fold induction relative to control cells, which were not transfected with effector plasmids mean ± S.D. ($n = 3$). For β-catenin, a T cell factor-responsive reporter gene plasmids was used instead of NF-κB-responsive reporter (see "Materials and Methods"). *F*, lysates (100 μg of total protein) were prepared from HEK293T cells transfected with 50 or 500 ng of PAN1-encoding plasmid, THP1 cells cultured with or without LPS for 4 h, or from MCF-7 cells and analyzed by SDS-PAGE/immunoblotting using anti-PAN1 and anti-β-actin antibodies.

or LRR domains and with mutants lacking the PYRIN or LRR domains. The plasmids encoding these PAN1 mutants were then transiently expressed in HEK293 cells in equal amounts using a plasmid dose at which full-length PAN1 suppresses TNFα and CD40 induction of NF-κB by ~50%.

TNFα-mediated NF-κB activation was inhibited to a similar extent by full-length PAN1, the PYRIN domain only, and PAN1 lacking the LRRs (Fig. 6*B*). In contrast, TNFα-induced NF-κB activity was not suppressed by the PAN1 mutant lacking only the PYRIN domain and by PAN1 mutants encompassing only the NACHT or LRR domains. The failure of the PAN1 (ΔPYR) and the PAN1 fragments comprised of only the NACHT or LRR domains to inhibit TNFα-mediated NF-κB induction was not due to a problem of protein expression, as demonstrated by immunoblot analysis (Fig. 6*C*). Each of these PAN1 mutants was expressed at levels equivalent to or higher than the full-length PAN1 protein. Thus, the PYRIN domain of PAN1 is necessary and sufficient for suppression of NF-κB activation induced by TNFα.

PAN1 Regulates Caspase-1 Activation—Some of the previously studied members of the PAN family of proteins have been shown to regulate activation of caspase-1 (6, 10, 12, 20, 21, 34), a protease responsible for proteolytic processing of pro-IL-1β (for review, see Ref. 15). We, therefore, explored the effects of

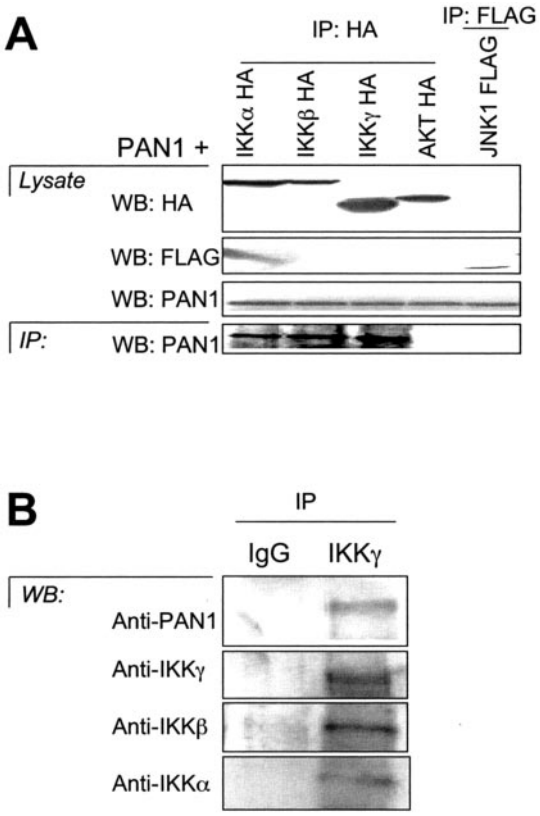

FIG. 4. **PAN1 associates with IKKs.** *A*, 293T cells were transfected with 1 μg of pcDNA3-PAN1 DNA together with 1 μg of plasmids encoding HA-IKKα, HA-IKKβ, HA-IKKγ, HA-AKT, or FLAG-JNK1. After 36 h, cell lysates were prepared and subjected to immunoprecipitation (*IP*) with anti-HA antibody or anti-FLAG antibody. The resulting immune-complexes were then analyzed by SDS-PAGE/immunoblotting (*WB*) using anti-PAN1 antibody. Alternatively lysates were run directly in gels and analyzed by immunoblotting using anti-PAN1 or anti-HA to confirm expression of proteins. *B*, co-immunoprecipitation assays were performed using lysates from THP1 cells that had been treated with LPS (1 μg/ml) for 25 min. Lysates were subjected to co-immunoprecipitation using either anti-IgG or anti-IKKγ antibodies, and the resulting immune-complexes were analyzed by Western blotting using various antibodies, as indicated.

