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COP, a Caspase Recruitment Domain-containing Protein and Inhibitor of Caspase-1 Activation Processing*

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The production of bio-active interleukin-1β (IL-1β), a pro-inflammatory cytokine, is mediated by activated caspase-1. One of the known molecular mechanisms underlying pro-caspase-1 processing and activation involves binding of the caspase-1 prodomain to a caspase recruitment domain (CARD)-containing serine/threonine kinase known as RIP2/CARDIAK/RICK. We have identified a novel protein, COP (CARD only protein), which has a high degree of sequence identity to the caspase-1 prodomain. COP binds to both RIP2 and the caspase-1 prodomain and inhibits RIP2-induced caspase-1 oligomerization. COP inhibits caspase-1-induced IL-1β secretion as well as lipopolysaccharide-induced IL-1β secretion in transfected cells. Our data indicate that COP can regulate IL-1β secretion, implying that COP may play a role in down-regulating inflammatory responses analogous to the CARD protein ICEBERG.

Interleukin-1β (IL-1β)[1] has been implicated in a wide variety of inflammatory conditions *in vivo* (reviewed in Ref. 1). Multiple animal model experiments performed with a naturally occurring IL-1 receptor agonist, neutralizing antibodies, and soluble IL-1 receptor have demonstrated a critical role of IL-1β for inflammation (2–4). The processing of inactive pro-IL-1β into its bio-active form is absolutely dependent on caspase-1, a cysteine protease formerly known as ICE (IL-1β-converting enzyme) (5, 6). Experiments involving caspase-1 knock-out mice have provided firm evidence for an important role for this protease in pro-inflammatory responses (7–9). For example, caspase-1 knock-out mice fail to produce IL-1β and display marked resistance to endotoxic shock following challenge with high doses of lipopolysaccharide (LPS) (8).

Caspase-1 is a member of a family of homologous cysteine proteases known as caspases. These proteases are initially synthesized as inactive zymogens, becoming activated typically by proteolytic cleavage at aspartyl residues, thus generating the catalytic large and small subunits of the enzyme. Pro-caspase-1 is among the members of the caspase family of zymogens, which possess large N-terminal prodomains (10). The caspase-1 prodomain contains a protein interaction motif known as the caspase recruitment domain (CARD). CARD domains are found in several pro-caspases and also in multiple proteins that regulate these proteases either by inducing or suppressing their activation (11–18).

The molecular mechanisms responsible for pro-caspase-1 processing remain largely enigmatic. However, a CARD-containing serine/threonine kinase, RIP2/CARDIAK/RICK, has been described that can bind to the CARD of pro-caspase-1 and trigger caspase-1 proteolytic processing and activation (19), probably by the "induced proximity" mechanism (20). The CARD of RIP2 is required for its interaction with and activation of pro-caspase-1 involving heterotypic CARD-CARD interactions between these proteins (19).

Recently, an inhibitor of pro-caspase-1 activation has been described, termed ICEBERG, which consists essentially only of a CARD (21). The CARD of ICEBERG has considerable homology to the caspase-1 prodomain and binds to the corresponding CARD motif present in both pro-caspase-1 and RIP2. ICEBERG functions as a competitive antagonist of RIP2/pro-caspase-1 interactions and inhibits caspase-1-dependent pro-IL-1β secretion in mononuclear cells (21).

Using bioinformatics approaches, we have identified a human gene that encodes a novel CARD-containing protein, COP (CARD-only protein). Similar to ICEBERG, the COP protein is relatively short (97 amino acids), composed essentially of only a CARD that shares impressive sequence identity with the CARD of pro-caspase-1. COP binds to both pro-caspase-1 and RIP2, inhibiting caspase-1-induced IL-1β secretion.

MATERIALS AND METHODS

Data Base Search and Plasmid Construction—A *COP* cDNA was identified by searching the expressed sequence tag (EST) data bases of GenBank™ using the nucleotide sequence of the caspase-1 prodomain as a query with the BLASTn program. An EST (GenBank™ accession number AA070591) was found to have a high degree of sequence identity to the prodomain of caspase-1. The corresponding EST clone was obtained from IMAGE Consortium (Washington University School of Medicine, St. Louis, MO). The entire open reading frame of *COP* was amplified by PCR using a set of primers, 5′-CCAGAATTCATGGCCGACAAGGTCCTGAAG-3′ (forward) and 5′-CCACTCGAGCTAATTTCCAGGTATCGGACC-3′ (reverse). The PCR product was digested with *Eco*RI-*Xho*I and ligated into mammalian expression vectors pcDNA3-Myc, pcDNA3-HA, and pcDNA3-Flag at *Eco*RI-*Xho*I cloning sites. Plasmids encoding wild-type pro-caspase-1, RIP2, and pro-IL-1β have been described (19, 22, 23). A pro-caspase-1 C285A mutant was made from wild-type caspase-1 plasmid by site-directed mutagenesis using a commercially available kit (Stratagene, La Jolla, CA) and the primers 5′-GATCATCATCCAGGCCGCCCGTGGTGACAGCCCTGG-3-′ and 5′-CCAGGGCTGTCACCACGGGCGGCCTGGATGATGATC-3′. A truncation mutant of pro-caspase-1 in which a stop codon was introduced downstream of the CARD was created by PCR using primers

