INCA, a Novel Human Caspase Recruitment Domain Protein That Inhibits Interleukin-1β Generation*

Received for publication, July 13, 2004, and in revised form, September 16, 2004
Published, JBC Papers in Press, September 21, 2004, DOI 10.1074/jbc.M407891200

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Using in silico methods for screening the human genome for new caspase recruitment domain (CARD) proteins, we have identified INCA (Inhibitory CARD) as a protein that shares 81% identity with the prodomain of caspase-1. The INCA gene is located on chromosome 11q22 between the genes of COP/Pseudo-ICE and ICEBERG, two other CARD proteins that arose from caspase-1 gene duplications. We show that INCA mRNA is expressed in many tissues. INCA is specifically up-regulated by interferon-γ in the monocyte cell lines THP-1 and U937. INCA physically interacts with procaspase-1 and blocks the release of mature IL-1β from LPS-stimulated macrophages. Unlike COP/Pseudo-ICE and procaspase-1, INCA does not interact with RIP2 and does not induce NF-κB activation. Our data show that INCA is a novel intracellular regulator of procaspase-1 activation, involved in the regulation of pro-IL-1β processing and its release during inflammation.

Caspases are an evolutionarily conserved family of aspartate-specific cysteine-dependent proteases that are synthesized as pro-enzymes with a p20 and a p10 domain. Upon binding to the appropriate platform, these domains undergo a conformational change resulting in the formation of the active enzyme (1). Caspases are implicated in signaling to apoptosis and inflammation (2). For example, the processing of inactive pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into biologically active forms requires the activation of caspase-1 (3), and macrophages from caspase-1-deficient mice are incapable of producing mature IL-1β and IL-18 in response to lipopolysaccharide (LPS) treatment (4, 5). Although caspase-1 is mainly known for the maturation of pro-IL-1β and pro-IL-18, the marked resistance of caspase-1-deficient animals to endotoxin shock induced by high doses of LPS (4, 5) cannot be strictly due to a failure in the maturation of these cytokines. Mice deficient in IL-1β display no resistance to LPS-induced endotoxin shock (6, 7) and IL-18 knockout mice are hypersensitive to endotoxin shock (8). Additional caspase-1-mediated functions, such as the recently described caspase-1 CARD-dependent activation of NF-κB (9), may therefore help explaining the phenotype of caspase-1 knockout mice.

Nevertheless, pro-IL-1β and pro-IL-18 play major roles in a wide variety of inflammatory conditions in vivo (10). The protein complexes in which procaspase-1 is activated and lead to the proteolytic maturation of these cytokines have recently been identified as "inflammasomes" (11, 12). The molecular composition of the inflammasome complex depends on the identity of the members of the NALP protein family serving as scaffold proteins in the complex. For example, the NALP1 inflammasome uses the adaptor protein ASC/PYCARD to recruit procaspases-1 and -5 (12). In contrast to the NALP1 inflammasome, procaspase-5 is not part of the NALP2 and NALP3 inflammasomes (11). Except for NALP2 and -3, all components of the inflammasomes possess an oligomerization domain called “caspase recruitment domain” or CARD (13).

This oligomerization domain is also found in several procaspases and in multiple proteins involved in the assembly of large protein platforms that promote the recruitment and proteolytic activation of caspases in the context of apoptosis and inflammation (14, 15). ICEBERG and COP/Pseudo-ICE are two human CARD-only proteins that share a high degree of sequence homology with the prodomain of procaspase-1, reaching 93 and 73%, respectively (16–18). Both ICEBERG and COP/Pseudo-ICE are encoded by caspase-like genes that have acquired premature nonsense mutations leading to the production of essentially CARD-only molecules. These genes map to chromosome 11q22, adjacent to the caspase-1 gene and have probably arisen by recent gene duplication events. Both ICEBERG and COP/Pseudo-ICE bind to and prevent procaspase-1 activation and the subsequent generation of IL-1β (16–18). However, in contrast to ICEBERG, COP/Pseudo-ICE also interacts with RIP2 in a CARD-CARD-dependent manner and activates the transcription factor NF-κB (9, 16).