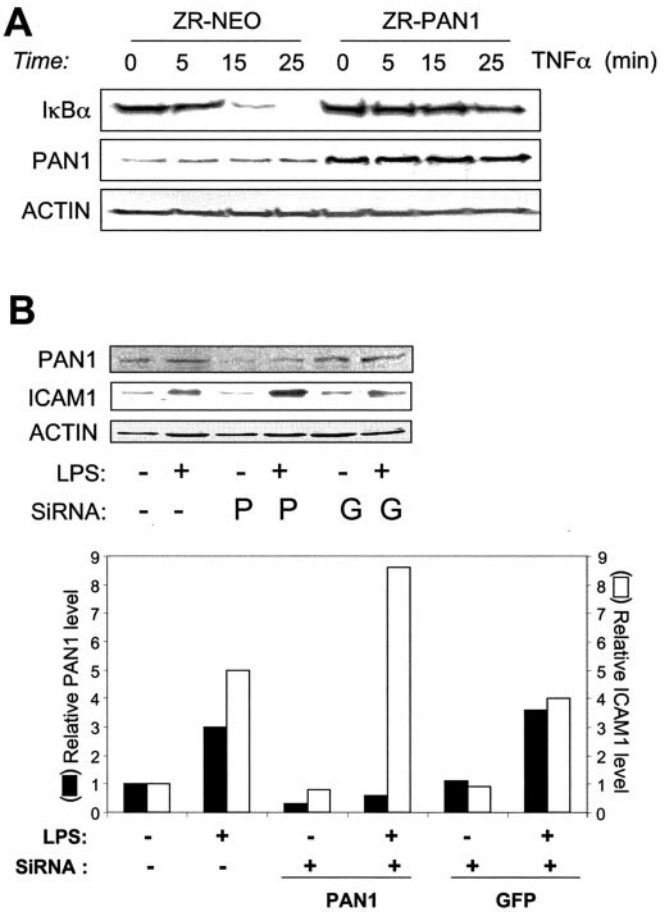

FIG. 5. **PAN1 inhibits TNFα-induced IκBα degradation and controls expression of endogenous NF-κB target gene ICAM-1.** *A*, endogenous IκBα was analyzed by immunoblotting using lysates from ZR-NEO and ZR-PAN1 stable transfectants prepared at various times after stimulation with 20 ng/ml TNFα. Cell lysates were normalized for protein content and analyzed by immunoblotting with antibodies specific for IκBα (*top*), PAN1 (*middle*), or β-actin (*bottom*). *B*, 12-*O*-tetradecanoylphorbol-13-acetate differentiated THP-1 cells were transfected with siRNA-PAN1 (*P*) or siRNA-GFP (*G*), then cultured the following day with (+) or without (−) LPS for 10 h. Lysates were subject to SDS-PAGE/immunoblotting using anti-PAN1 (*top*), anti-ICAM-1, or anti-β-actin (*top*). Blot results were quantified by scanning densitometry analysis of autoradiograms, expressing data as fold expression compared with uninduced cells after normalizing to β-actin expression (*bottom*). Data are representative of two independent experiments.

PAN1 on caspase-1 activity using the same experimental approach previously applied to other PAN-family members (19). For these experiments, lysates from THP-1 monocytic cells, which served as a source of endogenous pro-caspase-1, were mixed with lysates from HEK293T cells that had been transfected with a plasmid encoding PAN1. Comparisons were made with lysates from HEK293T cells transfected with control (empty) or Cardiak-encoding plasmids, thus serving negative and positive controls, respectively. After incubation at 30 °C for 1 h, caspase-1 activity was measured using a fluorigenic substrate of this protease. As shown in Fig. 7*A*, the addition of extracts containing either PAN1 or Cardiak induced 6–9-fold increases in caspase-1 activity compared with control extracts (Fig. 7*A*).

Caspase-1-mediated cleavage of pro-IL-1β results in secretion of mature IL-1β. We, therefore, tested the effects of PAN1 expression on IL-1β secretion by gene transfection methods. For these experiments plasmids encoding pro-IL-1β and pro-caspase-1 were co-transfected into HEK293N with or without various amounts of a plasmid encoding PAN1, and levels of secreted IL-1β were measured 30 h later in culture supernatants. As shown in Fig. 7*B*, low amounts of PAN1 (10 ng) induced increases in IL-1β secretion, with progressively less effect as the amount of transfected plasmid DNA was increased. Immunoblot analysis confirmed dose-dependent expression of the PAN1-encoding plasmid. We conclude that PAN1 is capable of inducing IL-1β secretion in collaboration with caspase-1. The reduced IL-1β secretion observed at high concentrations of PAN1 plasmid is similar to some other PAN-family proteins (20) and suggests that an appropriate stoichiometry of PAN1 relative to caspase-1 is required for optimal signaling.

Finally, we performed a structure-function analysis of the PAN1 protein with respect to IL-1β secretion (Fig. 7*C*). Similar to the results obtained when using NF-κB as an endpoint, expression of the PYRIN domain of PAN1 was sufficient for inducing IL-1β secretion, producing amounts of IL-1β secretion that were comparable with cells expressing full-length PAN1 protein. Furthermore, deletion mutants of PAN1 lacking the PYRIN domain were inactive in terms of IL-1β secretion. We conclude, therefore, that the PYRIN domain of PAN1 is both necessary and sufficient for inducing IL-1β secretion in collaboration with caspase-1.

