

# Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain Is a Regulator of Procaspase-1 Activation<sup>1</sup>

Christian Stehlik,\* Sug Hyung Lee,\* Andrea Dorfleutner,<sup>†</sup> Angela Stassinopoulos,\* Junji Sagara,<sup>‡</sup> and John C. Reed<sup>2\*</sup>

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)/target of methylation-induced silencing/PYCARD represents one of only two proteins encoded in the human genome that contains a caspase recruitment domain (CARD) together with a pyrin, AIM, ASC, and death domain-like (PAAD)/PYRIN/DAPIN domain. CARDS regulate caspase family proteases. We show here that ASC binds by its CARD to procaspase-1 and to adapter proteins involved in caspase-1 activation, thereby regulating cytokine pro-IL-1 $\beta$  activation by this protease in THP-1 monocytes. ASC enhances IL-1 $\beta$  secretion into the cell culture supernatants, at low concentrations, while suppressing at high concentrations. When expressed in HEK293 cells, ASC interferes with Cardiak/Rip2/Rick-mediated oligomerization of procaspase-1 and suppresses activation this protease, as measured by protease activity assays. Moreover, ASC also recruits procaspase-1 into ASC-formed cytosolic specks, separating it from Cardiak. We also show that expression of the PAAD/PYRIN family proteins pyrin or cryopyrin/PYPAF1/NALP3 individually inhibits IL-1 $\beta$  secretion but that coexpression of ASC with these proteins results in enhanced IL-1 $\beta$  secretion. However, expression of ASC uniformly interferes with caspase-1 activation and IL-1 $\beta$  secretion induced by proinflammatory stimuli such as LPS and TNF, suggesting pathway competition. Moreover, LPS and TNF induce increases in ASC mRNA and protein expression in cells of myeloid/monocytic origin, revealing another level of cross-talk of cytokine-signaling pathways with the ASC-controlled pathway. Thus, our results suggest a complex interplay of the bipartite adapter protein ASC with PAAD/PYRIN family proteins, LPS (Toll family receptors), and TNF in the regulation of procaspase-1 activation, cytokine production, and control of inflammatory responses. *The Journal of Immunology*, 2003, 171: 6154–6163.

**P**roteins containing the death domain fold play pivotal roles in apoptosis and inflammatory responses. The death domain fold represents a protein interaction motif consisting of a bundle of (usually) six antiparallel  $\alpha$ -helices. This core structure comprises four families of evolutionarily conserved and closely related domain families, including the death domains (DDs),<sup>3</sup> death effector domains (DEDs), caspase recruitment do-

main (CARDS), and pyrin, absent in melanoma (AIM), apoptosis-associated speck-like protein containing a CARD (ASC), and DD-like (PAAD; Ref. 1; also known as domain homologous to the pyrin N-terminal domain (PYRIN)/domain in apoptosis and IFN response (DAPIN)) domains (1–3). CARD and DED family proteins have been implicated in the activation of a family of intracellular cysteine proteases called caspases. Homotypic interactions among CARD- and DED-containing adapter proteins occur with the inactive proforms of those caspases that possess N-terminal prodomains containing complementary CARDS or DEDs, resulting in formation of multiprotein complexes and leading to protease cleavage and activation by an induced proximity mechanism (4). Various DED- and CARD-containing proteins have also been identified that compete for binding to these procaspases or adapter proteins, thereby suppressing protease activation (reviewed in Ref. 5).

Although many caspases are involved in apoptosis induction, some members of this family, such as caspase-1/IL-1 $\beta$ -converting enzyme, are chiefly responsible for proteolytic processing and activation of proinflammatory cytokines, such as IL-1 $\beta$  (reviewed in Ref. 6). IL-1 $\beta$  is a proinflammatory cytokine implicated in various diseases, including septic shock, inflammatory bowel disease, diabetes mellitus, and rheumatoid arthritis. IL-1 $\beta$  is synthesized as an inactive precursor of 31 kDa without a classical signal sequence. Generating the 17.5-kDa bioactive, secreted form of IL-1 $\beta$  requires proteolytic processing by active caspase-1. This cytokine is not activated in mice in which the gene encoding procaspase-1 has been ablated, attesting to the critical importance of caspase-1 for activation of pro-IL-1 $\beta$  (7, 8). Processing of pro-IL-1 $\beta$  is tightly

\*Burnham Institute, La Jolla, CA 92037; <sup>†</sup>Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037; and <sup>‡</sup>Department of Molecular Oncology, Research Center on Aging and Adaptation, Shinshu University School of Medicine, Nagano, Japan

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<sup>2</sup> Address correspondence and reprint requests to Dr. John C. Reed, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: jreed@burnham.org

<sup>3</sup> Abbreviations used in this paper: DD, death domain; co-IP, coimmunoprecipitation; CARD, caspase recruitment domain; AIM, absent in melanoma; PYRIN, domain homologous to the pyrin N-terminal domain; DAPIN, domain in apoptosis and IFN response; Cardiak, CARD-containing IL-1 $\beta$ -converting enzyme-associated kinase; Rick, RIP-like interacting CLARP kinase; NACHT, neuronal apoptosis inhibitory protein/MHC class II transcription activator/incompatibility locus protein from *Podospira auserina*/telomerase-associated protein; CLAN, CARD, leucine-rich region, and NACHT-containing protein; Ipaf, caspase 1-activating protein related to Apaf-1; PYPAF1, PYRIN-containing Apaf1-like protein 1; NALP, NACHT and leucine-rich region protein; NAC, NACHT- and CARD-containing protein; DEFCAP, death effector filament-forming Ced4-like apoptosis protein; DED, death effector domain; ASC, apoptosis-associated speck-like protein containing a CARD; PAAD, pyrin, AIM, ASC, and death domain-like; PAN, PAAD- and NACHT-containing protein; VSV, vesicular stomatitis virus; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; GFP, green fluorescent protein; RFP, red fluorescent protein;

TLR, Toll-like receptor; TRAF, TNFR-associated factor; Flag, src homology domain-containing protein tyrosine phosphatase-2.

controlled by various molecules that either inhibit or enhance activation of the zymogen (inactive) form of caspase-1. CARD-containing IL-1 $\beta$ -converting enzyme-associated kinase (Cardiak)/Rip2/RIP-like interacting CLARP kinase (Rick) and CARD, leucine-rich region, and neuronal apoptosis inhibitory protein/MHC class II transcription activator/incompatibility locus protein from *Podospira auserina*/telomerase-associated protein (NACHT)-containing protein (CLAN)/caspase 1-activating protein related to Apaf-1 (Ipaf)/CARD12, for example, are known activators of procaspase-1, which bind procaspase-1 via CARD-CARD interactions, and which directly or indirectly activate procaspase-1 by an induced proximity mechanism involving protein oligomerization (9–13). In contrast, the CARD-only proteins Iceberg and CARD-only protein/pseudocaspase-1 compete for interaction with the CARD of procaspase-1, thus interfering with oligomerization and suppressing protease activation (14–16).

The PAAD/PYRIN/DAPIN domain is found in diverse proteins implicated in apoptosis, inflammation, and cancer, although their molecular mechanisms of action are largely unknown. The founding member of this family, Pyrin, is mutated in families with familial Mediterranean fever, a hereditary hyperinflammatory response syndrome (17). Mutant alleles of the *CIAS1* gene encoding another PYRIN family protein, cryopyrin/PYRIN-containing Apaf1-like protein 1 (PYPAF1)/NACHT and leucine-rich region protein (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 PAADs in control of inflammatory responses (18, 19). A role for some PAAD-containing proteins in regulation of apoptosis (20–23) or NF- $\kappa$ B activation (20–25) has also been demonstrated recently.

