



# The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor- $\kappa$ B and pro-caspase-1 regulation

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Proteins containing PAAD {pyrin, AIM (absent-in-melanoma), ASC [apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain)] and DD (death domain)-like} (PYRIN, DAPIN) domains are involved in innate immunity, regulating pathways leading to nuclear-factor- $\kappa$ B (NF- $\kappa$ B) and pro-caspase-1 activation. Many PAAD-family proteins have structures reminiscent of Nod-1, a putative intracellular sensor of lipopolysaccharide. Hereditary mutations in some of the PAAD-family genes are associated with auto-inflammatory diseases. Several of these proteins utilize the bipartite PAAD- and CARD-containing adapter protein ASC/TMS-1 (target of methylation-induced silencing) for linking to downstream signalling pathways. In the present paper, we describe characterization of human PAAD-only protein-1 (POP1)/ASC2, which is highly homologous with the PAAD domain of ASC, and which probably originated

by gene duplication on chromosome 16. We demonstrate that POP1/ASC2 associates with ASC via PAAD–PAAD interactions and modulates NF- $\kappa$ B and pro-caspase-1 regulation by this adapter protein. In gene transfer experiments, POP1/ASC2 suppressed cytokine-mediated NF- $\kappa$ B activation similar to other PAAD-family proteins previously tested. Immunohistochemical studies showed expression of POP1/ASC2 predominantly in macrophages and granulocytes. We propose that POP1/ASC2 functions as a modulator of multidomain PAAD-containing proteins involved in NF- $\kappa$ B and pro-caspase-1 activation and innate immunity.

**Key words:** cryopyrin, nuclear factor (NF-) $\kappa$ B inhibitor kinase complex (IKK complex), innate immunity, interleukin-1 $\beta$ , pyrin.

## INTRODUCTION

The death domain (DD) fold represents a protein-interaction motif consisting of a bundle of five or six antiparallel  $\alpha$ -helices, which comprises four families of evolutionarily conserved domains: the DD, death-effector domain (DED), caspase-recruitment domain (CARD) and PAAD [from pyrin, AIM (absent-in-melanoma), ASC (apoptosis-associated speck-like protein containing a CARD) and DD-like] domain [1–7]. PAAD domains, also known as PYRIN or DAPIN, are found in diverse proteins implicated in apoptosis, inflammation and cancer, although their molecular mechanisms of action remain poorly defined. The founding member, pyrin, is mutated in families with familial Mediterranean fever, a hereditary hyper-inflammatory response syndrome [8]. In addition, versions of cryopyrin, another PAAD-family protein, have been associated with familial cold auto-inflammatory syndrome, Muckle–Wells syndrome and chronic infantile neurological cutaneous and articular ('CINCA') syndrome [9,10]. Both pyrin and cryopyrin utilize the adapter protein ASC, also termed TMS-1 (target of methylation-induced silencing) to activate nuclear factor  $\kappa$ B (NF- $\kappa$ B) and pro-caspase 1 [11,12].

Although capable of inducing NF- $\kappa$ B, many PAAD-family proteins also suppress NF- $\kappa$ B activation when induced by pro-inflammatory stimuli, such as tumour necrosis factor (TNF),

interleukin-1 $\beta$  (IL-1 $\beta$ ) and bacterial lipopolysaccharide (LPS), by unknown mechanisms. Interestingly, some PAADs associate with the NF- $\kappa$ B inhibitor kinase (IKK) complex, suggesting a dual activity of these proteins as either activators or inhibitors of IKK activation [11,13]. Some PAAD-containing proteins have also been implicated in the regulation of apoptosis [14–17].

NF- $\kappa$ B is a family of dimeric transcription factors containing the Rel-homology ('RH') domain, which have critical roles in regulating the expression of genes involved in inflammatory and immune responses (reviewed in [18]). NF- $\kappa$ Bs are sequestered and inactivated in the cytoplasm by proteins called inhibitory  $\kappa$ Bs (I $\kappa$ Bs). I $\kappa$ B molecules are degraded by the proteasome following cytokine-mediated phosphorylation by the IKK complex, thus freeing NF- $\kappa$ B-family transcription factors to enter the nucleus and transactivate promoters of various target genes. The IKK complex consists of two related kinases, IKK $\alpha$  and IKK $\beta$ , and a scaffold subunit IKK $\gamma$  or NF- $\kappa$ B essential modulator ('NEMO'; reviewed in [19]).

ASC was shown to bind the pro-domain of caspase 1 [IL-1 $\beta$ -converting enzyme (ICE)], thereby regulating its activity [20,21]. Although many caspases are involved in apoptosis, some members of this family, including caspase 1, are chiefly responsible for proteolytic processing and activation of pro-inflammatory cytokines, such as IL-1 $\beta$ , which is implicated

Abbreviations used: CARD, caspase-recruitment domain; ASC, apoptosis-associated speck-like protein containing a CARD; DD, death domain; DED, death-effector domain; DEFCAP, death effector filament-forming Ced-4-like apoptosis protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL<sup>®</sup>, enhanced chemiluminescence; EST, expressed sequence tag; FCS, fetal calf serum; FLIP, Fas-associated DD-like IL-1 $\beta$ -converting enzyme ('FLICE')-inhibitory protein; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; ICE, IL-1 $\beta$ -converting enzyme; I $\kappa$ B, inhibitory  $\kappa$ B; IKK, I $\kappa$ B kinase; IL-1 $\beta$ , interleukin-1 $\beta$ ; LPS, lipopolysaccharide; NAC, nucleotide-binding domain and CARD-containing protein; NACHT, NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospira anserina*) and TP1 (telomerase-associated protein); NALP, NACHT, LRR (leucine-rich repeat) and PYRIN protein; Neo, neomycin; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NP40, Nonidet P40; PAAD, pyrin, AIM (absent-in-melanoma), ASC and DD-like; PAN2, PAAD- and NACHT-containing protein; POP1, PAAD-only protein-1; PYPAF, PYRIN-containing Apaf1-like protein; RFP, red fluorescent protein; RT, reverse transcriptase; TBK1, TRAF-associated NF- $\kappa$ B activator (TANK)-binding kinase 1; TNF, tumour necrosis factor; TRAF, TNF-receptor-associated factor.

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in various inflammatory diseases (reviewed in [22]). The bioactive 17.5 kDa mature IL-1 $\beta$  is secreted following proteolytic processing of the inactive 31 kDa precursor by active caspase 1.

In the present paper, we report characterization of the first PAAD-only protein, POP1/ASC2, and demonstrate that POP1/ASC2 associates with the IKK complex, preventing its activation by cytokines and LPS. Moreover, POP1/ASC2 associates with ASC and modulates its ability to collaborate with pyrin and cryopyrin in NF- $\kappa$ B and pro-caspase-1 activation. Expression of endogenous POP1/ASC2 in macrophages and neutrophils suggest a role for this PAAD-only protein in the regulation of inflammatory responses.

## EXPERIMENTAL

### Bioinformatics

Using the sequence of the approx. 100-amino-acid N-terminal region of pyrin, a cascade of PSI-BLAST searches was performed using new hits as queries for subsequent searches until no new hits were found. This procedure, called saturated BLAST [23], revealed several genomic loci and expressed sequence tag (EST) clones potentially capable of encoding PAAD-domain proteins in the publicly available nucleotide databases [High Throughput Genomic Sequences (HTGS), Genome Survey Sequences, EST and draft human genome]. For genomic data, the amino-acid sequences of the predicted PAAD-proteins were tentatively deduced using the GENSCAN program for intron-exon prediction [24].

### Plasmids

The complete open reading frame of POP1 was amplified by high-fidelity PCR (Stratagene) from an EST (accession number W73558) and subcloned into pcDNA3 plasmid (Invitrogen) containing a N-terminal Myc-epitope tag. A glutathione S-transferase (GST)-ASC-PAAD fusion protein was generated by subcloning a modified cDNA encoding residues 1–100 of ASC into pGEX-4T1 (Amersham Biosciences). Green fluorescent protein (GFP) and red fluorescent protein (RFP) fusion-protein vectors were generated by subcloning cDNAs encoding ASC into pEGFP and POP1 into pDS-Red2 plasmids (Clontech). Expression plasmids encoding ASC, cryopyrin {PYRIN-containing Apaf1-like protein 1 (PYPAF1), NACHT [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospira anserina*) and TP1 (telomerase-associated protein)], leucine-rich repeat (LRR) and PYRIN protein 3 (NALP3)}, IKK $\alpha$ , IKK $\beta$ (K44M), IKK $\beta$ , IKK $\beta$ (K44A) IKKi, nucleotide-binding domain and CARD-containing protein (NAC) {death effector filament-forming Ced-4-like apoptosis protein (DEFCAP), CARD7, NALP1}, pro-caspase 1, pro-IL-1 $\beta$ , pyrin, TNF-receptor-associated protein (TRAF)-associated NF- $\kappa$ B activator ('TANK')-binding kinase 1 (TBK1), p65, TNF-receptor-1-associated DD protein (TRADD), TRAF2 and TRAF6, have been described in [11,14,25]. MyD88 (myeloid differentiation factor 88), and IL-1 receptor and IL-1 accessory protein were gifts from Dr Bruce Beutler (Department of Immunology, The Scripps Research Institute, La Jolla, CA, U.S.A.) and Dr Marta Muzio (DRO-Oncology, Pharmacology Department, Pharmacia Corp., Nerviano, Italy) respectively. The pRSV-NF- $\kappa$ B1 reagent was obtained through the AIDS Research and Reference Reagent Database, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health

(NIH) from Dr Gary Nabel and Dr Neil Perkins [26,27]. The authenticity of all constructs was confirmed by DNA sequencing.