Using bioinformatic approaches, we have identified a novel human gene that encodes a CARD-containing protein, which we termed INCA (INhibitory CARD). Similar to ICEBERG and COP/Pseudo-ICE, the INCA protein is relatively...
short (110 amino acids), composed essentially of only a CARD domain that shares 81% sequence identity with the prodomain of procaspase-1. We demonstrate that INCA binds to procaspase-1 and inhibits LPS-induced release of IL-1β from macrophages. In contrast to COP/Pseudo-ICE and procaspase-1, INCA does not bind to RIP2 and does not induce NF-κB activation. Moreover, INCA does not prevent NF-κB activation by either COP/Pseudo-ICE or the prodomain of procaspase-1. All together, INCA is a newly identified intracellular regulator of procaspase-1 activation implicated in the regulation of the proteolytic maturation of pro-IL-1β and its release during inflammation.

**EXPERIMENTAL PROCEDURES**

*Isolation of INCA cDNA—* The INCA gene was found by searching the GenBank™ High Throughput Genomic Sequence data base for sequences similar to the prodomain of procaspase-1, using the BLASTn program. The hypothetical cDNA sequence coding for INCA was assembled using several bioinformatics programs. The cDNA coding for INCA was amplified by RT-PCR from different human tissues and cell lines using the forward primer 5'-CGAGGGAGCT-3' and the reverse primer 5'-TGAACCTTCGAGAACTTGGAAATCTTTAGG-3'. The obtained cDNAs were cloned into pCAGGS and sequenced, confirming the in silico prediction.

**RNA Isolation and RT-PCR—** HeLa cells and the human monocytic cell lines U937 and THP-1 were cultured according to the supplier’s instructions. THP-1 cells were seeded at 4 x 10⁶ cells/ml medium and U937 cells at 2 x 10⁶ cells/ml medium in a six-well plate. After 36 h, the cells were either left untreated or stimulated with LPS (1 μg/ml), human TNF-α (1000 IU/ml), human IFN-γ (1000 IU/ml), or combinations of these stimuli for an additional 12 h. Total RNA was isolated from cells with the RNeasy isolation kit (Qiagen). First strand cDNA libraries were made according to the instructions for the SuperScript PreAmplication system (Invitrogen). Levels of RNA were normalized using glyceraldehyde 3-phosphate dehydrogenase as an exogenous control primers. For RT-PCR analysis of INCA mRNA, cDNA samples derived from multiple human adult tissues (OriGene Technologies, Rockville, MD) were amplified using INCA-specific primers 5’-GGATCTTAGCATGGCCACAAGCTCGTGAAGGG-3’ (INCA-forward) and 5’-TGAACCTTCGAGAACTTGGAAATCTTTAGG-3’ (INCA-reverse). The resultant PCR products were size-fractionated by electrophoresis in 1.5% agarose gels, and then stained with ethidium bromide for UV photography. To verify the identity of the amplified product, the amplified band was excised from gels, purified, and sequenced.

**Expression Plasmids—** pNF-conLuc, encoding the luciferase reporter gene driven by a minimal NF-κB responsive promoter, was a gift from Dr. A. Israel (Institut Pasteur, Paris, France). pCAGGS-FLAG-COP/Pseudo-ICE, encoding β-galactosidase, was from Eugerontec (Seraing, Belgium). The plasmid encoding a dominant negative I KK-β was a gift from Dr. J. Schmid (University of Vienna, Vienna, Austria). Plasmids encoding TT-epitope-tagged COP/Pseudo-ICE and ICEBERG have been described previously (16) and were kindly provided by Dr. E. S. Alnemri (Thomas Jefferson University, Philadelphia, PA). The cDNAs for FLAG-epitope-tagged COP/Pseudo-ICE and INCA were generated by PCR and cloned into the pEFbneo retroviral vector (Stratagene, La Jolla, CA). The PCR-generated cDNAs encoding human RIP2, COP/Pseudo-ICE, ICEBERG, human caspase-2 CARD, and the enzymatically inactive human procaspase-1 C285A mutant were all cloned in-frame with the E-epitope tag of the pCAGGS-E vector. All the PCR products described above were checked by sequencing to ensure that no errors had been introduced by PCR.