The protein, ASC, consists of a N-terminal PAAD followed by a C-terminal CARD. It derives its name from its reported ability to trigger apoptosis when overexpressed in some tumor cell lines and from its localization to punctate cytosolic structures (specks) (23). ASC is also known as target of methylation-induced silencing-1 and becomes inactive in  $\approx$ 40% of breast cancers (21). Expression of ASC is found predominantly in monocytes and mucosal epithelial cells (26).

We report here that ASC uses its CARD to interact with the CARD of procaspase-1 and certain caspase-1 activators, thereby regulating activation of this protease and secretion of IL-1 $\beta$ . The phenotype of ASC changed from caspase-1 activator to inhibitor, depending on the levels of its expression and on whether certain other PAAD family proteins were coexpressed or other inflammatory pathways were activated (e.g., LPS/Toll family receptor; TNF). ASC thus represents a potential modulator of inflammatory responses that may assist in coordinating the activity of cytokine-activating caspases in mammalian cells.

## Materials and Methods

### Expression plasmids

The complete open reading frame and segments corresponding to PAAD or CARD of ASC were amplified by high fidelity PCR (Stratagene, La Jolla, CA) from pcDNA3-ASC (23) and subcloned into pcDNA3 vectors (Invitrogen, Carlsbad, CA) containing N-terminal Myc or hemagglutinin (HA) epitope tags. ASC (L122Q) was generated using the QuickChange mutagenesis kit (Stratagene). GST fusion proteins were generated by subcloning into pGEX-4T1 (Amersham Pharmacia Biotech, Piscataway, NJ). Green fluorescent protein (GFP) and red fluorescent protein (RFP) fusion protein vectors were generated by subcloning into pEGFP and pDS-Red expression vectors (Clontech Laboratories, Palo Alto, CA). For generating a recombinant retrovirus, Myc-tagged ASC was subcloned into pMSCV-hph (Clontech). Expression plasmids encoding procaspase-1, procaspase-1 (C285A), pro-IL-1 $\beta$ , Cardiak/Rip2/Rick, NACHT- and CARD-containing protein (NAC)/death effector filament-forming Ced4-like apoptosis protein

(DEFCAP)/CARD7/NALP1, and CLAN/Ipaf/CARD12 have been described (9, 16, 20). The complete reading frames of pyrin/marenostrin, cryopyrin/PYPAF1/NALP3, and PAAD- and NACHT-containing protein (PAN1)/NALP2/PYPAF2/NBS1 were amplified by high fidelity RT-PCR from pooled RNA extracted from several cell lines and cloned into pcDNA3-Myc (Invitrogen). Authenticity of all constructs was confirmed by DNA sequencing.

### RT-PCR

THP-1 cells were treated with 600 ng/ml LPS for various times and lysed in Trizol reagent (Life Technologies), and total RNA was isolated according to the instructions provided by the manufacturer. DNase I-treated RNA (1  $\mu$ g) was transcribed into single-stranded cDNA using Superscript II (Life Technologies, Gaithersburg, MD) and amplified for 30 cycles using Ampliqaq (Clontech Laboratories).

### Cell culture and transfection

COS-7, HEK293N, HEK293T, and HeLa cells were cultured in DMEM, and THP-1 cells were cultured in RPMI 1640, respectively, supplemented with 10–20% heat-inactivated FBS. Where indicated, cells were treated with 600 ng/ml LPS for various times. Transfection of HEK293 cells was accomplished using Superfect (Qiagen, Alameda, CA), whereas COS-7 and HeLa cells were transfected with LipoFectamine Plus (Life Technologies), holding total DNA content constant.

### Retroviral infections

The 293GP packaging cell line (Clontech) was stably transfected with either pMSCV-Myc-ASC or pMSCV-Neo, and stable clones were selected by culturing in 200 ng/ml hygromycin (Calbiochem, La Jolla, CA). These cells were then transiently transfected with pVSV-G (Clontech Laboratories) in 60-mm dishes and vesicular stomatitis virus (VSV)-G-pseudotyped retrovirus-containing culture supernatants (3 ml/dish) were collected 48 h later, clarified by filtration, and used to infect  $10^7$  THP-1 cells for 90 min at 2500 rpm at 32°C followed by 5 h at 32°C. Infection was repeated twice on successive following days. Infection efficiency was monitored with a pMSCV-GFP VSV-G-pseudotyped retrovirus. To achieve clones of THP-1 cells with different levels of ASC, we isolated retrovirus from 293GP2 cells stably expressing either GFP or GFP-ASC and transiently expressing VSV-G as above. This VSV-G-pseudotyped retrovirus was subsequently used to infect THP-1 cells either once with a low titer (1.5 ml of the cell supernatant) or four times with high titer (3 ml of the cell supernatant) virus as above. At 20 h postinfection, cells were differentiated into macrophages by culture overnight in medium containing 50 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO) and (where indicated) stimulated with 600 ng/ml LPS before analysis.

### Caspase activity and IL-1 $\beta$ secretion assays

Cells were directly lysed in caspase lysis buffer (10 mM HEPES (pH 7.4), 25 mM NaCl, 0.25% Triton X-100, and 1 mM EDTA), normalized for protein content, and protease activity was continuously measured by monitoring the release of fluorogenic Ac-YVAD-AFC (Bachem, Torrance, CA) at 37°C, as described (27). IL-1 $\beta$  secreted into culture supernatants of 24-well plates was measured by ELISA using a commercial kit (R&D Systems, Minneapolis, MN), normalizing data for cell numbers, and performing assays in triplicate (16).

### In vitro protein interaction assays and coimmunoprecipitations

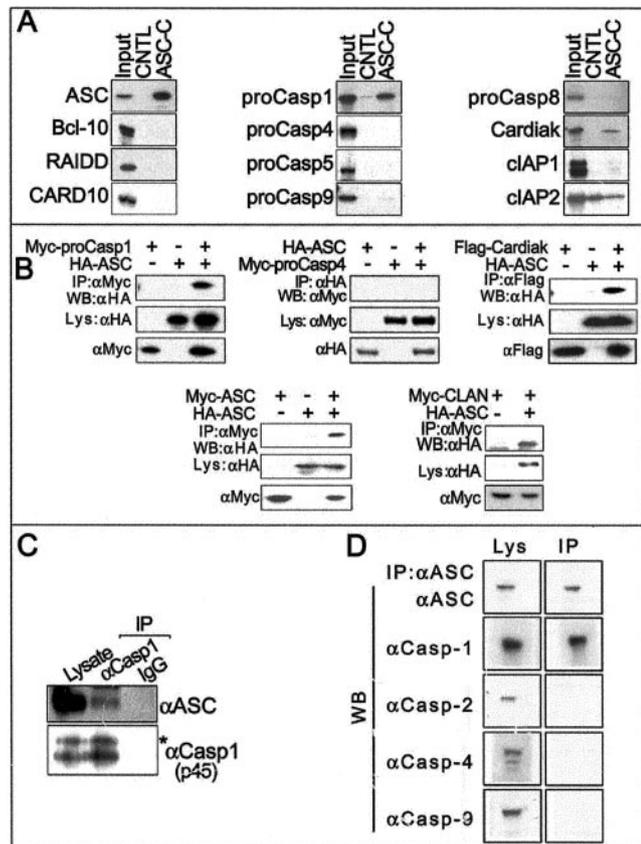
GST-ASC-CARD was expressed in XL-1 blue cells (Stratagene) and affinity purified using GSH-Sepharose (Amersham Pharmacia Biotech). GST-ASC-CARD or various GST control proteins (0.1  $\mu$ g) immobilized on 10  $\mu$ l of GSH-Sepharose were incubated with 1 mg/ml BSA in buffer A (142.4 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 1 mM EDTA, and 0.2% Nonidet P-40, supplemented with 1 mM DTT, 12.5 mM  $\beta$ -glycerol phosphate, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, and 1 $\times$  protease inhibitor mix; Roche, Basel, Switzerland) for 30 min at room temperature. Beads were washed twice and incubated overnight at 4°C with 1  $\mu$ l of in vitro translated (Promega, Madison, WI) and <sup>35</sup>S-labeled proteins in buffer A supplemented with 0.5 mg/ml BSA. Bound proteins were washed four times in 1 ml of buffer A, separated by SDS-PAGE, and detected by fluorography.