PAN1 Interactions with Other PYRIN Domain-containing Proteins—The PYRIN fold is a homotypic interaction domain

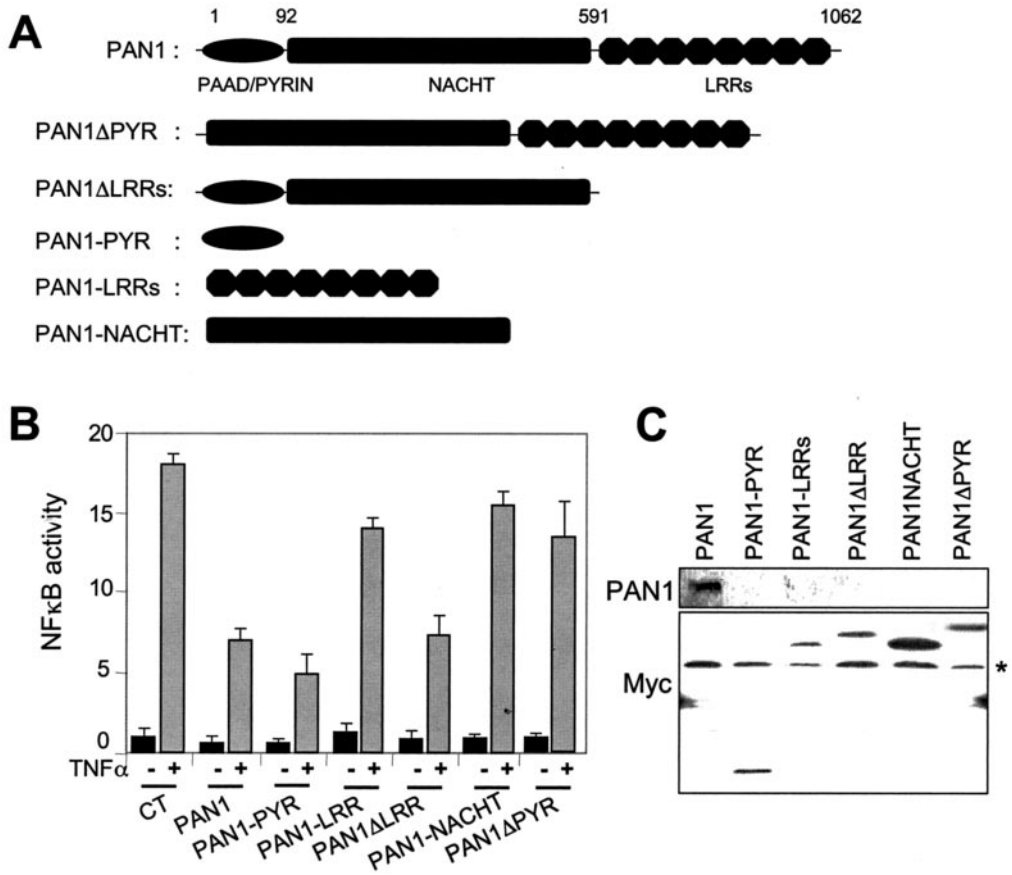

FIG. 6. **The PYRIN domain of PAN1 is sufficient for suppression of NF-κB activity.** *A*, a schematic representation is provided of Myc-tagged PAN1 deletion mutants that were constructed in expression plasmids. *B*, HEK293 cells were transfected with 50 ng of pNF-κB-luc, 10 ng of pTK-RL reporter vectors, and either 400 ng of pcDNA3Myc empty vector or the same amount of the indicated Myc-tagged PAN1 deletion mutants. At 36 h after transfection, cells were either left untreated (*black bar*) or stimulated for 8 h with TNFα (*gray bar*). Cells were then harvested, and luciferase activity was determined expressing data as -fold induction above control (*C*)-transfected, unstimulated cells (mean ± S.D., $n = 3$). *C*, immunoblot analysis of PAN1 mutants was performed using lysates from transfected cells, normalized for total protein (50 μg). Blots were incubated with either anti-PAN1 antiserum (*top*) or anti-Myc antibody (*bottom*). The *asterisk* indicates a nonspecific band, served as an internal loading control.

allowing for networks of PYRIN-PYRIN interactions. We, therefore, tested PAN1 for interactions with a few of the better-studied PYRIN domain-containing proteins, ASC, NAC (NALP1), Cyropryin (NALP3), and Pyrin (Fig. 8, *A* and *B*, and data not shown) by co-immunoprecipitation assays after transient co-transfection in HEK293T cells. PAN1 associated with the full-length ASC protein and with the isolated PYRIN domains of ASC and NAC but not with the full-length NAC, Cryopyrin, or Pyrin proteins (Fig. 8*B* and not shown). The specificity of these interactions was confirmed by control immunoprecipitations involving unrelated proteins that lack PYRIN domains (*e.g.* pro-caspase-1; cIAP1) (Fig. 8*B*). Thus, PAN1 displays selective interactions with other members of the PYRIN domain family.