**Transfection, Co-immunoprecipitation, and Immunoblotting Assay—** 293T cells were transduced using the calcium phosphate precipitation method (19). Cells were seeded the day before transfection at 2 x 10⁶ cells/well. Cells were transfected for 4 h, washed, and incubated for another 24 h, before lysis was prepared by harvesting the cells and lysing them in a buffer containing 1% Nonidet P-40, 10 mM Tris-cl, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 10% glycerol for 30 min at 4 °C. Cell lysates (0.5 ml) were clarified by centrifugation at 14,000 × g for 5 min and subjected to immunoprecipitation using anti-FLAG antibodies (Sigma) in combination with 15 μl of protein G-Sepharose beads, prewashed with lysis buffer containing 5% bovine serum albumin. Immunocomplexes were fractionated by SDS-PAGE and transferred to nitrocellulose mem-

**RESULTS**

**Identification of INCA—** To identify new CARD-containing proteins, we searched the GenBank™ High Throughput Genomic Sequence data base for sequences that share significant homology with the prodomain of human caspase-1 (residues 1–106). Using this approach, four different genomic clones (GenBank™ accession numbers AF020787, AC027011, AF001024, and AC021452) containing a new CARD-containing gene were found. The identified gene, which we named INCA (Inhibitory CARD), maps to human chromosome 11q22. Interestingly, the genes coding for caspase-1, the related CARD-proteins, ICEBERG and COP/Pseudo-ICE and caspases-4, -5 and -12 all reside on this locus. According to the public data base of the Human Genome Browser (genome.ucsc.edu/), the order of these genes from centromere to telomere is caspase-12, caspase-4, caspase-5, caspase-1, COP/Pseudo-ICE, and ICEBERG (Fig. 1A). Because INCA shares high sequence homology with the genes encoding caspase-1, COP/Pseudo-ICE, and ICEBERG, it is likely that INCA arose from a duplication

branes. Blots were subsequently incubated with anti-E-tetrapeptide tag antibody (Amersham Biosciences), followed by horseradish peroxidase-conjugated secondary antibodies, and detection by an enhanced chemiluminescence (ECL) method. Alternatively, after normalization for total protein content, lysates were analyzed directly by immunoblotting using various antibodies, including anti-caspase-1 CARD antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-77 tetrapeptide tag antibodies (Novagen, Madison, WI).

**Retroviral Infection of THP-1 Cells—** Retroviral particles were produced by transfecting the amphotrophic packaging cell line Phoenix (kindly provided by Dr. G. P. Nolan, Stanford University Medical Center, Stanford, CA) with pBFneo, pBFneo-FLAG-INCA, or pBFneo-FLAG-COP/Pseudo-ICE vectors using the calcium phosphate/chloroquine method. The next day cultures were refreshed. Culture supernatants containing retroviral particles were collected 24 h later and filtered through a 0.45-μm membrane. 1 ml of viral supernatant was mixed with 10 μl of DOTAP (Roche Applied Science) for 10 min on ice. The monocytic cell line THP1 was cultured at 37 °C under 6% CO₂ in RPMI 1640 supplemented with 10% fetal calf serum, l-glutamine (2 mM), penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), sodium pyruvate (1 mM), β-mercaptoethanol (5 x 10⁻⁵ M). For infection, THP1 cells (10⁶ cells/well) were centrifuged in the presence of 1 ml of retrovirus enriched with DOTAP in a 6-well plate for 45 min at 1,200 rpm and washed with 1 ml of fresh medium. After 1 h, the later fresh medium was added, and the cells were kept in culture for 18 h. THP1 cells were subjected to a total of three cycles of infection followed by 1 week of culture. Cells were then selected using 15 mg/ml neomycin (Invitrogen). After 4 weeks of selection, the cultures were expanded, and expression of FLAG-INCA and FLAG-COP/Pseudo-ICE was verified by Western blot analysis. flag-RIP1 cells, or those expressing FLAG-INCA or FLAG-COP/Pseudo-ICE, were stimulated with the indicated amounts of LPS for 48 h. Release of biologically active IL-1β was determined using growth factor-dependent D10(N4)M cells as described previously (9, 20, 21).