For immunoprecipitations, cells were lysed in isotonic lysis buffer (150 or 500 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.2% Nonidet P-40, 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; Roche), using  $\sim 5 \times 10^5$ – $1 \times 10^6$  cells for epitope-tagged proteins and  $\sim 10^8$  cells for endogenous proteins. Clarified lysates

were subjected to immunoprecipitation using agarose-conjugated anti-c-Myc, anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FlagM2 (Sigma-Aldrich) Abs, or protein A- or G-conjugated anti-caspase-1 or anti-ASC Abs (26). After incubation at 4°C for 4–12 h, immune complexes were washed three times in lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting using various Abs as indicated in conjunction with the ECL detection system (Amersham-Pharmacia). Where indicated, cell lysates (10% volume) were run alongside immune complexes. Alternatively, lysates were directly analyzed by immunoblotting after normalization for total protein content.

#### Immunofluorescence analysis

HEK293 or HeLa cells were transfected with plasmids encoding GFP or RFP fusion proteins or epitope-tagged proteins, transferred to four-well polylysine-coated chamber slides (LabTec, Andover, MA) the following day, fixed in 4% paraformaldehyde 1 day later, and analyzed by confocal laser-scanning microscopy (Bio-Rad, Hercules, CA).



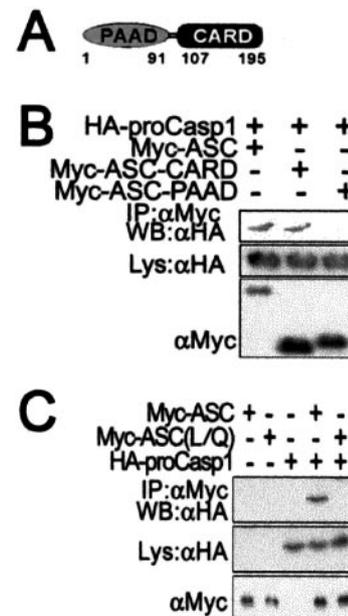
**FIGURE 1.** ASC binds procaspase-1. *A*, In vitro GST-binding assays are shown with 10% of input as indicated. *B*, Co-IP assays were performed using transiently transfected HEK293T cells and either anti ( $\alpha$ )-Myc or anti-Flag Abs and then analyzed by SDS-PAGE/immunoblotting (Western blot (WB) using indicated Abs. Alternatively, cell lysates (Lys) were normalized for total protein content and analyzed by Western blot. IP, Immunoprecipitation. *C*, THP-1 cells were stimulated with LPS for 4 h, and lysates were immunoprecipitated using either 1  $\mu$ g of anti-caspase-1 polyclonal Ab or control (CNTL) IgG immobilized to protein A-Sepharose. Bound proteins were analyzed by Western blot using mouse anti-ASC antiserum (*top*). Blots were reprobed with Abs to caspase-1 (*bottom*). \*, Cross-reactive protein. *D*, Anti-ASC immune complexes were prepared from THP-1 monocytes using a mouse monoclonal anti-ASC Ab, divided into five equal fractions, and then separated by SDS-PAGE, transferred to nitrocellulose; the resulting blots were incubated with rabbit or goat polyclonal Abs recognizing ASC, caspase-1, caspase-2, caspase-4, or caspase-9. Ab detection was accomplished by ECL. For purposes of presentation, only the region of blots corresponding to the relevant protein band is shown.

## Results

### ASC binds procaspase-1 and caspase-1 activators

To seek binding partners for the CARD of ASC, we expressed the CARD as a GST fusion protein and performed in vitro protein interaction experiments with various other CARD family proteins, which were produced by in vitro translation. These experiments revealed specific binding of ASC CARD to procaspase-1, but not to several other caspases, including the closely related caspases caspase-4 and caspase-5 (Fig. 1*A*). Interestingly, ASC-CARD also demonstrated specific interaction with Cardiak/Rip2/Rick and CLAN/Ipaf/CARD12, proteins known to bind and activate procaspase-1, but did not reveal specific interactions with multiple other CARD family proteins (Fig. 1*A* and data not shown) (9–12). Protein interactions were confirmed by coimmunoprecipitation (co-IP) experiments, in which the candidate binding proteins were expressed by transient transfection in HEK293T cells (Fig. 1*B*). ASC did not induce apoptosis under these conditions (not shown).

To determine whether the endogenous ASC protein can bind procaspase-1, co-IP experiments were performed using lysates from LPS-treated THP-1 monocytic leukemia cells. Immune complexes were prepared using anti-caspase-1 Ab or control IgG, followed by SDS-PAGE-immunoblot analysis using an ASC-specific antiserum, thus providing evidence that endogenous ASC can associate with endogenous procaspase-1 (Fig. 1*C*). To demonstrate the specificity of the ASC-procaspase-1 interaction, we coimmunoprecipitated ASC from THP-1 monocytes followed by SDS-PAGE-immunoblot analysis of several CARD-containing caspases, including caspase-1, -2, -4, and -9. Again, selective interaction of ASC with procaspase-1 (but not procaspases-2, -4, or -9) was detected (Fig. 1*D*).



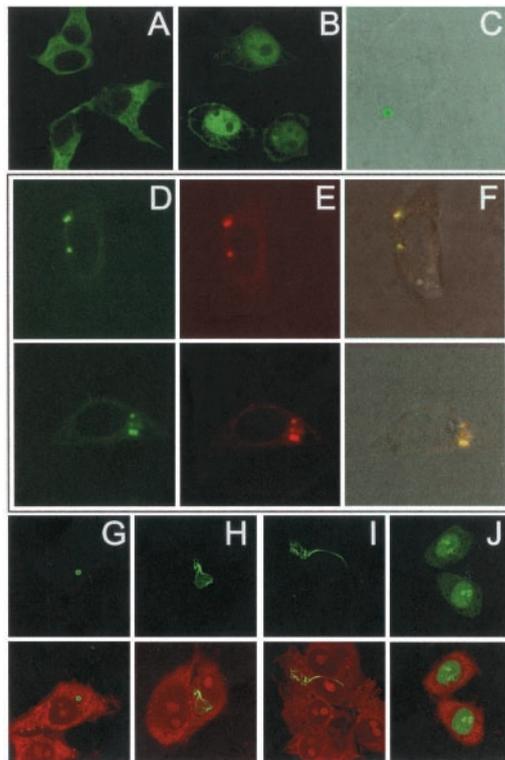
**FIGURE 2.** The CARD of ASC is responsible for binding to procaspase-1. *A*, Domain structure of ASC; amino acid residues demarcate boundaries of the PAAD and CARD. *B*, HEK293T cells were transiently transfected with plasmids as indicated and cell lysates were either loaded directly into gels after normalization for total protein content or subjected to immunoprecipitation using anti ( $\alpha$ )-Myc-Sepharose. Cell lysates (Lys) and immune complexes (IP) were analyzed by Western blotting (WB) using anti-HA Abs, as indicated. *C*, Co-IP assays were performed as above, using HEK293T cells transiently transfected with plasmids encoding the indicated proteins.