### Cell culture and transfections

COS-7, HEK-293N and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). Stable clones of HEK-293N cells expressing POP1 were selected at 48 h post-transfection in 800  $\mu$ g/ml G418 (Calbiochem). Where indicated, cells were treated with 600 ng/ml LPS, 20 ng/ml TNF or 20 ng/ml IL-1 $\beta$  for various times. HEK-293 cells were transfected using Superfect (Qiagen), whereas COS-7 cells were transfected with LIPOFECTAMINE<sup>TM</sup> Plus (Life Technologies Inc.).

### Antibody generation

A polyclonal anti-POP1 antiserum (AR-94) was generated by repeated immunization of rabbits with recombinant POP1 protein. The antiserum was pre-adsorbed three times with GST-ASC-PAAD before use at 0.1% (v/v) for immunoblotting.

### Reverse transcriptase (RT)-PCR

RNA was isolated from THP-1 cells treated with 600 ng/ml LPS for various times. Reverse-transcribed (Superscript II; Gibco BRL) RNA from LPS-treated THP-1 cells or panels of first-strand cDNAs from various tissues (Clontech) were amplified by PCR using Amplitaq (Clontech) with POP1-specific primers [5'-AGGCGCAGGGCTGAGCCATGGGA-3' (forward); 5'-TGCTGTTGTCTTACGCGACTG-3' (reverse)] for 45 cycles, with addition of new polymerase after 30 cycles, producing a 384 bp fragment. HEK-293N-Neo (neomycin) or -POP1 stably transfected cells were treated with 20 ng/ml TNF for 4 h. Total RNA was isolated (TRIzol<sup>®</sup> reagent, Gibco BRL), and 1  $\mu$ g was treated with DNase I before reverse transcription to produce cDNA. The resulting DNA was amplified for 30 cycles using Amplitaq with either TRAF1- or glyceraldehyde-3-phosphate-dehydrogenase-specific primers [11]. PCR products were excised from agarose gels and were sequence-verified.

### In vitro protein-interaction assays and co-immunoprecipitations

GST-ASC-PAAD was expressed in XL-1 blue cells (Stratagene) and was affinity-purified using GSH-Sepharose (Amersham Biosciences). GST-ASC-PAAD or various GST control proteins (0.1  $\mu$ g) immobilized on 10  $\mu$ l of GSH-Sepharose were incubated with 1 mg/ml BSA in buffer A [10 mM Hepes (pH 7.4), 142.4 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 1 mM EDTA and 0.2% (v/v) Nonidet P40 (NP40), 1 mM dithiothreitol (DTT), 12.5 mM  $\beta$ -glycerophosphate, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF and 1  $\times$  protease inhibitor mix (Roche)] for 30 min at room temperature (20  $^{\circ}$ C). Beads were washed twice in buffer A and were incubated overnight at 4  $^{\circ}$ C with 1  $\mu$ l of *in vitro* translated (Promega) and <sup>35</sup>S-labelled proteins in buffer A containing 0.5 mg/ml BSA. Bound proteins were washed four times in 1 ml of buffer A, separated by SDS/PAGE (15% gel), and were detected by fluorography.

For immunoprecipitations, cells were lysed in isotonic lysis buffer [20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.2% (v/v) NP40, 12.5 mM  $\beta$ -glycerophosphate, 2 mM NaF, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF and 1  $\times$  protease inhibitor mix]. Clarified lysates

were subjected to immunoprecipitation using agarose-conjugated anti-haemagglutinin (HA), anti-Myc (Santa Cruz Biotechnology), anti-IKK $\alpha$  (Pharmingen) antibodies or purified IgG. After incubation at 4 °C for 4–12 h, immune complexes were washed three times in lysis buffer, separated by SDS/PAGE (8–16% gradient gel), and were analysed by immunoblotting using various antibodies in conjunction with an enhanced chemiluminescence (ECL<sup>®</sup>) detection system (Amersham Biosciences). Where indicated, cell lysates (10% volume) were included alongside immune complexes. Alternatively, lysates were analysed directly by immunoblotting after normalization for total protein content.

### Cell imaging

COS-7 cells were transfected with plasmids encoding GFP or RFP fusion proteins, transferred on to four-well polylysine-coated chamber slides (LabTec) the following day, fixed in 4% (w/v) paraformaldehyde a day later, and analysed by confocal laser-scanning microscopy (Bio-Rad).

### Immunohistochemical analysis

Normal human tissues for immunohistochemical analysis were obtained from biopsy and autopsy specimens. Mouse tissues were obtained from embryos and adult mice and rats, after killing by CO<sub>2</sub> inhalation [28]. All procedures were approved by the Institute Review Board (IRB). Tissues were fixed in Bouin's solution (Sigma), and were embedded in paraffin. Tissue sections were immunostained using a diaminobenzidine-based detection method employing the Envision-Plus-horseradish peroxidase system (Dako), as described in [28], using pre-adsorbed anti-POP1 antiserum. As controls, pre-immune serum or anti-POP1-specific antiserum that had been incubated with recombinant POP1 protein was used. Nuclei were counterstained with haematoxylin.

### In vivo phosphorylation assay

HEK-293N cells were transfected with HA-POP1 or HA-ASC-PAAD in 6 cm diameter dishes. At 30 h post-transfection, cells were washed twice in phosphate-free DMEM, then cultured in phosphate-free DMEM containing 5% (v/v) dialysed FCS for 1 h, followed by incubation in phosphate-free DMEM/5% (v/v) dialysed FCS containing 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub> (Amersham Biosciences) for 3 h. Where indicated, cells were treated with TNF (20 ng/ml) for the last 20 min of incubation in [<sup>32</sup>P]P<sub>i</sub>-containing medium. Cell were washed and then cleared lysates were immunoprecipitated using lysis buffer [20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.2% (v/v) NP40, 12.5 mM  $\beta$ -glycerophosphate, 2 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF and 1  $\times$  protease inhibitor mix], with agarose-conjugated anti-HA antibodies for 4 h. Immune complexes were washed four times in lysis buffer, separated by SDS/PAGE (10% gel), blotted on to membranes and exposed to X-ray film. Membranes were analysed by immunoblotting using anti-HA antibodies to control for protein expression.

### In vitro kinase assays

IKK $\alpha$  or IKK $\beta$  were immunoprecipitated from cell lysates as described in [11], using 5  $\times$  10<sup>5</sup> cells for IKK transfectants and 10<sup>6</sup> cells for endogenous IKKs. Immune complexes were washed twice in lysis buffer, once in lysis buffer containing 2 M

urea, followed by two washes in kinase buffer [20 mM Hepes (pH 7.6), 50 mM NaCl, 20 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 0.5 mM DTT]. Immunoprecipitates were then equilibrated for 5 min in kinase buffer, adjusted to 10 mM MgCl<sub>2</sub> and 1 mM DTT, and were finally incubated in 20  $\mu$ l of kinase buffer supplemented with 35  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 1  $\mu$ g of GST-Ik $\beta$  (Santa Cruz Biotechnology) at 30 °C for 30 min.

### Luciferase reporter gene assays

Typically, 10<sup>5</sup> HEK-293N or HEK-293T cells cultured in 5% (v/v) serum in 24-well plates were transfected using SuperFect transfection reagent (Qiagen) with a total of 1  $\mu$ g of plasmid DNA (normalized for total DNA), including 150 ng of pNF- $\kappa$ B-LUC or p53-LUC (Clontech) and 6 ng of a *Renilla luciferase* gene driven by a constitutive thymidine kinase promoter (pRL-TK; Promega). At 36 h post-transfection, cells were treated where indicated with 20 ng of TNF or IL-1 $\beta$  for 8 h before analysing lysates using the Dual Luciferase kit (Promega).

### IL-1 $\beta$ secretion assays

IL-1 $\beta$  secreted into culture supernatants of 24-well plates was measured by ELISA using a commercial kit (R&D Systems), normalizing data for cell numbers, and performing assays in triplicate [25].