**Quantification of NF-κB Activity—** 293T cells were transduced with the indicated expression vectors in combination with 100 ng of NF-κB reporter and β-galactosidase reporter plasmid pBDGAL. In other experiments, cells were treated for 6 h with 500 IU/ml of TNF-α prior to harvesting. Forty-eight hours after transfection the cells were collected, washed in phosphate-buffered saline, and lysed in Tris phosphate buffer (25 mM, pH 7.8) containing 2 mM diethiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100. After addition of 50 μl of substrate buffer (658 μM luciferin, 378 mM co-enzyme A, and 742 μM ATP) to 20 μl of cell lysate, NF-κB activity was assayed in a TopCount NXT microplate scintillation reader (Packard Instrument Co., Meriden, CT). To normalize transfection efficiency, cell lysates were also subjected to β-galactosidase colorimetric assay. In brief, cell lysate (20 μl) was incubated for 5 min at room temperature with 200 μl of a solution containing 0.9 mg/ml o-nitrophenyl-β-d-galactopyranoside, 1 mM MgCl₂, 45 mM β-mercaptoethanol, 50 mM sodium phosphate, pH 7.5. The optical density was read at a wavelength of 595 nm. Results are expressed as relative luciferase units per second/optical density for β-galactosidase activity. The data represent the average ± S.E. of triplicates and are representative of at least three independent experiments.

**RESULTS**

**Identification of INCA—** To identify new CARD-containing proteins, we searched the GenBank™ High Throughput Genomic Sequence data base for sequences that share significant homology with the prodomain of human caspase-1 (residues 1–106). Using this approach, four different genomic clones (GenBank™ accession numbers AF020787, AC027011, AF001024, and AC021452) containing new CARD-containing gene were found. The identified gene, which we named INCA (Inhibitory CARD), maps to human chromosome 11q22. Interestingly, the genes coding for caspase-1, the related CARD-proteins, ICEBERG and COP/Pseudo-ICE and caspases-4, -5 and -12 all reside on this locus. According to the public data base of the Human Genome Browser (genome.ucsc.edu/), the order of these genes from centromere to telomere is caspase-12, caspase-4, caspase-5, caspase-1, COP/Pseudo-ICE, and ICEBERG (Fig. 1A). Because INCA shares high sequence homology with the genes encoding caspase-1, COP/Pseudo-ICE, and ICEBERG, it is likely that INCA arose from a duplication...
of one of these homologous genes.

To deduce the putative cDNA sequence of INCA, we aligned its genomic sequence with the cDNA sequences of pro-caspase-1, COP/Pseudo-ICE, and ICEBERG to predict potential exons and intron/exon boundaries. The results of this approach largely matched those obtained using de novo prediction methods such as GenScan and GeneMark.hmm. The predicted INCA cDNA sequence is composed of four exons (Fig. 1B) with all intron/exon boundaries conforming to the consensus GT/AG rule (Fig. 1C). The open reading frame spans from the first to the third exon, the latter of which encodes an in-frame stop codon (Fig. 1B). Only the first two amino acids

Fig. 1. Gene organization, transcript, and protein sequences of INCA. A, organization of caspase-12, caspase-4, caspase-5, caspase-1, COP, INCA, and ICEBERG genes on human chromosome 11q22. B, nucleotide sequence of the INCA cDNA. The start and stop codons are indicated in bold letters. The positions of intron/exon borders are indicated by inverted triangles. C, a schematic structure of the INCA gene showing the intron/exon borders. Consensus splice donor (GT) and acceptor (AG) motifs are underlined, and the length of the introns is indicated in base pairs (bp). The start and stop codons are shown in bold letters. D, amino acid sequence alignment of INCA, COP/Pseudo-ICE, ICEBERG, and the first 110 residues of pro-caspase-1. Black and white boxes indicate identical and non-identical amino acids, respectively. Residue position numbers are indicated on the right. E, schematic representation of the CARD-proteins depicted in A. The CARD and caspase domain modules are indicated with an arrow and are drawn to scale. Protein molecular mass is indicated in kilodaltons.
are encoded in the first exon, and the last 18 amino acids are encoded by exon 3. Therefore, exon 2 encodes most of the open reading frame, including the CARD domain. Exon 4 does not code for amino acids, because it resides downstream of the in-frame stop codon at the end of exon 3, and thus functions as a 3′-untranslated region (Fig. 1B). The deduced amino acid sequence of INCA shares 81% sequence identity with the CARD-domain of procaspase-1. These data show that the INCA gene probably encodes a protein of 110 amino acids (Fig. 1D), which essentially consists of a CARD domain (residues 1–91). INCA is therefore comparable to the related CARD-only proteins COP/Pseudo-ICE, ICEBERG, and human caspase-12 (Fig. 1E) (22–24), all of which are encoded by genes residing on the same chromosomal locus.