The NMR-derived solution structure of the PYRIN domain of ASC reveals two highly positively and negatively charged surfaces, predicted to mediate interactions with other PYRIN domains via electrostatic interactions (35, 36). We, therefore, used site-directed mutagenesis to substitute an acidic residue in the first α-helix of the PYRIN domain of ASC for a hydrophobic residue, making a E13W mutation. As shown by co-immunoprecipitation assays, the ASC(E13W) mutant failed to associate with PAN1, unlike the wild-type ASC protein (Fig. 8*B*). These data further confirm the specificity of the co-immunoprecipitation assay results, demonstrating specific association of ASC with PAN1.

To determine whether the endogenous ASC and PAN1 proteins are capable of interacting, we performed co-immunoprecipitations using lysates from LPS-stimulated THP-1 monocytes. Immune-complexes were prepared using anti-ASC or control IgG antibodies followed by immunoblot analysis using anti-PAN1 antiserum. As shown in Fig. 8*C*, ASC but not control immunoprecipitates contained associated endogenous PAN1, confirming interaction of the endogenous proteins.

PAN1 Collaborates with ASC in Inducing IL-1β Secretion—Certain PAN-family proteins such as Cryopyrin cooperate with the bipartite adapter protein ASC to induce caspase-1 activation and IL-1β secretion (20). We, therefore, compared the effects of PAN1 with Cryopyrin in co-transfection assays where either of these PAN-family proteins was co-expressed with ASC in cells. For these experiments, HEK293 cells were transfected with plasmids encoding pro-IL-1β and pro-caspase-1 alone or in combination with various other plasmids encoding ASC, PAN1, or Cryopyrin, then IL-1β secretion was measured in culture supernatants 36 h later. Cells transfected with control (empty) plasmid or Cardiak-encoding plasmid DNA served as negative and positive controls, respectively. As expected from prior reports, ASC induced a modest increase in IL-1β secretion by itself, which was strikingly augmented by co-expression of Cryopryin (Fig. 9*A*). The combination of ASC and PAN1 also resulted in more IL-1β secretion that either protein alone, with more than additive effects. However, the combination of ASC and PAN1 was not frankly synergistic, unlike the combination