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[1] The abbreviations used are: IL-1β, interleukin-1β; CARD, caspase recruit domain; COP, CARD-only protein; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair(s); LPS, lipopolysaccharide; NF-κB, nuclear factor κB; TLR4, Toll-like receptor 4; HA, hemagglutinin.

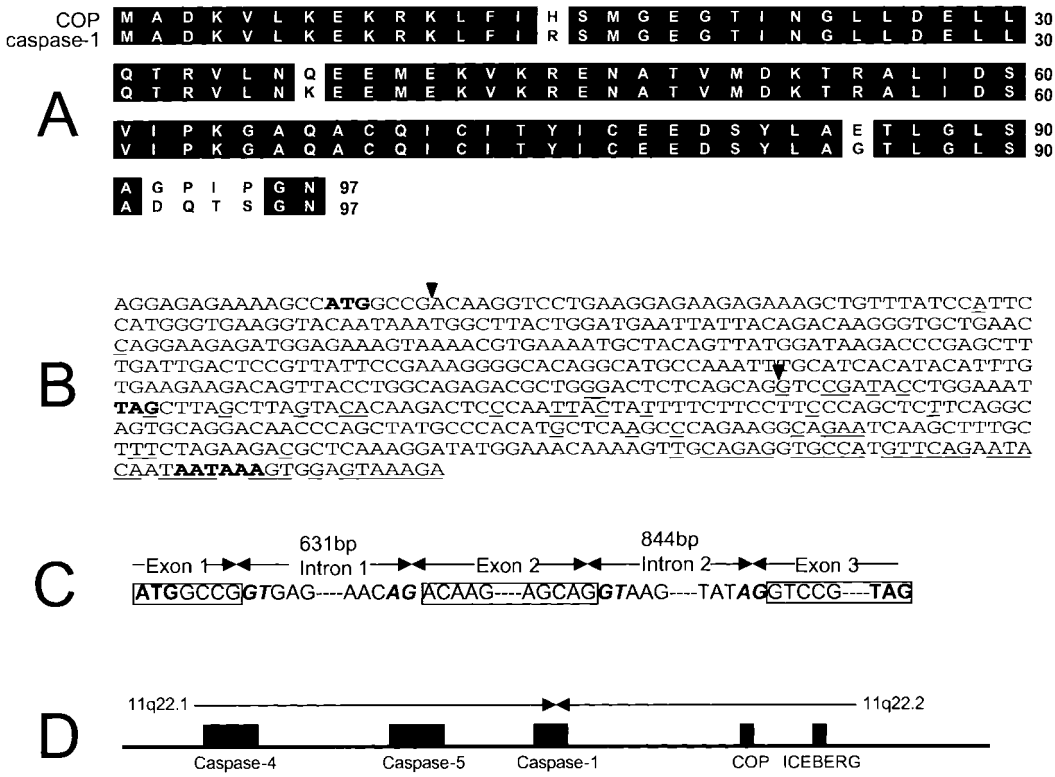

FIG. 1. ***COP* gene, transcripts and protein.** A, an amino acid sequence alignment of COP and the first 97 residues of pro-caspase-1 is presented. *Black* and *white boxes* indicate the identical and nonidentical amino acids, respectively. Residue *position numbers* are indicated at the *right*. *B*, the nucleotide sequence of the COP cDNA is presented. The start codon, stop codon, and polyadenylation signal sequence are indicated in *bold letters*. The positions of the intron/exon borders are indicated by *inverted triangles*. Nucleotide sequences that are different from those of pro-caspase-1 are *underlined*. *C*, the structure of the *COP* gene is indicated, showing the intron/exon borders. Consensus splice donor (*GT*) and acceptor (*AG*) motifs are shown in *italic letters*. The start and stop codons are shown in *bold letters*. The length of the introns in base pairs is indicated. *D*, genomic organization of *CASPASE-4*, *CASPASE-5*, *CASPASE-1*, *COP*, and *ICEBERG* genes on human chromosome 11q.