**Tissue Expression of INCA**—Using the nucleotide or amino acid sequences of INCA as a query for BLAST searches of the GenBank™ data base, no expressed sequence tag sequences corresponding to INCA could be identified (data not shown). To experimentally confirm the existence of the predicted INCA mRNA and to study its tissue distribution, we performed RT-PCR analysis using INCA-specific primers on a cDNA panel derived from several normal human tissues and from the human cervix carcinoma cell line HeLa. Parallel PCR analysis of procaspase-1 and β-actin mRNA served as a reference (Fig. 2). INCA-specific primers amplified a PCR product of about 470 bp from several tissues, with the highest expression levels detected in brain, heart, spleen, lung, and salivary gland (Fig. 2). Subsequent DNA sequencing of this PCR product confirmed the predicted INCA cDNA sequence (data not shown). INCA was absent or expressed at low levels in various other tissues, including stomach, thyroid, pancreas, prostate and skin, as well as in HeLa cells (Fig. 2). In general, INCA is expressed in most tissues where procaspase-1 is present. However, in a number of tissues, such as salivary gland, INCA is expressed in the absence of procaspase-1 (Fig. 2). This suggests that differential regulation mechanisms at the transcriptional or post-transcriptional level control these homologous genes.

**INCA Is Up-regulated by IFN-γ**—To investigate the possible existence of regulation mechanisms shared between procaspase-1 and INCA, we compared the modulation of INCA and procaspase-1 mRNA levels in response to various pro-inflammatory stimuli. Since caspase-1 mRNA levels are known to be up-regulated when cells are stimulated with IFN-γ but remain unchanged following LPS or TNF stimulation (25–28), we stimulated monocytic cell lines U937 and THP-1 with IFN-γ, TNF-α, LPS, or combinations of these stimuli, for an additional 12 h. Total RNA was isolated, and cDNAs were amplified using specific primers for procaspase-1, INCA, and β-actin. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Fragment size is indicated in kbp. The identities of the procaspase-1 and INCA PCR products were confirmed by DNA sequencing.
remaining largely unchanged in LPS- and TNF-α-stimulated cells (Fig. 3). These results confirm and extend published data on the induction profile of caspase-1 (25–28). Comparable to procaspase-1, treatment of U937 or THP-1 cells with IFN-γ leads to a significant up-regulation of INCA levels, which remain unchanged in LPS-stimulated cells (Fig. 3). These results indicate that procaspase-1 and INCA mRNA levels are both specifically up-regulated by IFN-γ. Interestingly, we noticed that TNF-α is capable of down-regulating the IFN-γ-induced up-regulation of procaspase-1 and INCA in both THP-1 and U937 cells (Fig. 3). In both cell lines, we observed that this IFN-γ-modulating effect of TNF-α is more pronounced for INCA than for procaspase-1 (Fig. 3). All together, these results suggest that INCA and procaspase-1 mRNA levels are modulated in similar ways.