### The CARD of ASC is responsible for binding procaspase-1

ASC contains both CARD and PAAD domains (Fig. 2A), which probably assume similar three-dimensional structures (28). We therefore contrasted the ability of the CARD and PAAD domains of ASC to associate with procaspase-1. To this end, the PAAD and CARD domains of ASC were expressed separately as epitope-tagged proteins by transient transfection in HEK293T cells, demonstrating that ASC-CARD, but not ASC-PAAD, binds procaspase-1 (Fig. 2B). Moreover, the region on procaspase-1 required for binding ASC was similarly mapped to the CARD-containing N-terminal prodomain (not shown). The role of the CARD in binding procaspase-1 was further confirmed by site-directed mutagenesis using ASC-CARD containing a leucine to glutamine mutation analogous to inactivating mutations previously described for other CARD proteins (29). Although wild-type ASC was capable of coimmunoprecipitating procaspase-1, mutant ASC (L122Q) did not, although both proteins were expressed at comparable levels (Fig. 2C).

### ASC and procaspase-1 colocalize in cells

Because ASC localizes to punctuate cytosolic structures (specks), we asked whether overexpression of ASC alters the intracellular distribution of procaspase-1. Fusion proteins of ASC with RFP and procaspase-1 with GFP were transiently expressed in HeLa cells



**FIGURE 3.** ASC recruits procaspase-1 into cytosolic specks. HeLa cells were transiently transfected with plasmids encoding various GFP or RFP fusion proteins and imaged by confocal microscopy. Panels present representative data: A–C, Cells transfected with (left to right) plasmids encoding GFP-PAN10 (used here as a control), GFP-procaspase-1 (C285A), or GFP-ASC individually; D–F, Cells transfected with the combination of GFP-procaspase-1 (C285A) and RFP-ASC, showing (left to right) green, red, and two-color (merge) fluorescence overlaid with phase contrast image; G–J, Cells transfected with plasmids encoding (left to right) GFP-ASC, GFP-ASC-PAAD, GFP-ASC-CARD, or GFP-ASC (L122Q). Data are representative of multiple experiments. Similar results were obtained using COS-7 cells (not shown).

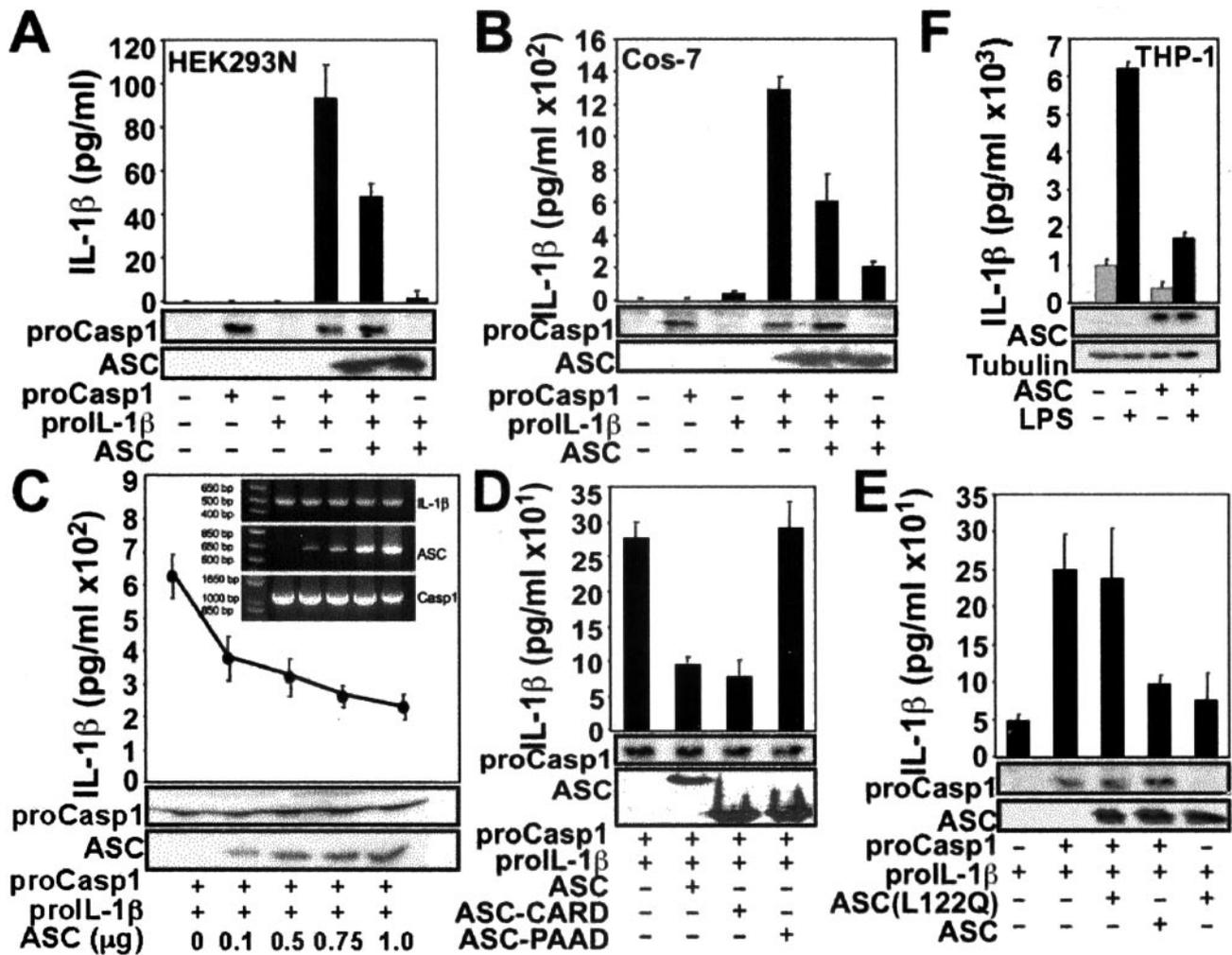
(Fig. 3) or COS-7 cells (not shown) and then imaged by confocal microscopy. To prevent caspase-1-induced apoptosis when overexpressed, cysteine 285 of the catalytic domain of procaspase-1 was mutated to alanine (30). GFP-tagged procaspase-1 was distributed mainly to the nucleus and cell membrane of cells, as reported previously (31–33). In contrast, a control protein consisting of GFP fused to the PAAD domain of PAN10 was located diffusely throughout the cytoplasm of cells (Fig. 3). As previously reported, ASC localized to distinct speckles within the cytosol of transfected cells (Fig. 3C; Ref. 23). Coexpression of GFP-procaspase-1 (C285A) with RFP-ASC resulted in recruitment of GFP-procaspase-1 (C285A) to specks (Fig. 3, D–F). In contrast, the control GFP protein was not recruited into specks by coexpression with ASC (not shown), thus demonstrating the specificity of these results. Similar results were obtained using both HeLa (Fig. 3) and COS-7 (not shown) cells, which both contain little or no endogenous ASC.

The structural features of ASC required for its targeting to cytosolic specks were addressed by contrasting the intracellular localization of GFP-tagged wild-type ASC (Fig. 3G) with various ASC mutants, including truncation mutants containing only the PAAD (Fig. 3H) or CARD (Fig. 3I) domains, or ASC containing the L122Q mutation (Fig. 3J). Full-length ASC localized to cytosolic specks, but the PAAD- and CARD-only fragments were associated with filament-like structures. In contrast, the CARD (L122Q) mutant was found predominantly throughout the cytosol and the nucleus (Fig. 3J). Thus, a combination of intact CARD and PAAD domains is required for localization of ASC to cytosolic specks. However, expression of ASC-CARD, but not ASC-PAAD, was sufficient to recruit procaspase-1 to filament-like structures, thus disrupting the normal pattern of procaspase-1 localization (data not shown).