5′-CGGAATTCATGGCCGACAAGGTCCTG-3′ and CGCTCGAGTTAG-TCTTGCATATTAAGGTAATTTCCAGA-3′.

Co-immunoprecipitations and Immunoblotting Assays—Human embryonic kidney 293T cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum. Cells in log phase were transfected in 60-mm-diameter dishes with expression plasmids (5 μg total DNA) using Superfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Cells were harvested 2 days later and lysed in ice-cold Nonidet P-40 lysis buffer (10 mM HEPES (pH 7.4), 142.5 mM KCl, 0.2% Nonidet P-40, 5 mM EGTA) supplemented with 1 mM dithiothreitol, 12.5 mM β-glycerophosphate, 1 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mix (Roche Molecular Biochemicals). Cell lysates (0.5 ml) were clarified by centrifugation at 16,000 × *g* for 5 min and subjected to immunoprecipitation using specific antibodies, including anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Flag antibodies (Sigma), in combination with 15 μl of protein A- or G-Sepharose (Zymed Laboratories Inc., South San Francisco, CA). Immunocomplexes were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The resulting blots were incubated with various antibodies, including anti-HA (1:1000 v/v, Roche Molecular Biochemicals), anti-Myc (1: 100 v/v, Santa Cruz Biotechnology), and anti-Flag antibodies (1:1000 v/v, Sigma), followed by horseradish peroxidase-conjugated secondary antibodies and detection by an enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech). Alternatively, lysates were analyzed directly by immunoblotting after normalization for total protein content.

RNA Blotting and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)—Blots containing poly(A)-selected mRNA from various adult tissues (CLONTECH, Palo Alto, CA) were hybridized using a ^{32}P-labeled *COP* cDNA probe. The probe represented a 570-base pair long cDNA containing portions of the 5′-untranslated region, the complete open reading frame, and portions of the 3′-untranslated region of *COP*. The *COP* probe (GenBank™ accession number AA070591) was excised from the plasmid by restriction digestion with *Eco*RI and *Xho*I, gel-purified, and radiolabeled by the random priming method using [α-^{32}P]dCTP and a kit from Ambion (Austin, TX). After hybridization, heat-denatured probe was annealed for 1 h at 68 °C with QuickHyb hybridization solution (Stratagene), and then blots were washed with solutions containing 2× SSC, 0.1% (w/v) SDS (twice each for 15 min at 25 °C) followed by 0.1× SSC, 0.1% (w/v) SDS (twice for 10 min at 40 °C). Bands were visualized by autoradiography.

For RT-PCR analysis of *COP* mRNA, cDNA samples derived from multiple human adult tissues (CLONTECH) were amplified using a set of COP-specific primers (a forward primer, 5′-GAAGACAGTTACCTG-GCAGA-3′, and a reverse primer, 5′-TTGTATTCTGAACATGGCACC-3′). The resulting PCR products were size-fractionated by electrophoresis in 1.5% agarose gels and then stained with ethidium bromide for UV photography. In some cases, bands were excised from gels, purified, and sequenced, thus verifying amplification of the correct product by this RT-PCR assay.

Assay for Pro-IL-1β Secretion—COS-7, 293T, or 293HEK cells were co-transfected in 12-well (22-mm diameter) plates using LipofectAMINE Plus reagent (Life Technologies, Inc.) with plasmids encoding mouse pro-IL-1β, human caspase-1, RIP2, or COP in various amounts (total DNA = 2.0 μg). Histiocytoma cells (human fibrosarcoma cell line) were transfected in 12-well plates using Superfect transfection reagent (Qiagen) with plasmids encoding Toll-like receptor 4 (TLR4) and/or COP in various amounts (total DNA = 1.5 μg). After transfection, the cells were incubated for 18 h with 10 μg/ml LPS from *E. coli,* serotype 0111:B4 (Alexis Biochemicals, San Diego, CA). At 2 days after transfection, supernatants were collected and stored at −80 °C or used immediately for an IL-1β ELISA according to the manufacturer's protocol (R&D systems, Minneapolis, MN).

RESULTS

Identification of COP—Using the amino acid sequence of the caspase-1 prodomain as a query for BLASTn searches of the public data bases, we found a human EST clone (GenBank accession number AA070591) containing an open reading frame encoding a 97-amino acid protein predicted to share 92% sequence identity with the CARD of pro-caspase-1 (Fig. 1*A*).