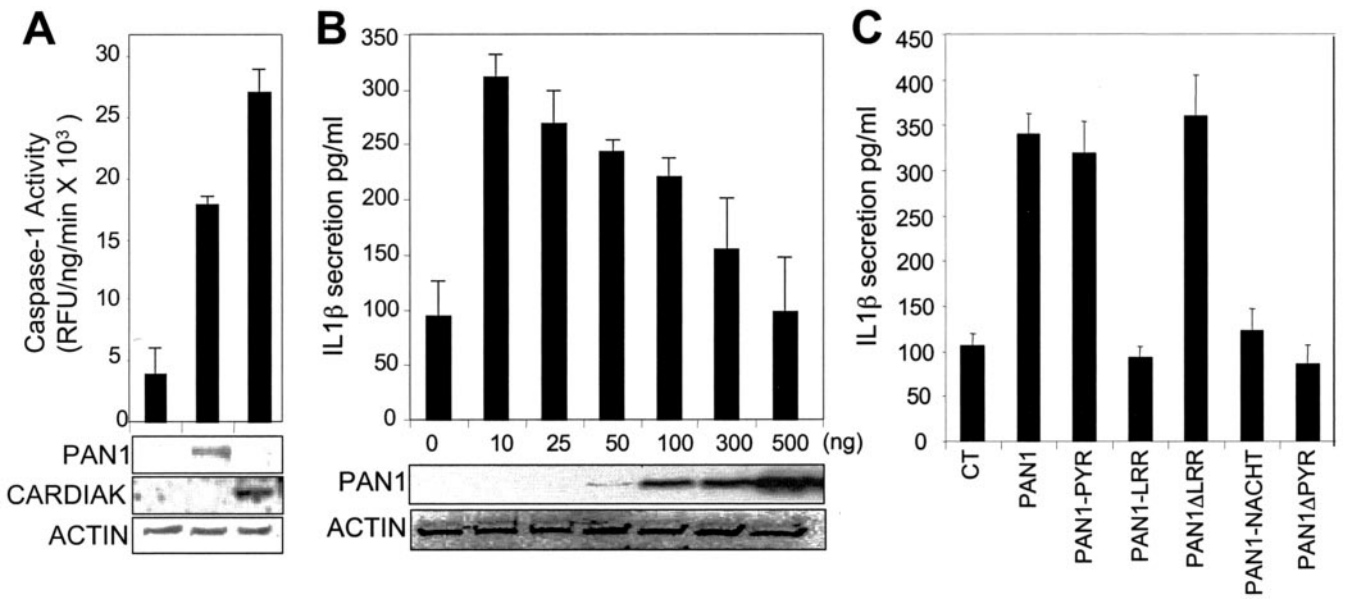

FIG. 7. **Regulation of pro-caspase1 activation by the PYRIN domain of PAN1.** *A*, HEK293T cells were transiently transfected with 1 μg of control DNA (*left lane*) or 1 μg of plasmids encoding Myc-PAN1 (*middle lane*) or FLAG-Cardiak (*right lane*), maintaining total DNA at 1 μg by the addition of empty plasmid. After 24 h HEK293 lysates were prepared (10 μg) and mixed with 50 μg of cell lysate from THP-1 cells (providing a source of pro-caspase-1 and other proteins). The mixed lysates were assayed for caspase-1 protease activity using fluorigenic substrate Ac-YVAD-aminofluoromethylcoumarin. Data represent relative fluorescence units/ng of protein (mean ± S.D.; $n = 3$) and are representative of several independent experiments. At the *bottom*, immunoblot analysis is shown using 20-μg aliquots of the HEK293T cell lysates and applying antibodies specific for PAN1 (*top*), Cardiak (*middle*), or β-actin (*bottom*). *B*, HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1β (200 ng), pro-caspase-1 (50 ng), or PAN1 (0, 10, 25, 50, 100, 200, 300, 500 ng), maintaining total DNA at 1 μg by the addition of empty plasmid. After one day, protein lysates were prepared from the transfected cells, normalized for total protein content (50 μg), and analyzed by immunoblotting using anti-PAN1 (*top*) and anti-β-actin (*bottom*) antibodies. Cell culture supernatants were analyzed by ELISA for secreted IL-1β at 36 h post-transfection (*C*). Plasmids encoding Myc-tagged PAN1 deletion mutants (50 ng) were transiently transfected into HEK293T cells in 24-well plates together with plasmids encoding pro-IL-1β (200 ng), pro-caspase-1 (50 ng), or empty vector (control *CT*). Supernatants were analyzed for IL-1β at 36 h post-transfection. Data shown represent pg/ml IL-1β, normalized for cell number (mean ± S.D.; $n = 3$), and are representative of several experiments.