### ASC modulates IL-1 $\beta$ secretion

Because caspase-1 is absolutely required for processing of pro-IL-1 $\beta$  and secretion of IL-1 $\beta$ , we tested whether ASC might have an effect on IL-1 $\beta$  production. For initial experiments, plasmids encoding pro-IL-1 $\beta$  and procaspase-1 were cotransfected with or without ASC into HEK293N (Fig. 4A) and COS-7 (Fig. 4B) cells, and levels of secreted IL-1 $\beta$  were measured 30 h later in culture supernatants. ASC significantly suppressed IL-1 $\beta$  secretion in these experiments. Immunoblot analysis of lysates prepared from these transfected cells showed that ASC did not interfere with procaspase-1 production, excluding a trivial explanation for the results. The effect of ASC on suppression of caspase-1-induced secretion of IL-1 $\beta$  was concentration dependent and correlated with the amount of ASC protein produced in transfected cells, whereas the mRNA level of pro-IL-1 $\beta$  was unaltered (Fig. 4C). Mapping studies indicated that the CARD of ASC is necessary and sufficient for suppression of IL-1 $\beta$  secretion (Fig. 4D). In contrast, the L122Q mutant of ASC failed to suppress caspase-1-mediated production of IL-1 $\beta$  (Fig. 4E), correlating with the binding studies.

To determine whether ASC is capable of regulating production of IL-1 $\beta$  induced by endogenous caspase-1 in response to a physiologically relevant stimulus, we used THP-1 monocytic cells that had been infected with recombinant retrovirus expressing Myc-tagged ASC (or with control retrovirus) and then differentiated these cells into adherent macrophages using phorbol ester, TPA. Cells were then stimulated with LPS for 12 h to trigger caspase-1 activation and induce IL-1 $\beta$  secretion (34). LPS stimulation of control virus-infected THP-1 cells resulted in a >6-fold increase of secreted IL-1 $\beta$ , whereas ASC expression strongly suppressed this response (>80% inhibition; Fig. 4F). Thus, ASC is capable of



**FIGURE 4.** Inhibition of IL-1 $\beta$  secretion by ASC. HEK293N (A, C, D, and E) or COS-7 (B) cells were transiently transfected in 24-well plates with plasmids expressing procaspase-1 (150 ng), pro-IL-1 $\beta$  (200 ng), ASC (500 ng), ASC-PAAD (500 ng), or ASC-CARD (500 ng) as indicated, maintaining total DNA constant at 1  $\mu$ g by addition of empty plasmid. Supernatants were analyzed for IL-1 $\beta$  at 36 h posttransfection. C, inset, RT-PCR analysis of ASC, caspase-1, and IL-1 $\beta$  mRNAs, under identical conditions. Left, molecular mass markers. F, THP-1 cells were infected three times sequentially with a recombinant retrovirus expressing Myc-tagged ASC or with Neo virus. After 1 day, cells were differentiated into adherent macrophages by culture overnight in TPA; then, where indicated, cells were stimulated with LPS for 12 h, before collecting culture supernatants for analysis of secreted IL-1 $\beta$ . Data represent picograms per milliliter of IL-1 $\beta$ , normalized for cell number (mean  $\pm$  SD;  $n = 3$ ).

modulating IL-1 $\beta$  production that results from activation of endogenous procaspase-1.

#### ASC expression is inducible

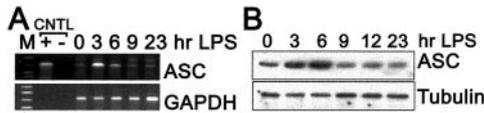
In the course of our studies, we observed that ASC expression is induced in myeloid lineage hemopoietic cells by LPS and TNF- $\alpha$ . For example, in THP-1 monocytic cells, levels of ASC mRNA (as determined by RT-PCR) and ASC protein (as determined by immunoblot analysis) increased after LPS stimulation, reaching maximum levels at 3 and 6 h, respectively, and then declining (Fig. 5). These findings suggest possible cross-talk of LPS- and TNF-signaling pathways involved in caspase-1 activation.

#### ASC disrupts Cardiak/procaspase-1 interactions

The CARD-containing protein Cardiak/Rip2/Rick has been shown to mediate procaspase-1 activation by the induced proximity

mechanism (11). The observation that ASC is capable of binding both Cardiak/Rip2/Rick and procaspase-1 therefore led us to hypothesize that ASC might prevent Cardiak/Rip2/Rick from bringing procaspase-1 molecules into proximity. To explore this possibility, HEK293T cells were transiently transfected with plasmids encoding Myc- and HA-tagged procaspase-1 (C285A mutant), alone or together with src homology domain-containing protein tyrosine phosphatase-2 (Flag)-tagged Cardiak, with or without HA-ASC; then, co-IP assays were performed. As shown in Fig. 6A, procaspase-1 self-association was enhanced in the presence of Cardiak/Rip2/Rick but profoundly decreased when ASC was co-expressed with procaspase-1 and Cardiak/Rip2/Rick. Immunoblotting demonstrated that the levels of procaspase-1 (C285A) and Cardiak were not changed by expression of ASC, excluding an influence on protein expression as an explanation for the results.

Next co-IP experiments were used to test whether ASC competes with Cardiak for procaspase-1 binding. As shown in Fig. 6B, ASC expression interfered with association of procaspase-1 and



**FIGURE 5.** ASC expression is regulated by inflammatory stimuli. *A*, RT-PCR was performed using RNA derived from THP-1 cells at various times after LPS stimulation, using primers specific for either ASC (*top*) or GAPDH (*bottom*). *B*, Western blot analysis of ASC protein levels was performed using lysates from LPS-treated THP-1 cells and Abs specific for either ASC (*top*) or  $\alpha$ -tubulin (*bottom*). CNTL, Control.

Cardiak, resulting in reduced recovery of procaspase-1 in Cardiak-containing immune complexes. To further confirm these interactions, we used fluorescence confocal microscopy to localize RFP-procaspase-1 (C285A) and GFP-Cardiak proteins in HeLa and HEK293 cells when expressed alone or together with Myc-ASC (Fig. 6C). Cardiak by itself localized to dot-like structures in the cytoplasm (Fig. 6C, *a*). Coexpression of GFP-Cardiak with RFP-procaspase-1 resulted in colocalization in the cytoplasm (Fig. 6C, *b–d*). Additional expression of ASC resulted in dissociation of RFP-procaspase-1 (Fig. 6C, *e* and *g*) and GFP-Cardiak (Fig. 6C, *f* and *g*), with GFP-Cardiak being distributed throughout the cytosol but RFP-procaspase-1 localizing to cytosolic specks (Fig. 6C, *g*). Because overexpression of Cardiak did not dissociate RFP-procaspase-1 and GFP-ASC from specks (Fig. 6C, *h–j*), these results suggest that ASC is the dominant binding partner. These findings also correlated with effects of Cardiak and ASC on caspase-1 protease activity, as measured by cleavage of a fluorogenic caspase-1 substrate peptide (Fig. 6D).

#### Dual role of ASC as a caspase-1 activator and inhibitor

After completion of this work, two reports appeared suggesting that ASC is an activator rather than an inhibitor of procaspase-1 and pro-IL-1 $\beta$  (13, 35). We therefore repeated experiments using the same conditions used by Wang et al., in which less plasmid DNA was used for transfections. These experiments revealed that the amount of ASC is critical for its function, showing that ASC enhances procaspase-1 activation at low concentrations of transfected plasmid DNA, while suppressing it at high concentrations (Fig. 7A).