The CARD region of COP (91 amino acids from its first amino acid) showed 97% identity to the CARD of pro-caspase-1. To confirm these results, cDNAs were amplified using specific primers from various adult human tissues and sequenced, revealing the same nucleotide sequence as the original EST, which shares 97% sequence identity at the nucleotide level with the prodomain region of pro-caspase-1 (Fig. 1*B*). The start codon initiating the open reading frame in these cDNA clones resides in a favorable context for translation (24) and is preceded by an in-frame stop codon. The 3′-untranslated region contains TAAA and TATA motifs, typical of short-lived mRNAs that are subject to post-transcriptional regulation (25), and a candidate polyadenylation signal sequence (AATAAA). The predicted protein contains a CARD (residues 1–91), which is followed by six amino acids and then a stop codon. Thus, this protein contains essentially only a CARD, prompting the moniker CARD-only protein (or COP).

To determine the genomic organization of the *COP* gene, the COP cDNA nucleotide sequence was employed for searches of the High Throughput Genomic Sequence (HTGS) data base, resulting in identification of three genomic clones containing the *COP* gene (GenBank™ accessions numbers AC027011, AP001153, and AP002787). Comparison of the COP cDNA and genomic DNA sequences suggests a three exon structure, in which only the first two amino acids are encoded in exon 1 and only the last five residues are encoded in exon 3, such that most of the coding regions (including the entire CARD) are derived from exon 2. The introns separating exons 1, 2, and 3 are 631 and 844 base pairs in length, respectively, containing consensus dinucleotide splice donor (GT) and splice acceptor (AG) motifs (Fig. 1*C*).

The *COP* genomic clones identified in the HTGS data base have been mapped to human chromosome 11q22, which is the same chromosomal region where the *PRO-CASPASE-1* gene resides, as well as *PRO-CASPASE-4, PRO-CASPASE-5,* and *ICEBERG*. To address the genomic localization of *COP, PRO-CASPASE-4, PRO-CASPASE-5,* and *ICEBERG* genes in chromosome 11, we searched the public data base of Human Genome Project Working Draft (genome.cse.ucsc.edu) and found that the order of these genes from centromere to telomere may be *pro-caspase-4, pro-caspase-5, pro-caspase-1, COP,* and *ICEBERG* (Fig. 1*D*). This result also suggested that *COP* is a separate gene, presumably arising from duplication of other homologous genes in this locus.

Tissue Expression of COP—To study the expression of *COP*, Northern blot analysis was performed using RNA derived from several adult human tissues and a 0.57-kbp ^{32}P-labeled *COP* cDNA probe. Hybridizing bands of ~0.6, 1.5, and 2.6 kbp were identified, with the 0.6-kbp band representing the most abundant of these transcripts and presumably corresponding to the fully spliced *COP* mRNA. The less abundant larger 1.5- and 2.6-kbp transcripts could represent unspliced precursors. However, based on previous reports (6), the 2.6-kbp mRNA also potentially could represent *pro-caspase-1* mRNA resulting from probe cross-hybridization because of the 87% nucleotide sequence homology shared between the *COP* cDNA probe and *pro-caspase-1*.

The 0.6-kbp *COP* mRNA was most abundant in spleen, followed by liver, placenta, and peripheral blood leukocytes. However, most tissues contain at least some detectable 0.6-kbp *COP* mRNA (Fig. 2*A*).

To corroborate the Northern blot analysis, we also analyzed *COP* mRNA expression in adult human tissues by RT-PCR methods using *COP*-specific primers. Successful amplification of *COP* was confirmed by DNA sequencing of PCR products (not shown). The RT-PCR analysis showed that *COP* mRNA is

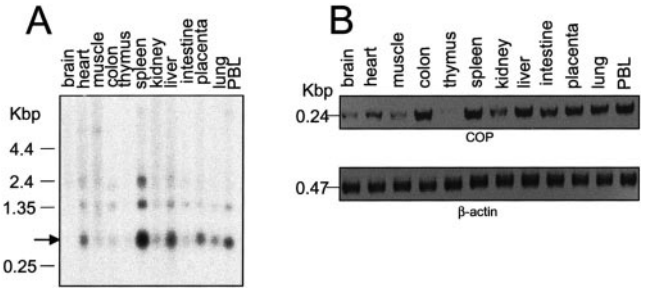

FIG. 2. **Tissue distribution of COP expression.** The expression of *COP* mRNA in human adult tissues was determined by Northern blot analysis (*A*) and RT-PCR (*B*). *A*, the *arrow* indicates the presumed fully processed *COP* mRNA of ~0.6 kbp in length. Molecular weight markers are indicated to the *left* in kilobase pairs (kbp). *PBL* represents mRNA from peripheral blood leukocytes. *B*, cDNAs were amplified using specific primers for COP or β-actin, and the resulting PCR products ~0.24 and ~ 0.47 kbp long, respectively, were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of the *COP* PCR product was confirmed by DNA sequencing (not shown).

expressed in all tissues analyzed except thymus (Fig. 2*B*). Parallel RT-PCR analysis of β-actin mRNA served as a control. In general, the relative levels of *COP* mRNA detected by RT-PCR were in agreement with the Northern blot data (Fig. 2, *A* and *B*).