Identification of INCA-interacting Proteins—The prodomain of procaspase-1 is required for dimerization and activation of the zymogen (29). Because INCA shares a high degree of amino acid sequence identity with the prodomain of procaspase-1 (Fig. 1), we tested the possibility that INCA interacts with procaspase-1 in co-immunoprecipitation assays. Interactions with several CARD-containing proteins were also tested, including INCA itself, and the related CARD-only proteins ICEBERG and COP/Pseudo-ICE. The unrelated CARD domain of procaspase-2 was used as a negative control for the co-immunoprecipitation assays. For these experiments, 293T cells were transiently transfected with expression plasmids encoding FLAG-INCA, FLAG-procaspase-1, FLAG-COP/Pseudo-ICE, and E-RIP2. Immune Complexes were prepared using anti-FLAG antibody adsorbed to protein G-Sepharose and analyzed by SDS-PAGE/immunoblotting using anti-E epitope tag antibody and chemiluminescence-based detection. Aliquots of the same lysates were also analyzed directly by SDS-PAGE/immunoblotting as indicated. IP, immunoprecipitation; WB, Western blotting.

![Fig. 4](http://www.jbc.org/)

**FIG. 4.** INCA interacts with specific CARD proteins. Co-immunoprecipitation assays were performed using lysates from 293T cells that have been transiently transfected with plasmids encoding FLAG-INCA, E-INCA, E-procaspase-1, E-COP, E-ICEBERG, E-RIP2, and E-procaspase-2 CARD. Immune Complexes were prepared using anti-FLAG antibody adsorbed to protein G-Sepharose and analyzed by SDS-PAGE/immunoblotting using anti-E epitope tag antibody and chemiluminescence-based detection. Aliquots of the same lysates were also analyzed directly by SDS-PAGE/immunoblotting as indicated. IP, immunoprecipitation; WB, Western blotting.

![Fig. 5](http://www.jbc.org/)

**FIG. 5.** INCA does not interact with RIP2. Co-immunoprecipitation assays were performed using lysates from 293T cells that have been transiently transfected with plasmids encoding FLAG-INCA, FLAG-procaspase-1, FLAG-COP/Pseudo-ICE, and E-RIP2. Immune Complexes were prepared using anti-FLAG antibody adsorbed to protein G-Sepharose and analyzed by SDS-PAGE/immunoblotting using anti-E epitope tag antibody and chemiluminescence-based detection. Aliquots of the same lysates were also analyzed directly by SDS-PAGE/immunoblotting as indicated. IP, immunoprecipitation; WB, Western blotting.
E-procaspase-1 co-immunoprecipitated with FLAG-INCA (Fig. 4B), suggesting that INCA can bind to the prodomain of procaspase-1. Note that the active site cysteine of procaspase-1 was mutated to alanine for these co-immunoprecipitation experiments to avoid induction of apoptosis. Finally, the CARD-only proteins COP/Pseudo-ICE and ICEBERG also co-immunoprecipitated with FLAG-INCA (Fig. 4C and D), indicating that these highly related CARD domains that bind to the prodomain of procaspase-1 can also cross-associate with the similar CARD domain present in INCA. E-procaspase-2 CARD also did not co-immunoprecipitate with FLAG-INCA (Fig. 4E), thus demonstrating the specificity of these results. Because it has been demonstrated that procaspase-1 and COP/Pseudo-ICE interact with the CARD-containing kinase RIP2 to induce NF-κB activation (9, 16), we also tested the interaction of INCA with this kinase (Fig. 5). In contrast to COP/Pseudo-ICE (16, 18) and procaspase-1 (9, 17), but similar to ICEBERG (16, 17), E-RIP2 did not co-immunoprecipitate with FLAG-INCA (Fig. 5A). In the same experiment, E-RIP2 was present in immunoprecipitates of procaspase-1- and COP/Pseudo-ICE-expressing cells (Fig. 5, B and C). This result demonstrates that COP/Pseudo-ICE and procaspase-1 contain RIP2-binding interfaces at the surface of their CARD domains that are not present in the more distantly related CARD domains of INCA and ICEBERG.