## RESULTS

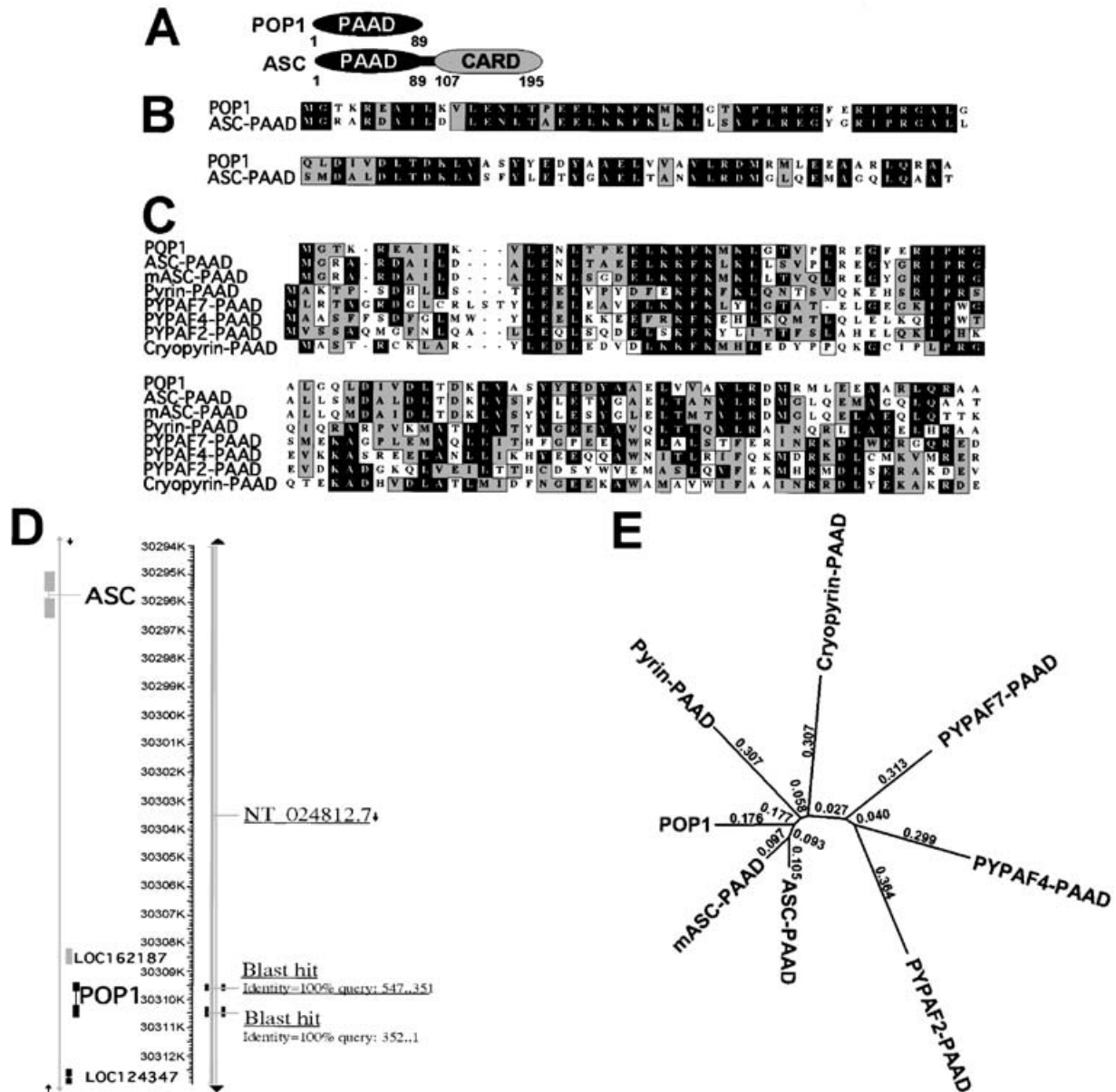
### Discovery and cloning of a PAAD-only protein, POP1/ASC2

Using bioinformatic approaches, we mined the human genome for additional PAAD-encoding genes. These efforts resulted in the discovery of a candidate gene in the human genome, which was also present in the EST database, predicted to encode a short protein consisting essentially only of a PAAD domain, which we designated initially ASC2, due to the high similarity to the PAAD of ASC [5]. Recently, the ASC2 protein was also referred to as POP1 and ASC1 [28a], although no characterization of the function of this protein has been previously reported. We propose using the name POP1 for PAAD-only protein-1 henceforth, since this term best describes this protein (Figure 1A).

The POP1 open reading frame is 270 bp in length, encoding a protein of 89 amino-acid residues, which is 64% identical (88% similar) in amino-acid sequence with the PAAD of ASC (Figure 1B). Lower homology is found with other PAAD-family members (Figure 1C). The *POP1* gene is located on chromosome 16p12.1, on the same chromosomal band as the *ASC* gene, approx. 14 kb away (Figure 1D). The structure of the *POP1* gene consists of two exons interrupted by an intron of 580 bp, where the entire PAAD is encoded in a single exon, which is spliced to a downstream non-coding exon corresponding to the 3' untranslated region of the *POP1* mRNA. This structure is highly similar to the 5' portion of the *ASC* gene. These findings, together with phylogenetic analysis (Figure 1E), suggest that the genes encoding POP1 and ASC arose by gene duplication.

### POP1 expression is found predominantly in monocytes, macrophages and granulocytes

Using RT-PCR, we surveyed adult and fetal human tissues for the presence of POP1 transcripts (Figure 2A). The expected 384 bp amplification product was observed in multiple tissues, including adult heart, liver, muscle, pancreas, fetal brain, fetal lung, fetal heart, fetal muscle and placenta. The requirement for



**Figure 1** *POP1* gene encodes a PAAD-only protein

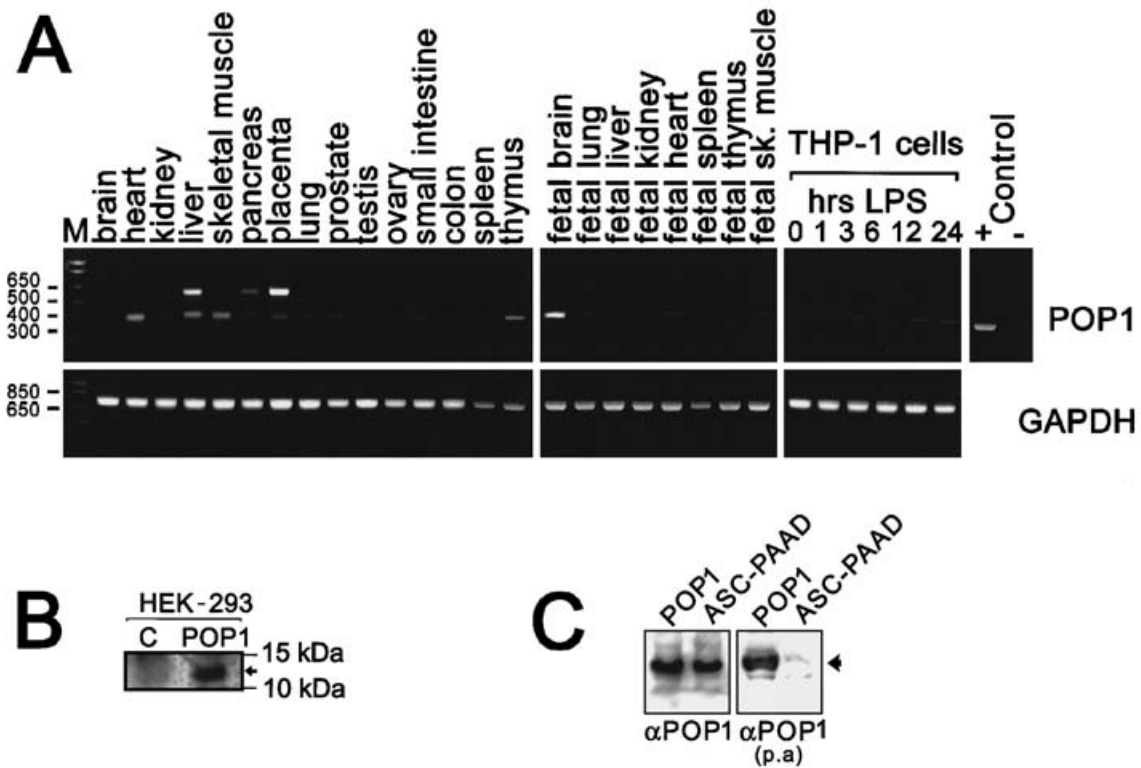
(A) Representation of the domain structure of POP1, compared with that of ASC, with amino-acid residues demarcating boundaries of the PAADs and CARD indicated. (B) Amino-acid-sequence alignment (ClustalW, standard PAM 250 parameters) is presented for the PAADs of POP1 and ASC-PAAD. Black and grey boxes indicate identical and similar (conserved) amino-acid residues respectively. (C) Amino-acid-sequence alignment is presented for the most similar PAADs as in (B). (D) Genomic location of *POP1* gene on contig NT\_024812.7 mapping to chromosome 16p12.1 by genomic BLAST search at NCBI, presented at map view. Note the close proximity of *ASC* and *POP1*, which are encoded on opposite strands. On this scale, the nearby *PYRIN* gene on 16p13.3 is not shown. The two exons of the *POP1* gene are indicated by rectangles. (E) Relations among the various PAAD-family members were calculated using the standard parameters of Ctree. Numbers represent changes per residue (distance).

45 PCR cycles, however, suggests very low expression of *POP1* in most tissues, probably resulting mainly from infiltrating blood cells (see below). We also detected *POP1* mRNA in the THP-1 monocyte cell line. In contrast to *ASC* (C. Stehlik and J. C. Reed, unpublished work), LPS-stimulation of these cells did not change levels of *POP1* mRNA (Figure 2A).

An antiserum was raised in rabbits using the GST-POP1 protein as an immunogen. This antibody detected the expected 12 kDa POP1 protein expressed in transfected HEK-293 cells (Figure 2B), but also cross-reacted with *ASC* (results not shown). We therefore pre-adsorbed this antiserum with a recombinant GST fusion protein containing the PAAD domain of *ASC* [11] and

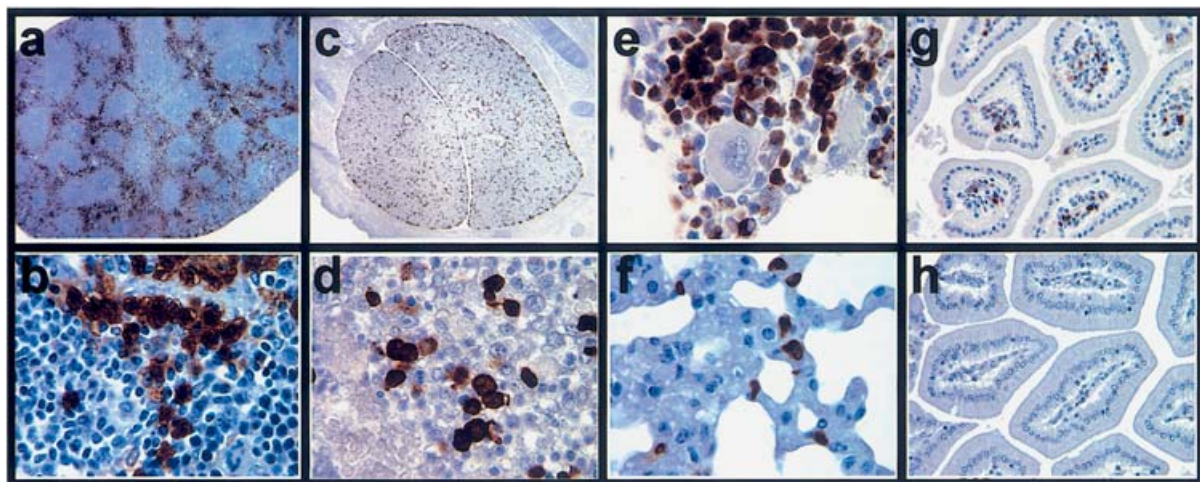
verified *POP1*-specific reactivity of the pre-adsorbed antiserum by immunoblot analysis of cells transfected with plasmids encoding either *POP1* or the PAAD domain of *ASC* (Figure 2C).