COP Binds to Caspase-1, RIP2, and Itself—The prodomain of pro-caspase-1 is required for dimerization and activation of this zymogen (26). Because the prodomain of COP shares a high degree of amino acid sequence identity with the prodomain of caspase-1 (Fig. 1), we tested the possibility that COP interacts with pro-caspase-1 in co-immunoprecipitation assays. Interactions with several other CARD-containing proteins were also tested, including COP itself, RIP2, Bcl-10, cIAP1, cIAP2, and pro-caspase-9. For these experiments, 293T cells were transiently transfected with expression plasmids encoding either HA- or Myc-tagged COP in combination with various other expression plasmids producing either Myc- or FLAG-tagged CARD proteins. Immunoprecipitations were then performed with anti-Myc antibody, and the resulting immunocomplexes were analyzed by SDS-PAGE/immunoblotting using anti-HA or anti-FLAG antibody. Lysates derived from these transfected cells were also analyzed directly by immunoblotting as a control to verify production of each protein. Fig. 3 shows selected representative results.

HA-COP co-immunoprecipitated with Myc-COP, indicating that this protein can self-associate (Fig. 3*A*). In addition, HA-COP co-immunoprecipitated with Myc-tagged pro-caspase-1 (C285A mutant) as well as with a fragment of pro-caspase-1 containing only its CARD-carrying prodomain (Fig. 3, *B* and *C*). Thus, COP binds pro-caspase-1 through its CARD domain. Note that the active site cysteine of pro-caspase-1 was mutated for these co-immunoprecipitation experiments to avoid induction of apoptosis, which can occur when this protease is overexpressed. Finally, Myc-COP co-immunoprecipitated with Flag-RIP2. In contrast, COP did not co-immunoprecipitate with the CARD-containing proteins Bcl-10, cIAP1, cIAP2, or pro-caspase-9 (Fig. 3*E* and data not shown), thus demonstrating the specificity of these results.

COP Inhibits RIP2-mediated Oligomerization of Pro-caspase-1—RIP2 has been shown to bind and activate caspase-1 through the interaction of their CARDs, resulting in oligomerization of pro-caspase-1 and its activation via the induced proximity mechanism (19, 20). The data demonstrating that COP binds to both pro-caspase-1 and RIP2 therefore led us to hypothesize that COP might function as a modulator of RIP2-induced pro-caspase-1 oligomerization. We therefore per-

formed experiments in which 293T cells were transiently transfected with expression plasmids encoding Myc-tagged pro-caspase-1 (C285A mutant) and HA-tagged pro-caspase-1 (C285A mutant), with or without Flag-tagged RIP2 and COP, and then monitored Myc-pro-caspase-1 and HA-pro-caspase-1 association by co-immunoprecipitation assays. As determined by this co-immunoprecipitation assay, pro-caspase-1 self-associated, and this was enhanced by co-expression of RIP2 (Fig. 4*A*). However, when COP was also co-expressed, this RIP2-mediated effect on pro-caspase-1 self-association was negated (Fig. 4*A*).