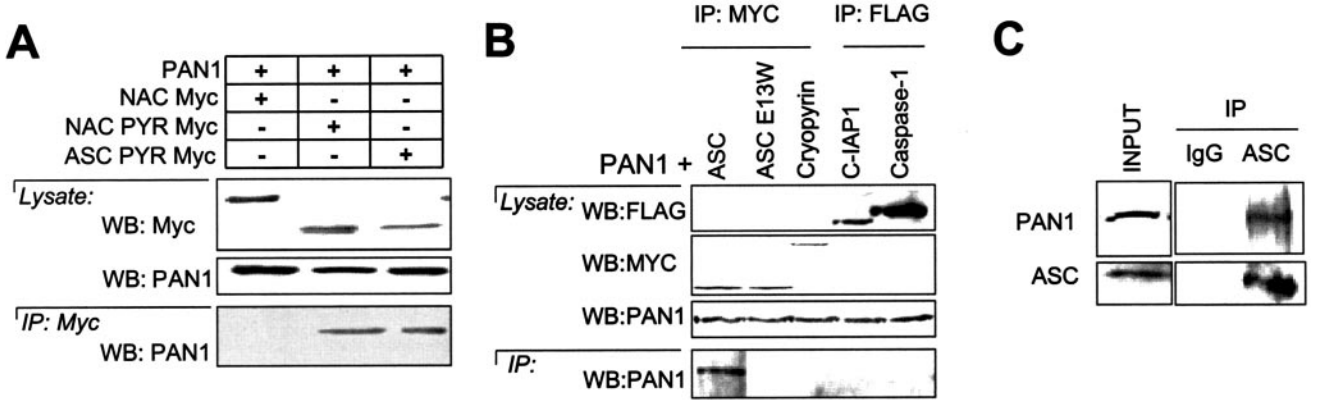

FIG. 8. **PAN1 interacts with PYRIN domain of ASC.** *A*, HEK293T cells were transfected with 1 μg of plasmid DNA encoding PAN1 together with 1 μg of plasmids encoding Myc-tagged NAC, Myc-tagged PYRIN domain of NAC, or Myc-tagged PYRIN domain of ASC. After 36 h cell lysates were prepared and subjected to immunoprecipitation (*IP*) with anti-Myc antibody. The resulting immune-complexes were then analyzed by SDS-PAGE/immunoblotting (*WB*) using anti-PAN1 antibody (*bottom*). Alternatively lysates were run directly in gels and analyzed by immunoblotting using anti-PAN1 (*middle*) or anti-Myc (*top*) antibodies to confirm expression of proteins. *B*, HEK293T cells were transfected with plasmids encoding untagged PAN1 together with Myc-ASC, Myc-ASC(E13W), Myc-Cryopyrin, FLAG-tagged cIAP-1, or FLAG-pro-caspase-1 in various combinations as indicated. Cell lysates were subjected to immunoprecipitation with anti-FLAG or anti-Myc antibodies and analyzed by SDS-PAGE/immunoblotting using anti-PAN1 antiserum (*bottom*). Alternatively, lysates (20 μg) were run directly in gels (*Lysate*) and probed with anti-FLAG (*top*), Myc (*middle*), or PAN1 (*bottom*) antibodies. *C*, co-immunoprecipitation assays were performed using lysates from THP-1 cells that had been treated with LPS (1 μg/ml) for 1 h and either IgG control or anti-ASC antibodies. The resulting immune-complexes (*IP*) were analyzed by immunoblotting using anti-PAN1 (*top*) or anti-ASC (*bottom*) antibodies as indicated. Lysates (50 μg) were also loaded directly in the gel as a control (*INPUT*).

of Cryopyrin and ASC, even when tested with a variety of concentrations of transfected plasmid DNA (Fig. 9 and data not shown).

To explore whether physical association of PAN1 and ASC is required for their collaborative effects on IL-1β secretion, we performed experiments with the ASC(E13W) mutant. When co-transfected with PAN1, ASC(E13W) failed to augment IL-1β production, consistent with a requirement for interaction of these proteins. Thus, PAN1 has at least some capacity to collaborate with ASC, and this cooperation correlates with the ability of these proteins to physically interact.

To further extend these observations to the endogenous PAN1 protein, we used the technique of siRNA (37, 38) to reduce PAN1 protein levels in differentiated THP1 cells, as-