To analyse *POP1* expression in more detail, we performed immunohistochemical staining of a wide variety of human, mouse and rat tissues, using the pre-adsorbed anti-POP1 antiserum. Abundant *POP1* immunostaining was found in monocytes, macrophages, and granulocytes (Figure 3). *POP1* expression was also observed in dendritic cells, pericytes of blood vessels, and occasionally in myoepithelial cells and perineurial cells, but was not found in other types of cells. In the developing mouse embryo, *POP1* is present in haematopoietic cells of the fetal liver, but is



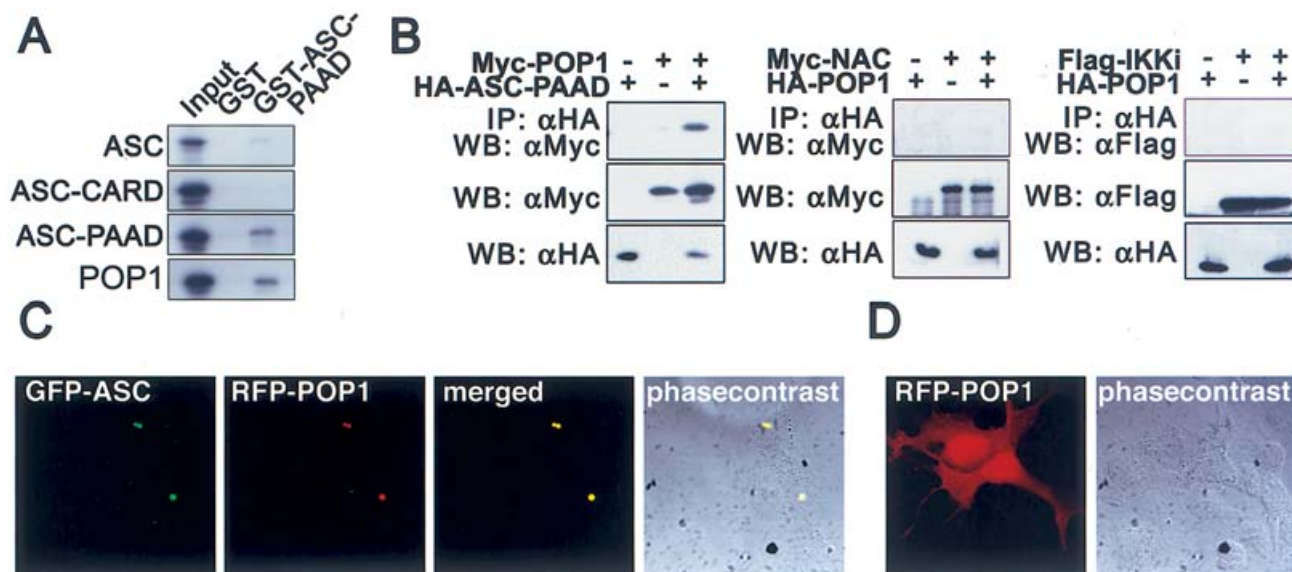
**Figure 2** Expression of *POP1* in human tissues

(A) RT-PCR analysis was performed on *POP1* (upper panels) and glyceraldehyde-3-phosphate dehydrogenase (lower panels) mRNA in various tissues or cells. Results are shown (from left to right) for adult tissues, fetal tissues and THP-1 monocytes. THP-1 cells were treated for the indicated times with 600 ng/ml LPS. Plasmid DNAs served as positive (pcDNA3-Myc-POP1) and negative (pcDNA3-Myc) controls (right panel). Note that in some tissues a larger transcript can be seen, probably representing unspliced RNA. M, molecular mass markers; sk., skeletal. (B) Immunoblot analysis of *POP1* protein was performed using lysates from HEK-293 cells transiently transfected with pcDNA or pcDNA-myc-POP1 plasmids. The blot was incubated with rabbit anti-POP1 polyclonal antibody. Arrow indicates *POP1*. (C) HEK-293 cells were transiently transfected with plasmids encoding *POP1* or ASC-PAAD. Cell lysates were normalized for total protein content and analysed by SDS/PAGE (15% gel) and immunoblotting using anti-POP1 ( $\alpha$ POP1) antiserum that was untreated (left panel) or pre-adsorbed (p.a.; right panel). Arrow indicates *POP1*.



**Figure 3** Immunohistochemical analysis of *POP1* expression

Representative results are presented showing immunohistochemical analysis of tissue sections stained with anti-POP1 antiserum that had been pre-adsorbed with GST-ASC-PAAD. Tissues represent mouse spleen at low (a) and high (b) power magnification, showing *POP1* immunoreactivity in granulocytes; mouse embryonic liver at low (c) and high (d) power magnification, showing *POP1* immunostaining in haematopoietic cells; mouse bone marrow (e), showing *POP1* staining in granulocytes; mouse lung (f), showing capillaries with blood cells, including *POP1*-positive granulocytes; human colon stained with anti-POP1 antiserum, showing *POP1*-positive inflammatory cells in submucosal tissue of villi in cross-section (g) or antiserum pre-adsorbed with GST-POP1 (h) (specificity control), lacking positive staining.



**Figure 4** POP1 associates with ASC

(A) GST or GST-ASC-PAAD proteins (1  $\mu$ g) were immobilized on GSH-Sepharose, and incubated with 1  $\mu$ l of various  $^{35}$ S-labelled, *in vitro* translated proteins, including ASC, ASC-CARD, ASC-PAAD and POP1. Beads were washed extensively and bound proteins were analysed by SDS/PAGE (15% gel), followed by exposure to X-ray film.  $^{35}$ S-labelled proteins (10%) were loaded directly on to gels ('Input'). (B) Co-immunoprecipitation assays were performed using lysates from HEK-293T cells that had been transiently transfected with plasmids encoding Myc-POP1, HA-ASC-PAAD or both plasmids together. Lysates were immunoprecipitated (IP) with anti-HA ( $\alpha$ HA) antibody, and the resulting immune complexes were analysed by SDS/PAGE (15% gel) and immunoblotting (WB) using anti-Myc ( $\alpha$ Myc) antibody with ECL<sup>®</sup>-based detection (upper panel). Alternatively, cell lysates were analysed directly by Western blotting using anti-Myc (middle panel) or anti-HA antibodies (lower panel). Also presented are results from co-immunoprecipitation experiments using NAC and IKKi, which do not associate with POP1. (C), (D) Confocal laser microscopy analysis was performed using COS-7 cells that were transiently transfected with plasmids encoding RFP-POP1 fusion protein in combination with GFP-ASC (C) or with RFP-POP1 fusion protein alone (D). Cells were imaged by two-colour fluorescence or phase-contrast microscopy. Results are representative of several experiments.

absent from erythrocyte lineage cells and most other types of cells (Figure 3). No POP1-immunostaining was found in human tumours including prostate, breast, ovarian, colon, oesophageal, renal, lung and gastric cancers, as well as lymphomas (results not shown). POP1 expression in tumours was found only in infiltrating inflammatory cells. The specificity of the staining was confirmed by antibody competition experiments using recombinant POP1 protein (Figure 3). POP1 immunostaining was predominantly cytoplasmic (Figure 3).

#### POP1 binds the PAAD of ASC

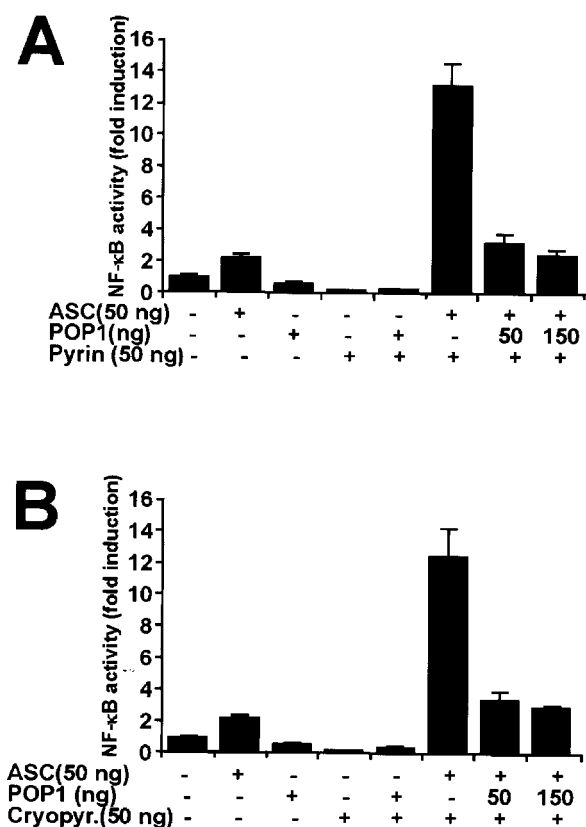
Since the PAAD of POP1 shares a high degree of sequence homology with the PAAD of ASC, we tested whether or not POP1 binds ASC. First, *in vitro* protein-interaction assays were performed in which the PAAD of ASC was produced as a GST fusion protein and purified from bacteria, and was used to test binding to  $^{35}$ S-labelled, *in vitro* translated POP1 protein. These experiments demonstrated that [ $^{35}$ S]POP1 binds to GST-ASC-PAAD, but not to GST control protein. The PAAD and CARD of ASC were included as positive and negative controls for GST-ASC-PAAD (Figure 4A).