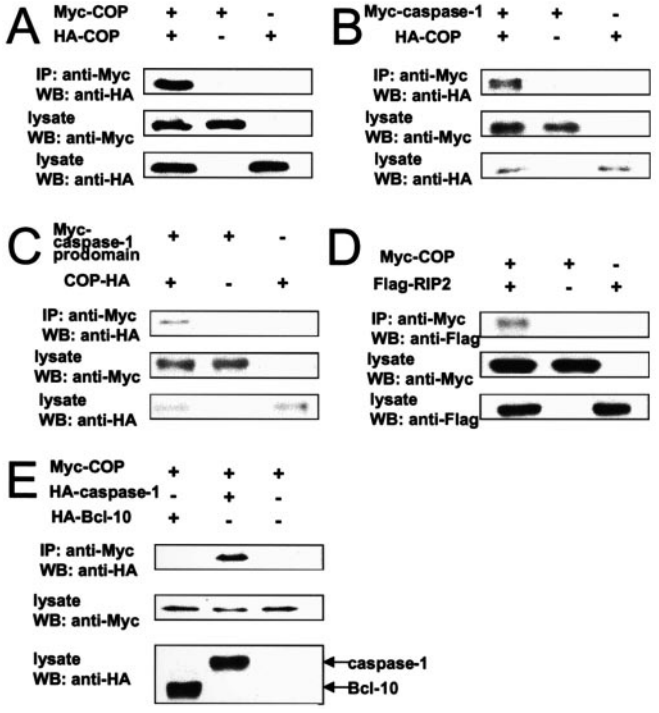

FIG. 3. **Interactions of COP protein with other CARD-containing proteins.** Co-immunoprecipitation assays were performed using lysates (normalized for total protein content) from 293T cells that had been transiently transfected with plasmids encoding various epitope-tagged proteins as indicated, including Myc-COP, HA-COP, Myc-pro-caspase-1(C285A), Myc-pro-caspase-1 prodomain (residues 1–102), Flag-RIP2, and HA-Bcl-10. Immunoprecipitates were prepared using anti-Myc antibody adsorbed to protein G-Sepharose and analyzed by SDS-PAGE/immunoblotting using anti-HA or anti-Flag antibodies with ECL-based detection. Aliquots of the same lysates (normalized for total protein content) were also analyzed directly by SDS-PAGE/immunoblotting as indicated. *IP*, immunoprecipitation; *WB*, Western blotting.

These findings suggested the possibility of a competitive mechanism in which COP competes with RIP2 for binding to pro-caspase-1. To test this hypothesis, therefore, transfection experiments were preformed in which Flag-RIP2 and Myc-tagged pro-caspase-1 (C285A mutant) were expressed in 293T cells in the presence of increasing amounts of HA-tagged COP. The effects of COP on association of RIP2 with pro-caspase-1 were then evaluated by co-immunoprecipitation assays in which immunoprecipitations were performed using anti-Flag antibody to recover Flag-RIP2 protein; the resulting immune-complexes were analyzed by SDS-PAGE/immunoblotting using anti-Myc antibody to detect associated Myc-pro-caspase-1. As shown in Fig. 4*B*, COP inhibited association of pro-caspase-1 with RIP2 in a dose-dependent manner. Immunoblot analysis of lysates from these same cells demonstrated that COP did not affect the total levels of pro-caspase-1 or RIP2 but rather just their association. These results therefore confirm that COP can interfere with binding of pro-caspase-1 to RIP2.

COP Inhibits Caspase-1-mediated Activation of Pro-IL-1β—Active caspase-1 cleaves pro-IL-1β, resulting in the generation of bioactive IL-1β, which is secreted from cells (6). We hypothesized that COP could suppress caspase-1-induced pro-IL-1β processing and thus reduce secretion of IL-1β. To test this idea, a transient transfection assay was employed in which a mouse pro-IL-1β expression plasmid was co-transfected into COS-7, 293T, or 293HEK cells together with plasmids encoding wild-type human pro-caspase-1 alone or in combination with RIP2 and COP. At 2 days after transfection, secretion of mature murine IL-1β into the culture medium was quantified by ELISA.

As shown in Fig. 5, co-expression of pro-caspase-1 and pro-IL-1β in COS-7 cells resulted in secretion of mature IL-1β ranging from 80 to 250 pg/ml, which was proportional to the amount of pro-caspase-1 plasmid used. This IL-1β secretion was enhanced by co-expression of RIP2 plasmid. In contrast, expression of COP together with pro-caspase-1, pro-IL-1β, and RIP2 resulted in a dose-dependent decrease in the amount of mature IL-1β secretion proportional to the amount of COP-encoding plasmid used. Similarly, in 293T and 293HEK cells, COP inhibited IL-1β secretion from cells transfected with pro-caspase-1 and RIP2 (data not shown).

We also tested whether COP inhibits IL-1β secretion in the absence of RIP2 over-expression. For these experiments, plasmids encoding COP and pro-caspase-1 were co-transfected at various ratios. IL-1β secretion was compared with that of cells transfected with pro-caspase-1 alone (Fig. 5). Transfection of COP resulted in a dose-dependent decrease of IL-1β secretion.