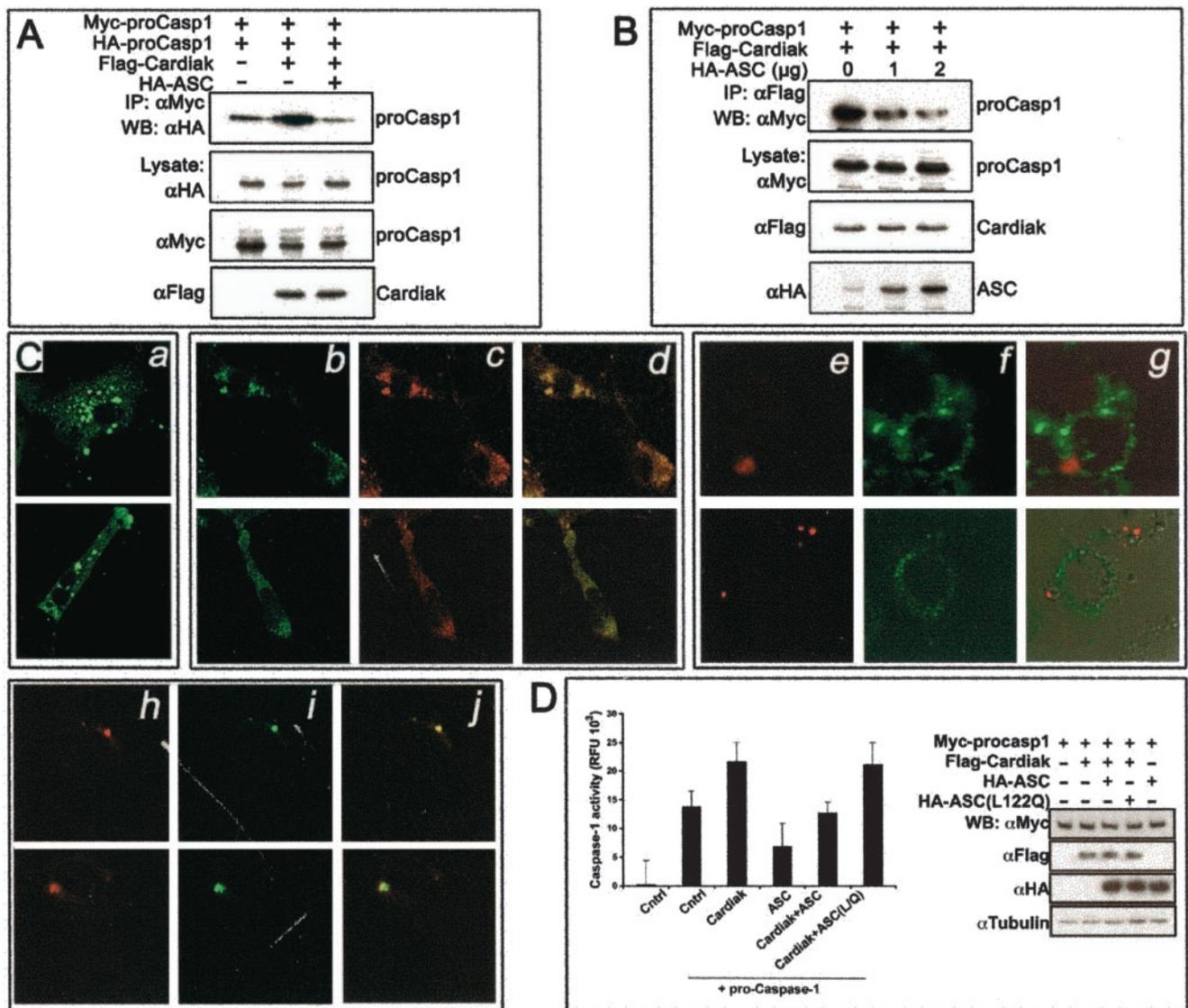
To preliminarily explore the physiological significance of these findings, we compared the levels of endogenous ASC protein produced in differentiated THP-1 monocytes before and after LPS treatment with the levels of ASC protein produced in transiently transfected HEK293 cells (Fig. 7A). All samples were normalized for total protein content and then analyzed by immunoblotting, normalizing data relative to a housekeeping protein ( $\alpha$ -tubulin). After LPS stimulation, endogenous ASC in THP-1 monocytic cells reached levels exceeding the amount of ASC corresponding to maximal procaspase-1 activation in transfected HEK293 cells (Fig. 7A). Thus, this comparison suggests that endogenous levels of ASC may become sufficiently high after activation of monocytic cells to place ASC protein concentrations into the inhibitory range, although relative amounts of procaspase-1 and other ASC-binding proteins are also likely to impact the net result.

These experiments were also extended to the monocytic cell line THP-1. We used the fact that multiple, sequential infections with a retrovirus result in the integration of multiple copies of the gene and thus in higher levels of expression. Therefore, we either infected THP-1 cells once with low titer VSV-G-pseudotyped retrovirus expressing GFP-ASC, or with high titer four times sequentially. Cell lysates were monitored for expression of GFP-ASC and endogenous ASC by SDS-PAGE immunoblotting using an anti-

ASC Ab (Fig. 7B), confirming production of stable infectants with intermediate vs high levels of GFP-ASC. After differentiation with TPA and stimulation with LPS, secretion of IL-1 $\beta$  into culture medium was compared. Only markedly elevated levels of GFP-ASC resulted in suppression of LPS-induced IL-1 $\beta$  production (Fig. 7B), suggesting that the endogenous levels of ASC found in THP-1 monocyte cells are inadequate to suppress caspase-1 activation that is stimulated by LPS treatment. Recently, it was reported that PYPAF7, a PAAD/PYRIN/DAPIN family protein can activate procaspase-1 when coexpressed with ASC (35). We therefore tested several PAAD/PYRIN/DAPIN family members for their effects on procaspase-1 activation individually and in combination with ASC. For this experiment, HEK293 cells were transiently transfected with a plasmid encoding pro-IL-1 $\beta$  and procaspase-1 alone or in combination with plasmids producing various PAAD family proteins, and IL-1 $\beta$  secretion was measured 2 days later. In fact, expression of procaspase-1 by itself was sufficient to induce some IL-1 $\beta$  secretion under these conditions. Pyrin and cryopyrin/PYPAF1/NALP3 inhibited procaspase-1 activation and IL-1 $\beta$  secretion (Fig. 7C). However, coexpression of ASC with these proteins overcame the inhibition by pyrin and cryopyrin/PYPAF1/NALP3 and even resulted in synergistic activation, dependent on the expression level of the PAAD/PYRIN proteins (Fig. 7, *C–E*). However, two other PAAD/PYRIN family member proteins, NAC/DEFCAP/CARD7/NALP1 and PAN1/PYPAF2/NALP2/NBS1, did not show an effect on IL-1 $\beta$  secretion, when coexpressed with procaspase-1 alone or in combination with ASC (Fig. 7C). Immunoblotting demonstrated production of all plasmid-derived proteins (not shown). These results suggest a complex interplay of the bipartite adapter protein ASC with other PAAD/PYRIN family proteins in the regulation of procaspase-1 activation. Thus, we conclude that ASC and certain other PAAD family proteins can modulate caspase-1-mediated secretion of IL-1 $\beta$ , depending on their relative levels of expression.