Secondly, co-immunoprecipitation assays were performed to determine whether POP1 could bind ASC also in cells. For these experiments, POP1 was expressed in HEK-293T cells as a Myc-tagged protein, whereas the PAAD of ASC was expressed as a HA-tagged protein, by transient transfection. Immunoprecipitation experiments using anti-HA antibody demonstrated the presence of associated Myc-tagged POP1 (Figure 4B). POP1 did not bind to NAC (DEFKAP, NALP1, CARD7), IKKi (IKK $\epsilon$ ) or a variety of other proteins, confirming the specificity of these results.

Thirdly, fluorescence confocal microscopy was used to localize the ASC and POP1 proteins in COS-7 cells that had been transfected with plasmids encoding RFP-tagged POP1 and GFP-tagged ASC. When RFP-POP1 was co-expressed with GFP-ASC, RFP-POP1 was found in discrete cytosolic specks, co-localizing with GFP-ASC (Figure 4C). In contrast, when RFP-POP1 was expressed by itself, the protein was located throughout the cytosol and nucleus of cells (Figure 4D). Thus ASC appears to pull POP1 into the cytosolic specks that are characteristic for the ASC protein [16], providing further evidence that POP1 and ASC associate in cells.

#### POP1 inhibits NF- $\kappa$ B induction by PAAD-family proteins

The PAAD of ASC binds PAADs in pyrin and cryopyrin, allowing these proteins to collaborate in induction of NF- $\kappa$ B when co-expressed in HEK-293T cells by transient transfection [11,12,29]. We therefore tested the effects of POP1 on ASC-mediated NF- $\kappa$ B induction using similar experimental approaches. ASC, POP1, cryopyrin or pyrin did not induce substantial amounts of NF- $\kappa$ B activity when transfected alone (Figures 5A and 5B). Co-transfection of ASC with either pyrin (Figure 5A) or cryopyrin (Figure 5B) resulted in >10-fold increases in NF- $\kappa$ B activity. In contrast, when POP1 was included in these transfections, NF- $\kappa$ B activity was markedly inhibited (Figures 5A and 5B). Immunoblot analysis confirmed the production of all proteins and excluded an effect of POP1 on the expression of ASC, pyrin or cryopyrin (results not shown). The inhibitory effect of POP1 on NF- $\kappa$ B activity was specific, since POP1 did not suppress the activity of other transcription factors (see below). We therefore conclude that POP1 can suppress NF- $\kappa$ B induction stimulated by the combination of ASC and pyrin or cryopyrin.



**Figure 5** POP1 inhibits NF- $\kappa$ B induction by PAAD-family proteins

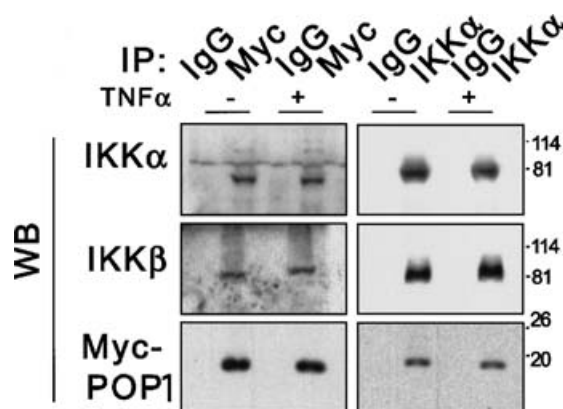
HEK-293T cells were transfected in 24-well plates with either 850 ng of control pcDNA3 plasmid or the indicated amounts and combinations of plasmids encoding ASC (50 ng), POP1 (50 or 150 ng), pyrin (50 ng) (A) or cryopyrin (cryopyr.; 50 ng) (B). Transfections also included 150 ng of pNF- $\kappa$ B and 6 ng of pRL-TK (thymidine kinase), maintaining the total DNA of transfections at 1  $\mu$ g by the addition of control plasmid DNA. At 36 h post-transfection, cells were analysed for NF- $\kappa$ B activity by reporter gene assay and results are represented as fold-induction relative to cells transfected with pcDNA3 control plasmid and are means  $\pm$  S.D. ( $n = 3$ ).

### POP1 associates with the IKK complex

We recently reported that two PAAD-family proteins, ASC and PAAD- and NACHT-containing protein 2 (PAN2, PYPAF4, NALP4), can be co-immunoprecipitated with components of the IKK complex [11,13]. We therefore performed experiments to explore whether or not POP1 also similarly associates with protein kinases associated with this complex. HEK-293N transfectants were generated that stably expressed POP1 and these cells were used to determine whether or not POP1 associates with endogenous IKK $\alpha$  and IKK $\beta$ , as measured by co-immunoprecipitation assays. These co-immunoprecipitation experiments demonstrated interaction of POP1 with both IKK $\alpha$  and IKK $\beta$  (Figure 6). Thus, like ASC and PAN2, the PAAD domain of POP1 can associate with kinases of the IKK complex. Since POP1 inhibits TNF-induced NF- $\kappa$ B activation, we tested whether or not TNF stimulation caused dissociation of POP1 from the IKK complex, but no change in binding was detected by co-immunoprecipitation assays (Figure 6).

### POP1 suppresses kinase activity of IKKs

The IKK complex becomes activated in cells following stimulation with TNF and other pro-inflammatory cytokines.



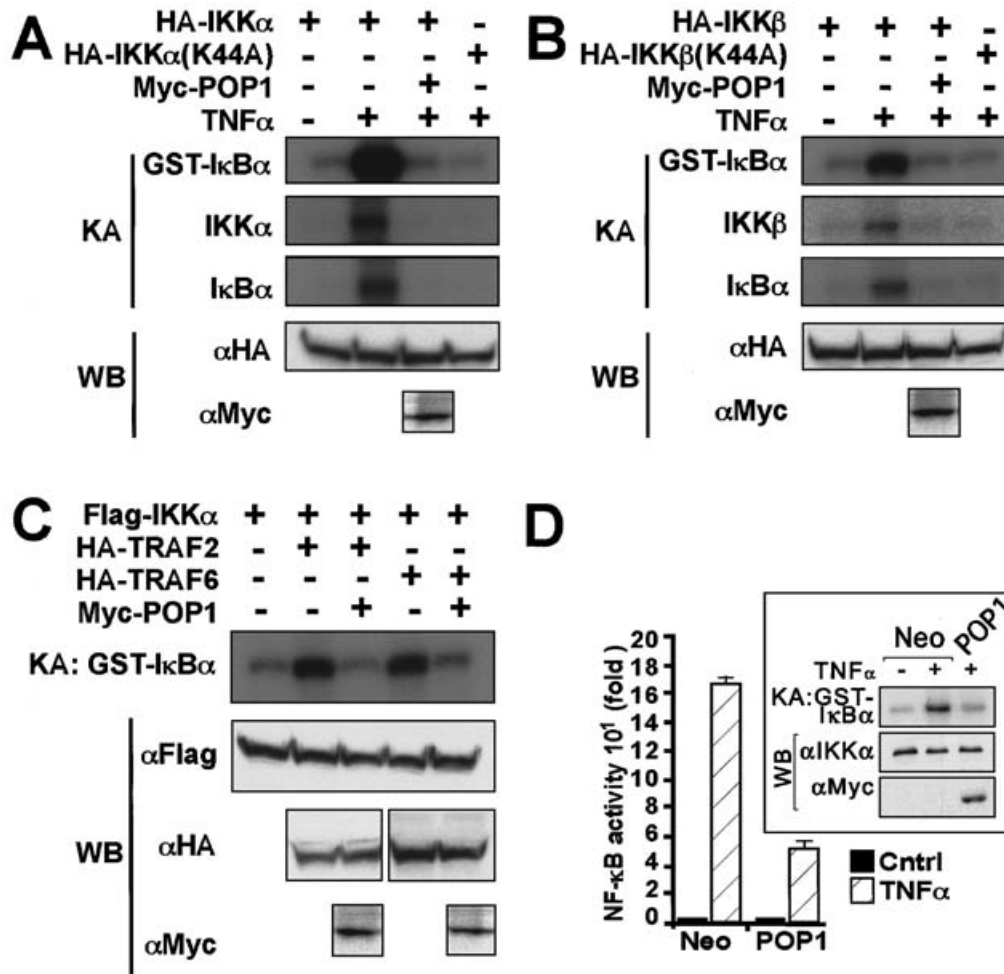
**Figure 6** POP1 associates with components of the IKK complex

Co-immunoprecipitation assays were performed using HEK-293N cells stably expressing POP1-encoding or Neo control plasmids. Cells were either treated with TNF for 15 min or left unstimulated. Cleared lysates were subject to co-immunoprecipitation using either IgG, anti-Myc, or anti-IKK $\alpha$  antibodies and the resulting immune complexes were analysed by Western blotting (WB) using various antibodies, as indicated. Molecular mass markers are indicated on the right.

We therefore determined the effect of POP1 on IKK activity in TNF-stimulated cells using *in vitro* kinase assays. For initial experiments, either HA-epitope-tagged IKK $\alpha$  (Figure 7A) or IKK $\beta$  (Figure 7B) was expressed in cells alone or in combination with plasmids encoding POP1. Then either HA-IKK $\alpha$  or HA-IKK $\beta$  was immunoprecipitated from transfected cells, and *in vitro* phosphorylation of GST-I $\kappa$ B substrate by the resulting immune complex was measured. *In vitro* phosphorylation of IKK $\alpha$  and IKK $\beta$ , as well as phosphorylation of associated endogenous I $\kappa$ B, was also measured in the kinase assays.