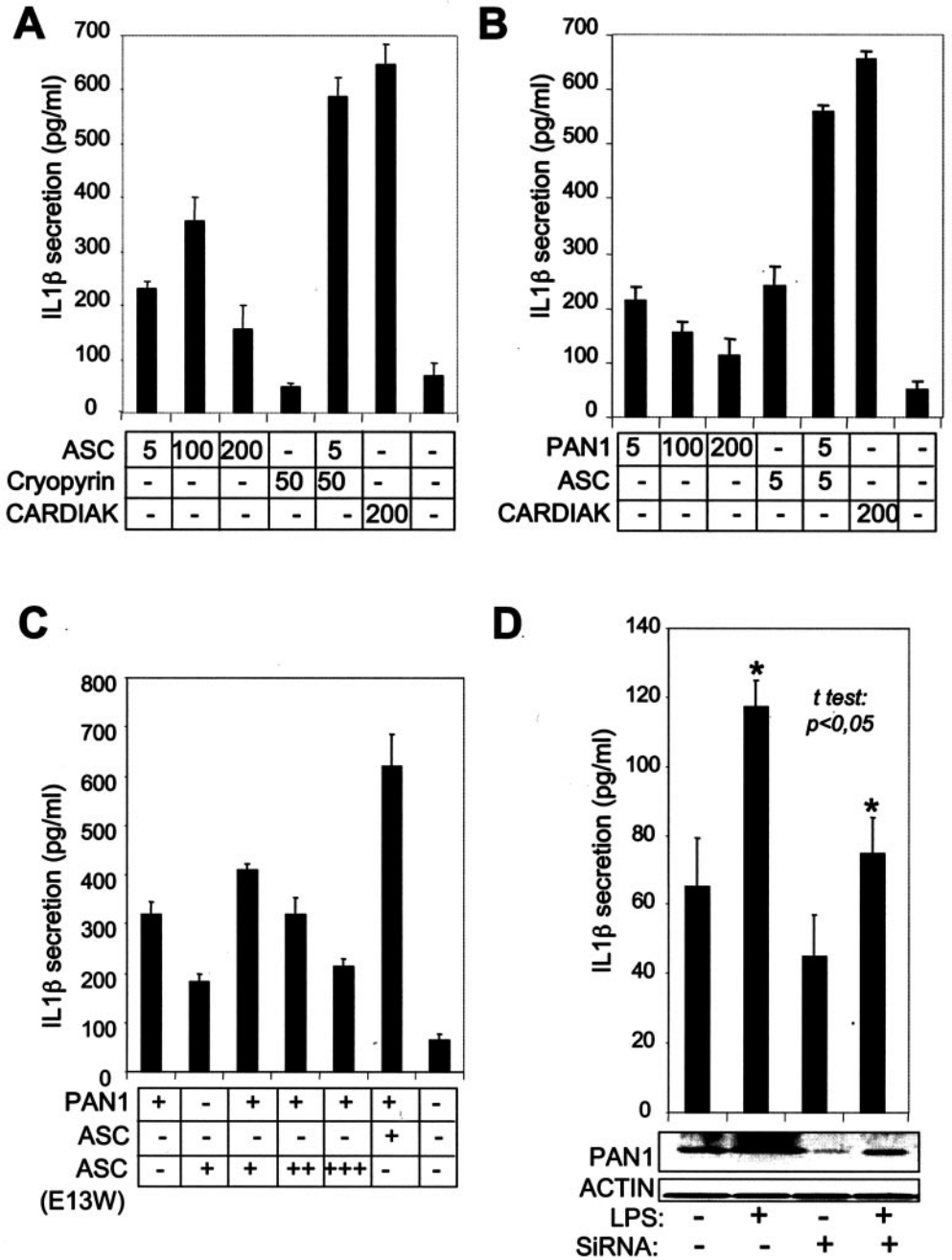

FIG. 9. **PAN1 collaborates with ASC to induce IL-1β secretion.** *A* and *B*, plasmids encoding PAN1, ASC, Cryopyrin, or Cardiak (amount indicated in ng) were transiently transfected into HEK293 cells in 24-well plates together with plasmids encoding pro-IL-1β (200 ng), pro-caspase-1 (50 ng), or empty vector (−). Supernatants were analyzed for IL-1β at 36 h post-transfection. *C*, plasmids encoding ASC, ASC(E13W), or PAN1 were transiently transfected into HEK293 cells in 24-well plates together with plasmids encoding pro-IL-1β (200 ng), pro-caspase-1 (40 ng), or empty vector (−). The amounts of ASC(E13W)-encoding plasmid DNA transfected are indicated: +, 5 ng; ++, 50 ng; +++, 100 ng. Supernatants were analyzed for IL-1β at 36 h post-transfection. *D*, 12-*O*-tetradecanoylphorbol-13-acetate-differentiated THP-1 macrophages were transfected with siRNA directed against PAN1 (+) or GFP (−), then cells were cultured the following day with (+) or without (−) 1 μg/ml LPS for 10 h as indicated before collecting culture supernatants for analysis of secreted IL-1β. In addition, lysates were prepared from transfected cells and analyzed by SDS-PAGE/immunoblotting using anti-PAN1 (*top*) and anti-β-actin (*bottom*) antibodies. Statistical significance of data was determined by unpaired *t* test, comparing LPS-treated PAN1 *versus* GFP siRNA-treated cells (*asterisk*). IL-1β production by unstimulated cells cultured without LPS was not significantly different. Data in *A–D* represent pg/ml of IL-1β, normalized for cell number (mean ± S.D.; $n = 3$).

sessing the impact on the production IL-1β induced by LPS. The siRNAs directed against PAN1 mRNA substantially reduced PAN1 protein levels in THP-1 cells, whereas a control siRNA molecule did not (not shown) (Fig. 9*D*). PAN1-targeting siRNAs also significantly reduced the amounts LPS-inducible IL-1β secretion in differentiated THP-1 macrophages (*t* test $p < 0.05$). We conclude, therefore, that endogenous PAN1 is capable of modulating IL-1β production.

DISCUSSION

PYRIN (PAAD, PYD, Dapin) domains are encoded in multiple genes within the human genome, including several implicated in hereditary hyper-inflammation syndromes, interferon responses, cancer suppression, and apoptosis induction (1–6). In this report, we have investigated the expression and function of the PAN family of PYRIN domain-containing proteins, PAN1. We show that expression of the PAN1 protein is induc-

ible by LPS and certain cytokines, suggesting a role for this protein in host-defense and inflammation. Previous studies have demonstrated that several PYRIN-family proteins are expressed in immune and inflammatory cells, including Pyrin, NALP1/NAC, PYPAF7, ASC, POP, and Cryopyrin (10–13, 20). Co-expression of PAN-1 in cell types previously shown to express other PYRIN-family proteins supports the concept that PAN1 exerts its functions or is regulated at least in part by interactions with other members of the PYRIN domain-containing family of proteins.

Interestingly, PAN1 is expressed in several human tumor cell lines. In this regard, the *ASC* gene is reportedly hypermethylated and silenced in human breast, ovarian, and colorectal cancers as well as human melanomas (39–42). These observations have prompted speculation that ASC operates as a tumor suppressor. It remains to be determined what role PAN1 plays in cancer. However, given evidence that chronic inflammation plays a role in carcinogenesis (for review, see Ref. 43), it is tempting to speculate a function for PAN1 in regulating aspects of tumor cell biology through effects on inflammatory cells.