## Discussion

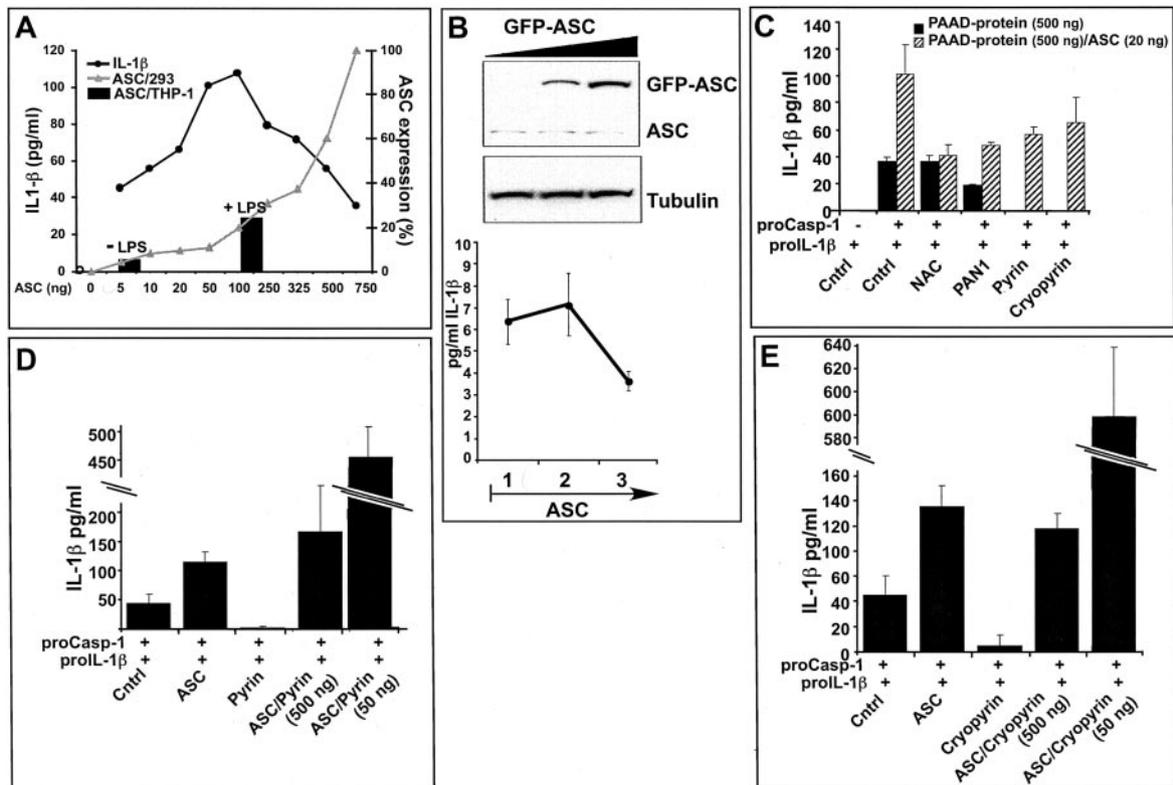
ASC is one of only two genes in the human genome encoding proteins that contain both a PAAD (PYRIN/DAPIN) and CARD. As such, it possesses a structure reminiscent of adapter proteins that bridge domain families, such as Fas-associated death domain protein/mediator of receptor-induced toxicity-1 which is the only protein encoded in the human genome to combine a DD with a DED, and RIP-associated protein with a death domain/CASP2 and RIPK1 domain-containing adaptor with a death domain, the only protein that possesses both a CARD and a DD (reviewed in Ref. 36). ASC is capable of collaborating with certain members of the PAAD family to induce either NF- $\kappa$ B activity or procaspase-1 activation (35, 37). In this report, we extend previous reports regarding the function of ASC. Our data concur with recent reports suggesting that ASC associates with procaspase-1 (13, 35). Moreover, in this report, we extend previous work by showing that this association is not merely limited to overexpression situations, because we could demonstrate for the first time specific interaction between endogenous ASC and endogenous procaspase-1, but not other CARD-containing caspases, using THP-1 monocytic cells. The CARD of ASC is necessary and sufficient for binding the CARD-containing prodomain of procaspase-1, and it competes for binding with the CARD family protein Cardiak, which functions as activator of this protease. ASC can also compete with the caspase-1 agonist CLAN/Ipaf/CARD12 for binding to procaspase-1 (not shown). Thus, ASC is capable of interfering with the association of certain known procaspase-1 agonists with their target protease, thereby modulating IL-1 $\beta$  secretion.



**FIGURE 6.** ASC inhibits procaspase-1 interaction with and oligomerization by Cardiak. *A* and *B*, HEK293T cells were transiently transfected in 6-cm dishes with 1  $\mu$ g of each of the indicated plasmids, maintaining total DNA constant by addition of empty plasmid, and IPs were performed using either anti ( $\alpha$ )-Myc-Sepharose (*A*) or anti-Flag-Sepharose (*B*) followed by analysis of immune complexes by Western blot (WB) using either anti-HA or anti-Myc Ab, respectively. Lysates were also analyzed directly by Western blot after normalization for protein content (30  $\mu$ g per lane) using indicated Abs. *C*, HEK293N (*a-g*, top panels) and HeLa (*a-g*, bottom panels; *h-j*, both panels) cells were transiently transfected with plasmids encoding various GFP or RFP fusion proteins and imaged by confocal microscopy. Panels present representative data, showing cells transfected with plasmids encoding (*a*) GFP-Cardiak alone, (*b-d*) GFP-Cardiak plus RFP-procaspase-1 (C285A), showing (left to right) green, red, and two color merge fluorescence; *e-g*, Cells transfected with RFP-procaspase-1 (C285A), GFP-Cardiak, and Myc-ASC showing (left to right) red, green, and two-color (merge) fluorescence; *h-j*, Cells transfected with plasmids encoding RFP-procaspase-1 (C285A), GFP-ASC, and Flag-Cardiak showing (left to right) red, green, and two-color (merge) fluorescence. *D*, HEK293N cells were transiently transfected in 6-cm dishes with 1  $\mu$ g of each of the indicated plasmids encoding Myc-procaspase-1, Flag-Cardiak, HA-ASC, HA-ASC(L122Q), or various combinations, as indicated, maintaining total DNA constant by addition of empty plasmid. *Left*, Cell lysates were analyzed for caspase-1 protease activity. Data represent relative fluorescence units per milligram of protein. *Right*, Cell lysates were normalized for protein content (30  $\mu$ g/lane) and analyzed by Western blot using the indicated Abs. Under these conditions, the amounts of proteolytically processed procaspase-1 produced are too small to be detected by immunoblotting of cell lysates.

A recent publication by Druihle et al. (13) suggested a model in which ASC functions as an activator of procaspase-1. These investigators used a heterologous oligomerization domain fused to the CARD of ASC, which resulted in enhanced IL-1 $\beta$  secretion upon inducing oligomerization. In contrast, oligomerization of the PAAD (PYRIN/DAPIN) of ASC did not alter procaspase-1 activity. Overexpression of the CARD alone strongly inhibited IL-1 $\beta$  secretion, which was interpreted to represent a dominant-negative effect of the truncated ASC protein (13). However, in our experiments, full-length ASC behaved in a manner similar to that of the

CARD alone, suggesting that a re-evaluation of the role of endogenous ASC is necessary. Also, Alnemri's group showed that a fragment of ASC comprising only the CARD domain suppressed LPS-induced IL-1 $\beta$  secretion in THP-1 monocytic cells, whereas (using the same approach) we observed suppression of LPS-induced IL-1 $\beta$  secretion using either the CARD of ASC or full-length ASC protein. Thus, in some cellular contexts, even full-length ASC may operate as an inhibitor rather than an activator of procaspase-1. Consistent with this hypothesis, testing of a range of ASC concentrations with respect to procaspase-1 activation in