In unstimulated cells, low levels of IKK $\alpha$  and IKK $\beta$  activity were detected, which increased in response to TNF stimulation (Figures 7A and 7B). Co-expression of kinase-dead IKK $\alpha$  or IKK $\beta$  mutants blocked this response, serving as a control. TNF-induced activation of IKK $\alpha$  and IKK $\beta$  was suppressed to essentially baseline levels by co-expression of POP1 (Figures 7A and 7B). This inhibitory effect of POP1 on IKK $\alpha$  and IKK $\beta$  activity was not attributable to a difference in the total levels of the IKK $\alpha$  or IKK $\beta$  proteins, as determined by immunoblot analysis. Similar results were obtained when IKK $\alpha$  or IKK $\beta$  was activated in cells by transient transfection of plasmids encoding intracellular signalling proteins such as TRAF2 and TRAF6 (Figure 7C).

To extend these studies involving expression of epitope-tagged proteins by transient transfection, the activity of endogenous IKK $\alpha$  was evaluated in HEK-293N cells that had been stably transfected with either control or POP1-encoding plasmids (Figure 7D). POP1 potently suppressed TNF-induced activation of endogenous IKK $\alpha$  in these cells, as determined by *in vitro* kinase assays where immunoprecipitated IKK $\alpha$  was tested for its ability to phosphorylate GST-I $\kappa$ B substrate *in vitro*. These differences in IKK $\alpha$  activity were not due to differences in the total levels of IKK $\alpha$  protein, as determined by immunoblotting (Figure 7D). Moreover, the reduction of IKK $\alpha$  activity was associated with reduced NF- $\kappa$ B activity, as measured by a NF- $\kappa$ B luciferase reporter gene assay (Figure 7D). Consistent with POP1-mediated inhibition of the IKK complex, we also observed enhanced stability of endogenous I $\kappa$ B $\alpha$  and reduced DNA binding of p65 in response to TNF (results not shown).



**Figure 7** POP1 suppresses IKK activation

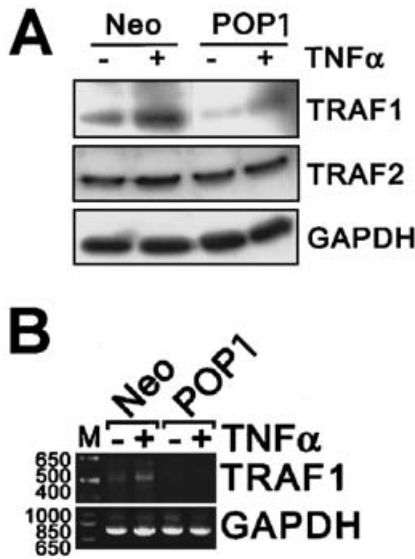
HEK-293N cells were transiently transfected with plasmids encoding either HA-IKK $\alpha$  (A) or HA-IKK $\beta$  (B), with or without POP1 as indicated. After 36 h, cells were either left unstimulated (-) or stimulated with 20 ng/ml TNF (+) for 15 min before lysing cells and immunoprecipitating IKKs using anti-HA ( $\alpha$ HA) antibody. Immune complexes containing IKK $\alpha$  or IKK $\beta$  were analysed for *in vitro* kinase activity, using GST-I $\kappa$ B $\alpha$  as a substrate, by SDS/PAGE (8–16% gradient gel), followed by autoradiography. Auto-phosphorylation of either IKK $\alpha$  or IKK $\beta$  and phosphorylation of associated endogenous I $\kappa$ B $\alpha$  in the immune complexes was also revealed by the kinase assays (KA). Western blot analysis (WB) of the same lysates was performed to verify equal amounts of HA-IKK proteins, and also to confirm expression of Myc-tagged POP1 ( $\alpha$ Myc). (C) HEK-293N cells were transiently transfected with plasmids encoding FLAG-IKK $\alpha$  and either HA-TRAF2, or HA-TRAF6, in the presence or absence of Myc-POP1, then IKK $\alpha$  was immunoprecipitated and *in vitro* kinase assays were performed 30 h later, using GST-I $\kappa$ B $\alpha$  as a substrate. Expression was controlled by immunoblot analysis of the lysates.  $\alpha$ Flag, anti-FLAG antibody. (D) Stably transfected HEK-293N-POP1 or -Neo cells were transiently transfected with 150 ng of pNF- $\kappa$ B-luc and 6 ng of pRL-TK (thymidine kinase) in 24-well plates, maintaining the total DNA of transfections at 1  $\mu$ g by the addition of control plasmid DNA. At 36 h post-transfection, cells were stimulated for 8 h with 20 ng/ml TNF, and then cell lysates were analysed for relative amounts of NF- $\kappa$ B-driven luciferase activity. Results are means  $\pm$  S.D. ( $n=3$ ) expressed as fold-induction relative to cells not stimulated with TNF. Inset, endogenous IKK $\alpha$  kinase activity (KA) was measured by *in vitro* kinase assay in stably transfected HEK-293N-POP1 or -Neo transfectants, using anti-IKK $\alpha$  ( $\alpha$ IKK $\alpha$ ) immunoprecipitates prepared from cell lysates before or after 15 min of TNF stimulation, employing GST-I $\kappa$ B $\alpha$  as an exogenous substrate (upper panel). Lysates were also analysed by Western blotting (WB) using anti-IKK $\alpha$  and anti-Myc ( $\alpha$ Myc) antibodies to confirm loading of equivalent amounts of IKK $\alpha$  (middle panel) and to verify expression of Myc-tagged POP1 protein (lower panel).

### POP1 suppresses expression of NF- $\kappa$ B-inducible genes

We evaluated whether stable expression of the ASC-PAAD protein in HEK-293N cells interfered with TNF-induced expression of an endogenous NF- $\kappa$ B target gene, TRAF1, which contains several NF- $\kappa$ B-binding sites in its promoter [30]. Whereas TNF stimulated marked increases in TRAF1 protein levels in HEK-293N-Neo control cells, this response was markedly blunted in POP1-expressing cells (Figure 8A). The specificity of these results was confirmed by re-probing the same blot with antibodies that recognize TRAF2 or tubulin, showing that the levels of these proteins did not change in response to TNF. POP1 also suppressed TRAF1 expression at the mRNA level (Figure 8B), consistent with a transcriptional mechanism.

### POP1 inhibits NF- $\kappa$ B activation induced by diverse stimuli

We explored in more detail the effects of POP1 on NF- $\kappa$ B regulation by examining the consequences of POP1 expression on NF- $\kappa$ B activation induced by a variety of stimuli, including TNF, IL-1 $\beta$ , Nod1 and Bcl-10. In transient transfection experiments, POP1 potently suppressed the induction of NF- $\kappa$ B activity by all these stimuli in HEK-293, COS-7, HeLa or other cells in a dose-dependent manner (Figures 9A–9F; and results not shown). Thus POP1 blocks NF- $\kappa$ B induction by multiple stimuli, consistent with an effect on the IKK complex, a point of convergence of multiple pathways leading to NF- $\kappa$ B activation [11,13]. In contrast, overexpression of POP1 did not interfere with activation of other transcription factors, including p53 (Figure 9G; and results not shown), thus confirming the specificity of these results.



**Figure 8** POP1 inhibits expression of NF- $\kappa$ B target genes

(A) HEK-293N cells were transiently transfected with either Neo control or Myc-POP1 plasmids, then, where indicated, cells were treated with TNF $\alpha$  for 4 h. Cell lysates were normalized for total protein content and were analysed by SDS/PAGE (10% gel) and immunoblotting using anti-TRAF1 (upper panel), anti-TRAF2 (middle panel) and anti-GADPH antibodies (lower panel). (B) Alternatively, total RNA was isolated from stably transfected HEK-293N-Neo or HEK-293N-POP1 cells and analysed by RT-PCR for TRAF1 (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (lower panel) as indicated.

**Functional mapping of the point of POP1-mediated suppression of NF- $\kappa$ B induction**

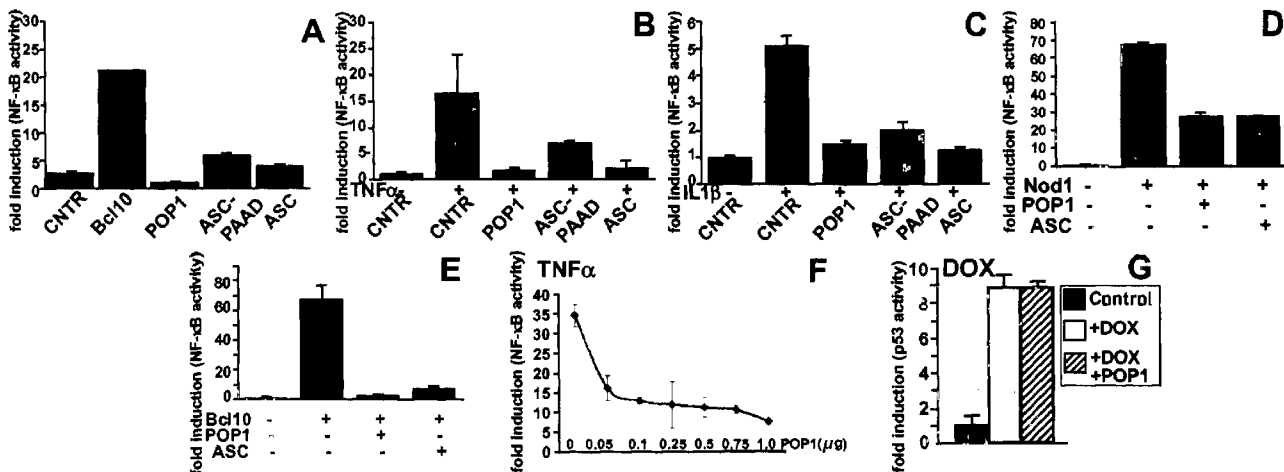
To map where POP1 affects pathways leading to NF- $\kappa$ B induction, we induced NF- $\kappa$ B activity in cells by transient transfection of plasmids encoding various intracellular-signal-transducing proteins that operate within cytokine receptor pathways leading

to IKK activation. Co-expression of POP1-encoding plasmid with these signal transducers revealed that POP1 blocks the induction of NF- $\kappa$ B activity by the adapter proteins TRAF2 and TRAF6, the TRAF-binding kinases TBK1 and Nik, by the IKK complex constituents IKK $\alpha$  and IKK $\beta$ , and by the related kinase IKKi (Figure 10). In contrast, co-expression of POP1 did not suppress reporter-gene activation induced by overexpression of the p65 or p50 subunits of NF- $\kappa$ B (Figure 10). These functional mapping studies thus suggest that POP1 blocks NF- $\kappa$ B induction at the level of the IKK complex, consistent with the results above demonstrating association of POP1 with the IKK complex and suppression of IKK kinase activity. Thus these reporter-gene results provide further evidence that POP1 can modulate NF- $\kappa$ B induction by directly affecting the activity of the IKK complex. Similar findings were recently reported for ASC and for the PAAD-family protein PAN2 [11,13].