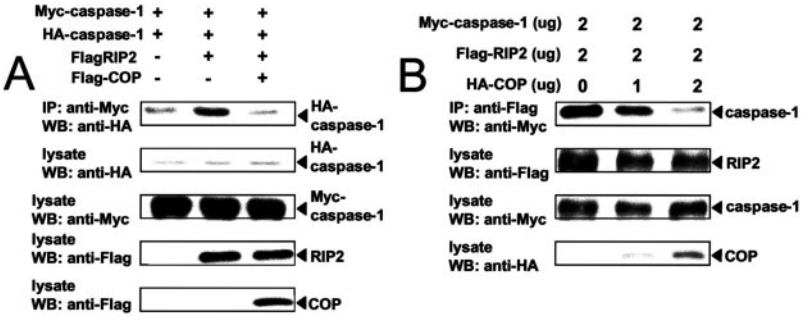

FIG. 4. **COP inhibits RIP2-mediated caspase-1 oligomerization.** *A*, 293T cells (~10^6 cells in 60-mm diameter dishes) were co-transfected with plasmids encoding Myc-pro-caspase-1 mutant (C285A, 1.5 μg) and HA-pro-caspase-1 mutant (C285A, 1.5 μg) together with: *left*, empty pcDNA3 DNA (3 μg); *middle*, pcDNA3 plasmids encoding Flag-tagged RIP2 (1.5 μg) and empty pcDNA3 (1.5 μg); or *right*, Flag-RIP2 (1.5 μg) and Flag-COP (1.5 μg). After transfection, cell lysates were normalized for total protein content and then employed for co-immunoprecipitation assay or direct immunoblot analysis as described for Fig. 3. *B*, 293T cells were co-transfected as described in *A* with plasmids encoding Myc-pro-caspase-1 mutant (C285A, 2 μg) and Flag-RIP2 (2 μg) together with increasing amounts of HA-COP plasmid (0, 1, and 2 μg). After transfection, cell lysates (normalized for total protein content) were employed for co-immunoprecipitation assays or loaded directly in gels for SDS-PAGE/immunoblot analysis. *IP*, immunoprecipitation; *WB*, Western blotting.

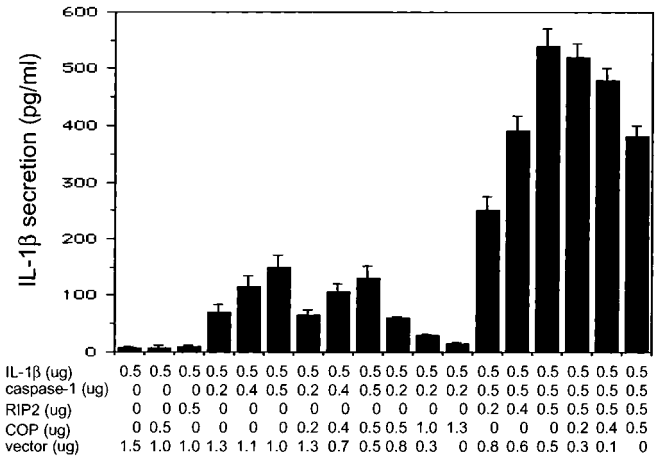

FIG. 5. **COP reduces IL-1β secretion.** COS-7 cells were co-transfected with plasmids encoding mouse pro-IL-1β in combination with various amounts of various expression plasmids encoding pro-caspase-1, RIP2, or COP as indicated (total DNA normalized to 2 μg with empty pcDNA3 DNA (*Vector*)). After 24 h, supernatants were collected and subjected to ELISA for IL-1β. Data represent the mean ± S.D. (n = 3).

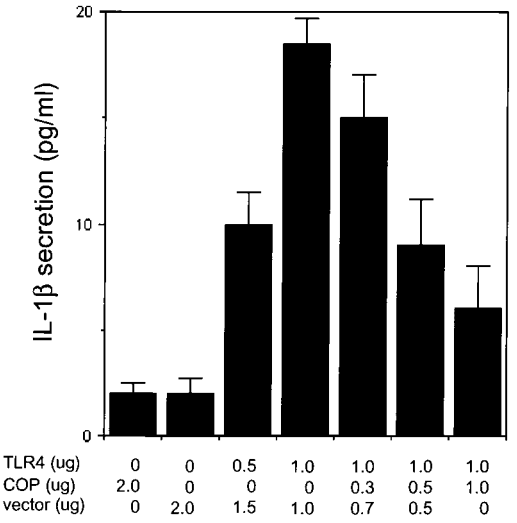

FIG. 6. **COP reduces LPS-induced IL-1β secretion.** HT1080 cells were transfected with plasmids encoding COP and TLR4 (total DNA normalized to 2 μg with empty pcDNA3 DNA (*Vector*)) followed by LPS treatment (10 μg/ml) after 18 h. After 48 h, supernatants were collected and subjected to ELISA for IL-1β. Data represent the mean ± S.D. (n = 3).

We infer therefore that COP is capable of suppressing the caspase-1-mediated secretion of IL-1β. The amounts of pro-IL-1β protein processed by caspase-1, however, were too small to be detected by immunoblotting, consistent with previous reports (6).