**FIGURE 7.** Regulation of IL-1 $\beta$  secretion by ASC and PAN-proteins. **A**, HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 $\beta$  (200 ng), procaspase-1 (30 ng; ●) or empty vector (○), and ASC (5, 10, 20, 50, 100, 250, 325, 500, and 750 ng), maintaining total DNA constant at 1  $\mu$ g by addition of empty plasmid. After 1 day, protein lysates were prepared from the transfected cells, normalized for total protein content (60  $\mu$ g), and analyzed by Western blot using anti-ASC and anti- $\alpha$ -tubulin Abs, followed by densitometric scanning of the resulting bands. Loaded on the same membrane were identical amounts (60  $\mu$ g) of protein lysates derived from TPA-differentiated THP-1 cells, which had been cultured with or without LPS for 6 h (■). Densitometry data for ASC were normalized relative to  $\alpha$ -tubulin and expressed as a percentage relative to the maximum level of ASC produced in transfected HEK293T cells. **B**, THP-1 cells were infected with either a control GFP-expressing VSV-G-pseudotyped retrovirus (*lane 1*) four times sequentially (twice a day) for 90 min at 2500 rpm at 32°C followed by 5 h at 32°C. Alternatively, cells were infected once with low titer (*lane 2*) or four times with high titer GFP-ASC-expressing VSV-G-pseudotyped retrovirus (*lane 3*). Efficiency of infection was monitored by GFP fluorescence. At 20 h postinfection, cells were differentiated into macrophages by culture overnight in medium containing 50 ng/ml TPA and (where indicated) stimulated with 600 ng/ml LPS before analysis. Cell culture supernatants from these cells were analyzed for secreted IL-1 $\beta$  by ELISA (BD Biosciences, San Jose, CA). Cells were also lysed and tested by SDS-PAGE-immunoblotting using a monoclonal anti-ASC Ab for expression of GFP-ASC compared with endogenous ASC. **C**, Using conditions similar to those described previously for PYPAF7 (35), HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 $\beta$  (200 ng), procaspase-1 (30 ng) or empty vector, and various PAAD family members (500 ng; ■), as indicated, or PAAD family members (500 ng) were coexpressed with 20 ng ASC (▨), maintaining total DNA constant at 1  $\mu$ g by addition of empty plasmid. **D**, HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 $\beta$  (200 ng), procaspase-1 (30 ng), and either 50 or 500 ng of pyrin alone or together with 20 ng of ASC, maintaining total DNA constant at 1  $\mu$ g by addition of empty plasmid. **E**, HEK293T cells were transiently transfected in 24-well plates with plasmids encoding pro-IL-1 $\beta$  (200 ng), procaspase-1 (30 ng), and either 50 or 500 ng of cryopyrin alone or together with 20 ng of ASC, maintaining total DNA constant at 1  $\mu$ g by addition of empty plasmid. Culture supernatants were analyzed for IL-1 $\beta$  at 36 h posttransfection by ELISA. Data represent picograms per milliliter of IL-1 $\beta$ , normalized for cell number (mean  $\pm$  SD;  $n = 3$ ).

HEK293 cells showed that ASC enhances caspase-1 activation at low concentrations, while suppressing at high concentrations. However, experiments with THP-1 monocyte cells suggest that the levels of this adapter protein required to suppress IL-1 $\beta$  production induced at least by LPS are unlikely to rise beyond optimal levels and enter into the inhibitory range. It remains to be determined whether the levels of ASC achieved adequate to suppress caspase-1 activation induced by other (non-LPS) types of stimuli. Attempts to further address this matter using small interfering RNA and antisense oligonucleotides failed due to inability to achieve more than ~50% reductions in endogenous ASC protein levels, despite testing several sequences (not shown). Thus, ultimate determination of the relative roles of ASC in regulating caspase-1 activation and pro-IL-1 $\beta$  processing await ablation of the gene in mice or analogous gene targeting experiments. However, this dual activity of ASC is reminiscent of FLIP long, a

modulator of procaspase-8 activation, which can either inhibit or stimulate activation of this protease depending on the levels of expression and cellular context (38).

Recently, it was suggested that PAAD-PAAD interactions between ASC and PYPAF7 mediate activation of procaspase-1 (35). Several PAAD family proteins have architectures reminiscent of the caspase activator CLAN/Ipaf/CARD12 with an N-terminal PAAD, followed by a NACHT domain, then multiple leucine-rich repeats. These proteins containing both PAAD and NACHT domains, PANs (also known as PYPAFs and NALPs), are thought to self-oligomerize via their nucleotide-binding NACHT domains (39), using the PAAD to bring associated proteins into close proximity for activation. In this way, the PAAD of ASC would presumably dock on the PAAD of PANs, whereas the CARD would allow these proteins to bind CARD-containing target proteins, such as procaspase-1. However, we found that some PANs, pyrin

and cryopyrin/PYPAF1/NALP3, potentially inhibited IL-1 $\beta$  secretion when expressed individually, whereas others such as NAC and PAN1 did not effect IL-1 $\beta$  secretion. Strikingly, coexpression of suboptimal amounts of ASC-encoding plasmid DNA reverted the inhibitory effects of pyrin and cryopyrin/PYPAF1/NALP3. This effect was again dependent on the expression level of the respective PANs, suggesting that the level of expression of these PAAD family members is a critical determinant of their function. Also, although an interaction of pyrin or cryopyrin/PYPAF1/NALP3 with ASC has been reported (37, 40), we were unable to detect association of ASC with NAC/DEFCAP/CARD7/NALP1 or PAN1 (our unpublished data), possibly explaining our observations. However, because others have suggested that ASC can bind NAC/DEFCAP/CARD7/NALP1 via PAAD-PAAD interactions and activate caspase-1 (41), perhaps cell context, posttranslational modifications, or other factors are important determinants of whether ASC can or cannot collaborate with various other PAAD family proteins.

Multiple pathways can lead to caspase-1 activation, including those initiated by Toll family receptors, TNF family receptors, CLAN/Ipaf/CARD12, and PAN family proteins (10, 11, 35). ASC appears to be capable of modulating caspase-1 activation mediated by some of these pathways, suggesting a mechanism for cross-talk between different inflammation pathways. For example, ASC and other PAAD family proteins suppressed IL-1 $\beta$  secretion induced by LPS (which binds Toll-like receptor (TLR) 2 and TLR4) and TNF, suggesting the existence of cross-talk between these caspase-1 activation pathways. In this regard, both TLRs and TNFRs use TNFR-associated factor (TRAF) family proteins for signal transduction (36). TRAFs bind Cardiak, linking these pathways to caspase-1 activation (11, 42). Because ASC competes with Cardiak for binding to procaspase-1, we presume that ASC pulls procaspase-1 away from proteins necessary for its activation within the context of LPS and TNF stimulation, while simultaneously drawing procaspase-1 into a pathway activated by PAAD family proteins such as pyrin and cryopyrin/PYPAF1/NALP3. This behavior of overexpressed ASC on caspase-1 activation induced by alternative initiators is analogous to the recently reported role of ASC in opposing NF- $\kappa$ B activation induced by similar types of stimuli, including TLRs, TNF, and TRAFs (24, 25). We hypothesize therefore that overexpression of ASC recruits procaspase-1 into specks, sequestering it from other activators involved in TLR, TNF, and TRAF signaling. Only when ASC-interacting proteins such as cryopyrin, pyrin, and PYPAF5 become activated, however, is the pool of procaspase-1 pulled into specks triggered.

In total, at least 19 genes encoding PAAD/PYRIN/DAPIN-containing proteins are predicted to reside in the human genome (3), including several implicated in hereditary hyperinflammation syndromes, IFN responses, cancer suppression, and apoptosis induction (3, 43). Fourteen of these proteins are PAN family members, having a conserved architecture of N-terminal PAAD/PYRIN/DAPIN, followed by a nucleotide-binding fold known as the NACHT domain (39), and then variable numbers of leucine-rich repeat domains, as well as other domains in some cases. The ultimate impact of ASC interactions with other PAAD (PYRIN/DAPIN) family proteins may therefore depend on their relative ratios, where ASC can function as either an inhibitor or an activator, depending on cell context. Homotypic interactions among PAAD family proteins thus may create opportunities for protein interaction networks that link various signaling pathways and permit fine tuning of inflammatory responses. Future studies, including targeted ablation of the gene encoding ASC

in mice, will reveal the *in vivo* roles of ASC in inflammation and innate immunity.

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