**TNF-induces phosphorylation of POP1**

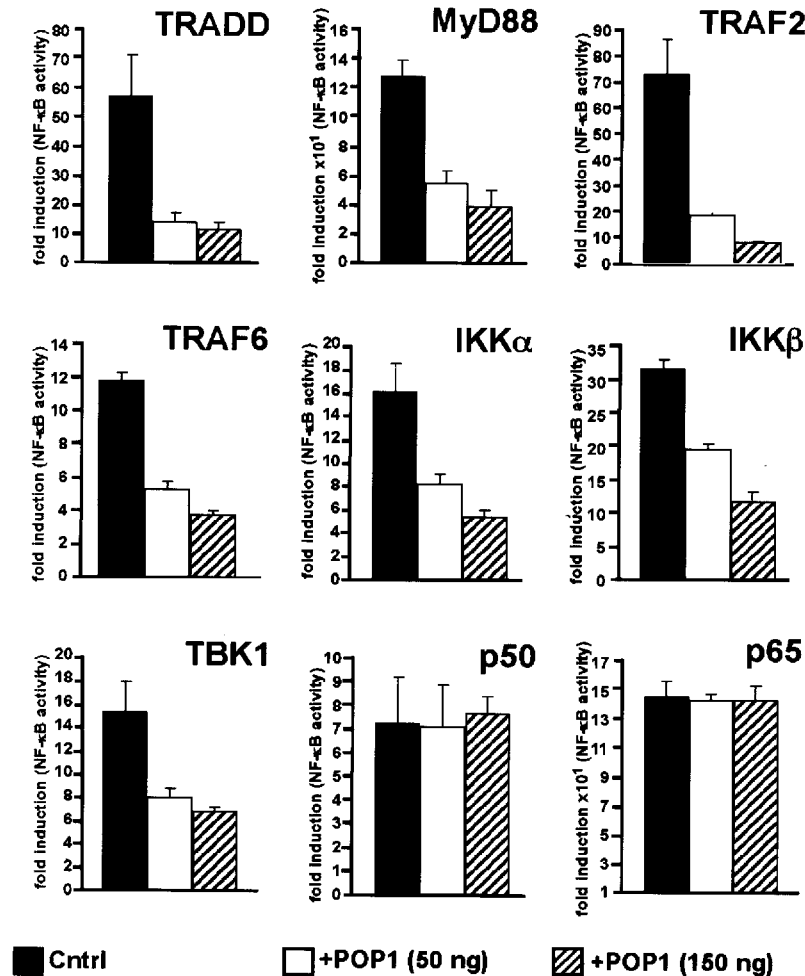
Although POP1 is expressed in inflammatory cells *in vivo* (Figure 3) and whereas it suppresses cytokine-mediated activation of IKK when overexpressed in cells *in vitro* (Figure 7), under normal circumstances, TNF is still able to activate NF- $\kappa$ B in inflammatory cells. We therefore speculated that TNF must have a mechanism for overcoming POP1-mediated suppression of IKK activation. We first considered that TNF might induce dissociation of POP1 from the IKKs but a co-immunoprecipitation experiment performed using lysates from unstimulated versus TNF-stimulated cells failed to substantiate this hypothesis (Figure 6). Similar results were obtained recently for the PAAD-family proteins, ASC and PAN2 [11,13].

We therefore explored the possibility that TNF induces post-translational modifications of POP1, focusing on phosphorylation. Either POP1 or the PAAD domain of ASC was expressed in HEK-293N cells, and the cells were metabolically labelled with [<sup>32</sup>P]P<sub>i</sub>, followed by culture for 20 min with or without TNF. POP1 and ASC-PAAD were immunoprecipitated and



**Figure 9** POP1 suppresses NF- $\kappa$ B induction by multiple stimuli

NF- $\kappa$ B activity as measured by reporter gene assays in transiently transfected HEK-293N cells. Cells were either cultured without further stimulation (A), (D) and (E), or cells were stimulated with 20 ng/ml TNF (B) and (F), or with 20 ng/ml IL-1 $\beta$  for 8 h (C). (C) HEK-293N cells were also co-transfected with IL-1RI and IL-1RacP, to render them IL-1-responsive. (D), (E) Cells were transfected with plasmids encoding Nod1 (D) or Bcl-10 (E) alone or in combination with POP1, and NF- $\kappa$ B activity was measured. (F) Increasing concentrations of POP1-encoding plasmid were transfected as indicated, demonstrating dose-dependent suppression of NF- $\kappa$ B activity. (G) To assess the specificity of reporter gene assays in HEK-293N cells, p53 transcriptional activity was determined after transfection with p53-responsive luciferase reporter plasmid pRL-p53 and stimulation the next day with the DNA-damaging drug doxorubicin (DOX; 40 ng/ml). NF- $\kappa$ B or p53 transcriptional activity was measured 2 days post-transfection by reporter gene assay. Results are presented as means  $\pm$  S.D. (n = 3) fold-induction relative to pcDNA3 control-transfected cells, or cells not treated with DOX and are representative of several experiments performed with HEK-293N, HeLa and HT1080 cells. CNTR, control.



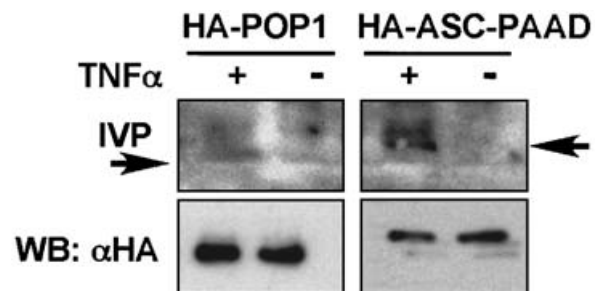
**Figure 10** POP1 inhibits NF- $\kappa$ B induction at the level of the IKK complex

NF- $\kappa$ B activity as measured by reporter gene assays in HEK-293N cells transiently transfected with 200 ng of plasmids encoding various NF- $\kappa$ B-inducing proteins, as indicated, and either 50 or 150 ng of POP1-encoding plasmid. NF- $\kappa$ B transcriptional activity was measured 2 days later by reporter gene assay, and results are presented as means  $\pm$  S.D. ( $n = 3$ ) fold-induction relative to control cells, which were not transfected with effector plasmids.

the resulting proteins were analysed by SDS/PAGE (15% gel) and autoradiography. As shown in Figure 11, TNF stimulation induced phosphorylation of POP1 and ASC-PAAD. Immunoblot analysis confirmed recovery of equivalent amounts of POP1 and ASC-PAAD from TNF-stimulated and -unstimulated cells. The functional significance of the phosphorylation remains to be determined.

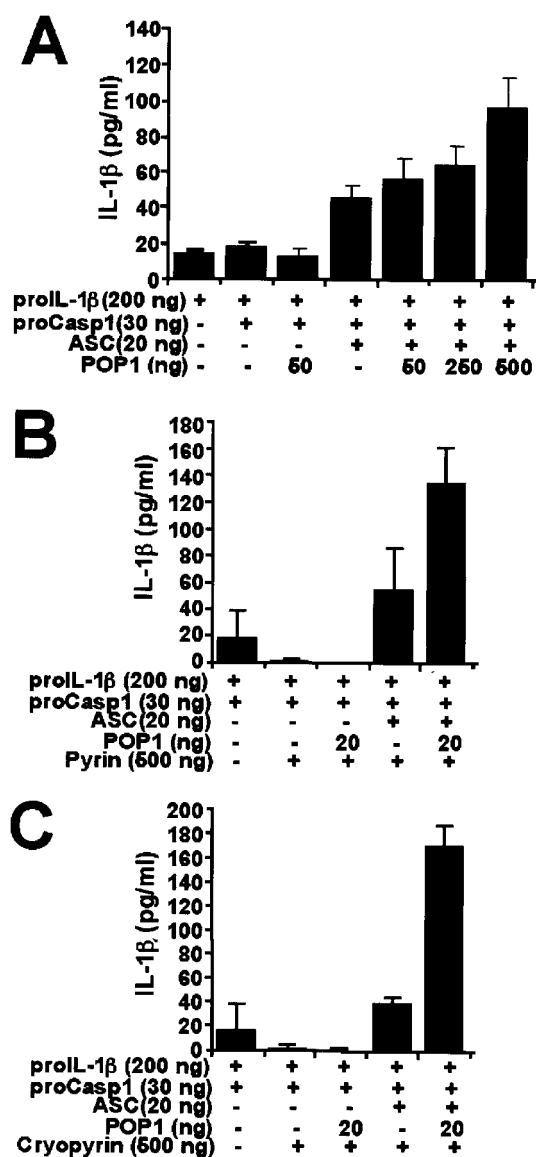
#### POP1 enhances ASC-mediated activation of IL-1 $\beta$