To explore whether COP is capable of suppressing IL-1β production induced by a physiological pathway, we transfected HT1080 cells with TLR4, a receptor for LPS that is known to trigger caspase-1 activation, resulting in IL-1β secretion (27). As shown in Fig. 6, TLR4 over-expression followed by LPS treatment resulted in mature IL-1β secretion from HT1080 cells, ranging from 10 to 20 pg/ml. However, co-expression of COP resulted in dose-dependent decreases in LPS-induced IL-1β secretion.

DISCUSSION

We have described a new member of the human CARD-containing family of proteins, which we have termed COP. This presents the 16th human CARD protein described in the literature (excluding CARD-containing caspases) in addition to APAF1, NOD1 (CARD4), NOD2, NAC (DEPCAP), RAIDD (CRAIDD), RIP2 (CARDIAK; RICK), ARC (NOP), ASC, BCL-10 (huE10), ICEBERG, Ipaf1(CLAN), cIAP1, cIAP2, CARD9, CARD11, CARD12, and CARD13, and thus we also propose the alternative designation, CARD16, for this protein. COP (CARD16) consists essentially of only a CARD, having only 91 residues upstream and 6 residues downstream of this protein interaction domain. The CARD of COP shares 97% amino acid identity with the corresponding domain in pro-caspase-1. COP interacts with the prodomain of pro-caspase-1 and also with RIP2, a protein previously demonstrated to bind the prodomain of pro-caspase-1. COP seems to compete with RIP2 for binding to pro-caspase-1, thereby inhibiting RIP2-mediated caspase-1 oligomerization. Consequently, COP interferes with the ability of RIP2 to enhance caspase-1-induced secretion of mature IL-1β. Moreover, COP diminishes IL-1β secretion induced by LPS stimulation of TLR4, demonstrating that COP can regulate at least one pathway known to be physiologically important in pathogen responses.

ICEBERG, another protein containing a CARD with striking homology to the prodomain of pro-caspase-1, shares the same mechanism of action in inhibiting pro-IL-1β processing induced by caspase-1 (21). In this regard, COP together with ICEBERG is likely to control IL-1β generation and thereby oppose IL-1β-induced inflammation. Interestingly, the genes encoding both COP and ICEBERG map to human chromosome 11q22 where the genes for caspases involved in inflammation (caspases 1, 4 and 5) also reside (28). Thus, chromosome 11q22 contains a cluster of pro-inflammatory caspase genes and their inhibitors, which probably arose from exon and gene duplication events during evolution.

Northern blot and RT-PCR assays revealed that *COP* is widely expressed in human tissues. Similarly, *PRO-CASPASE-1* mRNA is known to be expressed in multiple tissues, implying that *COP* and *pro-caspase-1* may be constitutively co-expressed under normal conditions. Consequently, COP may be present as a counter-measure to pro-caspase-1, ensuring against adventitial activation of the pro-inflammatory caspase. It remains to be determined, however, whether the expression of COP increases in response to inflammatory signals, analogous recent evidence implies that levels of ICEBERG become elevated as a relatively late event following cytokine signal transduction, presumably for the purpose of down-regulating inflammatory responses (21).

Several CARD-containing proteins are capable of regulating NF-κB induction, in addition to or instead of caspases, including NOD1, NOD2, CARDIAK, BCL-10, CARD-9, CARD-11, CARD-13, cIAP1, and cIAP2 (11–20). Although we observed that over-expression of COP can trigger NF-κB induction (not shown), the levels of NF-κB activity produced were only ~10-fold above background and thus not of the same magnitude as that seen under the same experimental circumstances using physiological activators of NF-κB such as TRAFs, TNFR1, and Toll-like receptors (29). We therefore speculate that NF-κB regulation is not a major function of COP. Similarly, although several CARD-containing proteins can either induce or suppress apoptosis (18, 28), we failed to find a significant role for COP in apoptosis regulation in pilot experiments (data not shown). These observations thus argue that the predominant role for COP is likely to be manifested in its capacity to function as a negative regulator of pro-caspase-1 activation.

IL-1β plays a critical role in septic shock (1), which currently represents the most common cause of lethality in patients treated in the intensive care setting (30). Thus, maintenance of IL-1β homeostasis to prevent systemic inflammatory reactions

is important for survival when challenged with Gram-negative bacteria or other inflammatory insults. It is perhaps not surprising therefore that several endogenous inhibitors of IL-1β have been identified, including IL-1 receptor antagonist, a soluble IL-1β-binding protein, ICEBERG, and now COP (2–4). Further understanding of the mechanisms that control the expression and function of these caspase-1 and IL-1β antagonists may therefore reveal new strategies for ameliorating inflammatory diseases and preventing lethal septic shock.

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