Homotypic interactions among PYRIN domains create a potential network of protein interactions linking members of this family of proteins to specific signaling pathways. We show here that PAN1 associates with selected other PYRIN domain-containing proteins, suggesting specificity in its interactions with members of this large family of proteins. However, the observation that PAN1 associates with the isolated PYRIN domain of NAC (NALP1) but not full-length NAC raises the possibility that PYRIN domains are masked in ways that limit interactions with PAN1. Perhaps conformational changes in PAN1, NAC, and related proteins are required to expose their PYRIN domains, allowing interactions via PYRIN-PYRIN binding.

Similar to some other members of the PAN family, PAN1 was shown to be capable of regulating both caspase-1 and NF-κB activity, placing it at the crossroads of two critically important pathways for host-defense and inflammation. Although this manuscript was in preparation, it was reported that PAN1 (NALP2) can associate with pro-caspase-1 via two different CARD-carrying adapter proteins, ASC and Cardinal, resulting in activation of this protease (6). However, those studies were based entirely on overexpression and did not examine the role of the endogenous PAN1 protein. We show here for the first time that endogenous PAN1 and ASC associate in cells. By using siRNA to reduce PAN1 expression, we also found evidence for a role for endogenous PAN1 in regulating IL-1β secretion after LPS stimulation of a macrophage line. Why PAN1 participates in LPS responses leading to IL-1β secretion is unclear. The principal LPS receptors in mammalian cells are TLR-family members, particularly TLR4 (44). However, PAN1 possesses LRRs analogous to those found in TLRs and conceivably could bind LPS as a stimulatory ligand. In this regard the only bacterial ligand identified so far for intracellular LRR-containing proteins with similarity to PAN1 is muramyl dipeptide, which reportedly binds the LRRs of Nod2 and activates this host-defense protein (45).

The recently reported role for PAN1 in assembling a multiprotein complex ("inflammasome") that includes pro-caspase-1 and adapter proteins ASC and Cardinal could explain the phenomenon that transfection of excess PAN1-encoding plasmid causes progressively less, rather than more, IL-1β secretion. Presumably a precise stoichiometry of protein concentrations is required for optimal assembly of multiprotein complexes that trigger caspase-1 activation, resulting in IL-1β secretion. However, our finding that the PYRIN domain of PAN1 is necessary and sufficient for inducing IL-1β secretion suggests that alternative mechanisms exist by which PAN1 can stimulate IL-1β production besides inflammasome assembly. The need for precise stoichiometric ratios of proteins could also account for why PAN1-mediated effect on caspase-1 and NF-κB was not previously detected by some groups (21).

We show here for the first time that PAN1 can bind components of the IKK complex and regulate NF-κB activity. The properties of PAN1 in regulating NF-κB are essentially identical to PAN2 and some other members of the PYRIN domain family of proteins such as ASC and POP (12–14). Like PAN2, the PYRIN domain of PAN1 appears to be necessary and sufficient for suppressing NF-κB activity. Also, like PAN2, the PAN1 protein suppresses NF-κB activation induced by multiple stimuli whose signals converge on the IKK complex. PAN1, however, did not suppresses NF-κB activity induced by overexpression of p65 c-Rel, thus mapping its action upstream of this transcription factor, consistent with an effect at the point of the IKK complex. Consistent with this hypothesis, overexpression of PAN1 suppressed TNFα-induced degradation of IκBα, an event known to follow as a direct consequence of IKK activation.

We presume the basis for PAN1-mediated suppression of NF-κB activation induced by multiple pathways is due to its sequestration of IKK complexes, thus leaving less of this kinase complex available for participation in pathways activated by TNFα, CD40, Bcl-10, TLRs, and other activators of the IKK complex. Although showing a phenotype as a suppressor of NF-κB when overexpressed, it is possible that PAN1 could be an inducer of NF-κB in other contexts. Perhaps if stimulatory ligands of the LRRs of PAN1 are present, then PAN1 converts from a suppressor to an activator of the IKK complex. Indeed, inducible oligomerization of NACHT domain-containing proteins stimulated by microbial or endogenous LRR ligands is thought to result in a multiprotein scaffold upon which recruited IKKs can become activated by an induced-proximity mechanism, as demonstrated for Nod1 and Nod2 (45). However, siRNA-mediated reductions in PAN1 expression correlated with increased LPS-inducible expression of a NF-κB target gene, ICAM-1, in THP-1 macrophages, providing indirect evidence that the endogenous PAN1 protein is a suppressor of NF-κB activation pathways in at least some contexts. Also, a similar protein, Nod2, has recently been shown by gene ablation studies to inhibit NF-κB activation driven by TLR2. In this regard it may be significant that expression of PAN1 is induced by LPS and interferons, peaking after a few hours. This observation raises the possibility that PAN1 participates in a negative feedback loop designed to blunt NF-κB activation pathways and, thus, limit the duration of IKK activity. Further analysis of the roles of PAN1 in regulating NF-κB in various physiological and pathological contexts is required to elucidate the full spectrum of functions of this protein.

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