The combination of ASC and either pyrin, cryopyrin or PYPAF7 (PAN6) can induce caspase 1 activation ([21]; and C. Stehlik and J. C. Reed, unpublished work). Since we found that POP1 could suppress NF- $\kappa$ B activation induced by the combination of ASC and pyrin or cryopyrin, we suspected it would also suppress IL-1 $\beta$  production. However, in co-transfection experiments, POP1 enhanced IL-1 $\beta$  production induced via ASC-dependent mechanisms. Although POP1 did not induce increases in IL-1 $\beta$  production by itself, when co-transfected with ASC, plasmids producing POP1 caused a dose-dependent increase in IL-1 $\beta$  secretion (Figure 12A). Moreover, in transient transfection assays where ASC was co-expressed with either pyrin (Figure 12B) or



**Figure 11** TNF induces phosphorylation of POP1

HEK-293N cells were transfected with plasmids encoding HA-POP1 or HA-ASC-PAAD in 6 cm diameter dishes. At 30 h post-transfection, cells were washed in phosphate-free DMEM, and *in vivo* phosphorylation (IVP) was initiated by adding 1 mCi/ml [ $^{32}$ P] $P_i$  for 3 h. Then cells were either treated with 20 ng/ml TNF $\alpha$  for 20 min or left untreated. Following washing, cleared lysates were used for immunoprecipitations using agarose-conjugated anti-HA ( $\alpha$ HA) antibodies. Following washing, immune complexes were analysed by SDS/PAGE (15% gel) and immunoblotting and exposed to X-ray film. Membranes were also incubated with anti-HA antibodies to control for protein expression and loading. Left panel, arrow indicates phospho-POP1; right panel, arrow indicates phospho-ASC-PAAD.



**Figure 12** POP1 enhances ASC-dependent IL-1 $\beta$  secretion

HEK-293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 $\beta$  (200 ng), pro-caspase 1 (30 ng), and (A) ASC (20 ng) and POP1 (50 ng, 250 ng or 500 ng), (B) ASC (20 ng), POP1 (20 ng or 500 ng) and pyrin (500 ng) or (C) ASC (20 ng), POP1 (20 ng or 500 ng) and cryopyrin (500 ng), as indicated, maintaining the amount of total DNA constant at 1  $\mu$ g by the addition of control (empty) plasmid. Cell-culture supernatants were analysed for secreted IL-1 $\beta$  at 36 h post-transfection by ELISA. Results shown are means  $\pm$  S.D. ( $n = 3$ ) of the concentration (pg/ml) of IL-1 $\beta$ , normalized for cell number.

cryopyrin (Figure 12C), POP1 again enhanced the amounts of IL-1 $\beta$  produced. We therefore conclude that POP1 can enhance IL-1 $\beta$  production while inhibiting NF- $\kappa$ B induction, at least under the conditions of these assays. These results also indirectly demonstrate the specificity of the aforementioned results for NF $\kappa$ B, inasmuch as POP1 is inhibitory for NF $\kappa$ B activation, but stimulatory for IL-1 $\beta$  production. Thus POP1 does not cause a generalized suppression of cellular activation processes.

## DISCUSSION

The present paper provides evidence that the first identified PAAD-only protein, POP1, modulates the activity of PAAD-

family proteins. PAAD (PYRIN, DAPIN) domains have been recently found in multiple proteins, including several implicated in hereditary hyper-inflammation syndromes, interferon responses, cancer suppression, and apoptosis induction [1–7]. PAADs are capable of homotypic interactions with themselves or other members of the PAAD family, suggesting opportunities for creating protein-interaction networks that link various signalling pathways and permit fine-tuning of responses [12,21,29]. Thus the PAAD of POP1 presumably affects NF- $\kappa$ B- and caspase-1-activation pathways by interacting with other PAAD-containing proteins. The close proximity of the *POP1* gene to *ASC* on chromosome 16 at band p12.1 as well as the striking sequence similarity of their PAAD domains (88%) suggests that these genes arose by gene duplication. Interestingly, the gene encoding pyrin is located on the same chromosomal arm at band 16p13. Of note, the genomes of some pox-family viruses also contain candidate PAAD-only proteins (C. Stehlik, A. Godzik and J. C. Reed, unpublished work), suggesting a role for these viral genes in the suppression of host inflammatory reactions. The structure of POP1 suggests a role as a regulator of multidomain PAAD-family proteins by acting as a dominant-negative inhibitor, similar to some CARD-only proteins, such as COP/pseudo-ICE or ICEBERG, which suppress caspase-1 activation [25,31,32], or the DED-only proteins PED (phosphoprotein enriched in diabetes)/PEA-15 (phosphoprotein enriched in astrocytes 15 kDa), FLIP-s [short-form Fas-associated DD-like IL-1 $\beta$ -converting enzyme ('FLICE')-inhibitory protein] and v-FLIP (viral FLIP) in regulating apoptosis by affecting recruitment of pro-caspase 8 to ligand-activated TNF-family death receptors (reviewed in [33,34]).

Predominant expression of POP1 in monocytes, macrophages and granulocytes lends supports to the idea that POP1 represents an immune-modulating protein. Expression of POP1 in pericytes may also be consistent with this hypothesis, inasmuch as these cells express macrophage markers and are capable of phagocytosis and antigen presentation. Importantly, previous studies have demonstrated that ASC is expressed in the same cells as POP1 *in vivo*, as would be expected if interactions of these two proteins are important for the regulation of inflammatory cell responses [35,36].

Our finding that POP1 is also recruited to ASC-containing punctate structures in the cytoplasm of cells suggests that both proteins also assemble into a complex, which is further supported by our ability to co-precipitate these two proteins from cells. Consistent with inhibitory functions, we speculated that POP1 might inhibit the association of ASC with other PAAD-containing proteins in these structures. Since POP1 was able to inhibit the collaboration of ASC with pyrin or cryopyrin in inducing NF- $\kappa$ B activation, we speculate that POP1 interferes with the binding of ASC to pyrin and cryopyrin.

POP1-mediated blocking of NF- $\kappa$ B activation was also observed when cells were treated with TNF, IL-1 $\beta$  or LPS. Mapping studies pointed to the IKK complex as a potential target, which is known to be the point of convergence for many pathways leading to NF- $\kappa$ B activation in inflammatory cells. Similar findings were recently observed for other PAAD-family members, including ASC, NAC and PAN2, suggesting a conserved function of this domain [11,13]. Although we did not detect any differences in the association of POP1 with the IKK complex after treatment of cells with TNF, we observed that POP1, as well as the PAAD of ASC, became phosphorylated in TNF-treated cells. It remains to be determined whether or not it is the pool of IKK-complex-associated POP1 that becomes phosphorylated following TNF treatment. Using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>), five potential phosphorylation sites with

scores of > 0.989 on a scale of 0 to 1 were predicted in POP1: Ser<sup>59</sup>, Thr<sup>3</sup>, Thr<sup>16</sup>, Tyr<sup>61</sup> and Tyr<sup>64</sup> [37], but the responsible kinase or kinases remains to be identified. Post-translational modification of the PAAD domain might explain the ability of TNF to successfully induce IKK activation in cells that contain normal levels of POP1, ASC and other PAAD-family proteins, in contrast to overexpression situations where this phosphorylation-dependent mechanism may be overwhelmed. Further investigations are required to determine the functional significance of PAAD domain phosphorylation.

Recently, we demonstrated that the PAAD of ASC associates with and suppresses components of the IKK complex, and proposed that cryopyrin or pyrin interaction with ASC dislodges ASC from the IKKs, relieving endogenous suppression of these kinases and permitting NF- $\kappa$ B activation [11]. Alternatively, PAAD-containing proteins, such as cryopyrin, which are thought to self-oligomerize via a nucleotide-binding NACHT domain [38], might employ ASC as an adaptor for bridging to the IKK complex, achieving kinase activation through an induced proximity mechanism [11,39]. POP1, on the other hand, presumably interferes with both models mimicking the inhibitory effect of ASC either on the IKK complex or, alternatively, by preventing pyrin and cryopyrin from utilizing ASC as an adaptor to link to the IKK complex. Moreover, the ability of POP1 and the PAADs of ASC and PAN2 to associate with IKKs suggests competition between these proteins for access to IKKs.

ASC synergistically collaborates with pyrin, cryopyrin and NAC to activate pro-caspase 1 and subsequently IL-1 $\beta$  secretion [12,40]. Thus we expected POP1 to suppress IL-1 $\beta$  secretion. However, POP1 enhanced ASC-mediated activation of pro-IL-1 $\beta$ , both when expressed with ASC alone and when expressed with ASC in combination with pyrin or cryopyrin. Because the CARD domain of ASC binds the CARD of pro-caspase 1, we speculate that POP1 functions in this context to induce oligomerization of ASC-pro-caspase 1 complexes via PAAD-PAAD interactions, resulting in enhanced pro-caspase 1 activation through the induced proximity mechanism of pro-caspase activation [41]. Consistent with this hypothesis, Srinivasula et al. [20] also demonstrated that enforced multimerization of the CARD of ASC results in an increase in pro-caspase-1 activation. In contrast, in the POP1-mediated mechanism blocking NF- $\kappa$ B pathway, the PAAD domain is the actual effector domain, associating with the IKK complex, thus resulting in competition with ASC or other PAAD-containing proteins.

Further work is necessary, especially gene-ablation experiments in mice, to determine the physiological role of POP1 in the context of inflammation and to elucidate the precise role of POP1 in inflammatory disorders caused by hereditary mutations in pyrin and cryopyrin.

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