Comparative Analysis of the Capacity of CARD-only Proteins to Modulate NF-κB—We have recently demonstrated that caspase-1 CARD also potently activates the transcription factor NF-κB in a RIP2-dependent manner (9). COP/Pseudo-ICE also interacts with RIP2 and induces NF-κB activation upon overexpression in 293T cells (16). However, ICEBERG does not interact with RIP2 and is unable to activate NF-κB (16). ICEBERG shares 53% sequence identity with caspase-1 CARD, whereas INCA and COP/Pseudo-ICE share 81 and 93% sequence identity with the prodomain of caspase-1, respectively. Thus, INCA is intermediate between COP/Pseudo-ICE and ICEBERG. Therefore, we tested whether INCA is capable of inducing NF-κB activity. 293T cells were co-transfected with an NF-κB-driven luciferase reporter plasmid and plasmids encoding either empty vector, enzymatically inactive caspase-1 C285A, COP/Pseudo-ICE, INCA, or ICEBERG. As expected, both procaspase-1 C285A and COP/Pseudo-ICE potently induced NF-κB activity (Fig. 6). Like ICEBERG, INCA was completely incapable of activating NF-κB (Fig. 6), even when very high plasmid concentrations were used (data not shown). This
result correlates with the observation that INCA does not interact with the NF-kB-activating kinase RIP2 (Fig. 5A). In conclusion, unlike pro-caspase-1 CARD and COP/Pseudo-ICE, INCA and ICEBERG are unable to induce NF-kB activation.

**INCA Does Not Inhibit NF-kB Activation Induced by TNF, Caspase-1, COP/Pseudo-ICE, or RIP2**—Several recently cloned CARD-containing proteins have been shown to inhibit rather than to induce NF-kB activity (30, 31). For example, CARD-8 is known to inhibit both RIP2- and TNF-induced NF-kB activation (31). Because both INCA and ICEBERG are unable to induce NF-kB activation (Fig. 6), we investigated whether they can inhibit NF-kB activity induced by TNF, caspase-1, COP/Pseudo-ICE, or RIP2. Because most NF-kB signaling pathways converge at the IKK complex, we used a dominant negative form of IKK-β (IKK-β DN) as a positive control for inhibition. As expected, IKK-β DN completely abolished NF-kB activity.

**Fig. 7.** INCA does not inhibit NF-kB activation by TNF, pro-caspase-1 C285A, COP/Pseudo-ICE, or RIP2. A, 293T cells were transiently co-transfected with a plasmid allowing NF-kB-dependent luciferase reporter expression, 0.2 μg of a plasmid encoding pro-caspase-1 C285A, COP/Pseudo-ICE, or RIP2, and 0.6 μg of a plasmid coding for INCA, ICEBERG, or IKK-β DN. In another setup, cells were transiently co-transfected with a plasmid allowing NF-kB-dependent luciferase expression and 0.6 μg of a plasmid encoding INCA, ICEBERG, or IKK-β DN, and treated with 500 IU/ml human TNF for induction of NF-kB activation. Total DNA was maintained at 1 μg by the addition of control plasmid DNA. 24 h after transfection, lysates were analyzed for NF-kB activity as described under “Experimental procedures.” B, aliquots of the same whole cell lysates were analyzed by SDS-PAGE/immunoblotting to confirm the appropriated expression of all constructs. Data represent the mean ± S.D. (n = 3).
from the four activating molecules (Fig. 7A). However, INCA and ICEBERG did not significantly affect TNF-, caspase-1-, COP/Pseudo-ICE-, or RIP2-induced NF-κB activation (Fig. 7A), although Western blotting analysis confirmed the appropriate expression of both CARD-proteins (Fig. 7B). All together, these data suggest that INCA and ICEBERG do not function as endogenous modulators of the studied NF-κB signaling pathways.

**INCA Inhibits the Release of IL-1β from THP-1 Cells**—THP-1 monocytes release IL-1β in response to inflammatory stimuli such as LPS. The processing of pro-IL-1β to the 17.5-kDa mature form and its release are well known consequences of caspase-1 activation (4, 32). The INCA-related CARD-only proteins ICEBERG and COP/Pseudo-ICE have been shown to significantly blunt IL-1β maturation following LPS stimulation of THP-1 cells (16, 17). To test whether INCA resembles ICEBERG and COP/Pseudo-ICE in this feature, we generated stable transfectants of THP-1 cells expressing FLAG-tagged INCA under the control of a retroviral promoter. Stable transfectants of THP-1 cells expressing FLAG-tagged COP/Pseudo-ICE were used as a positive control in this experiment. The stable transfectants expressed INCA and COP/Pseudo-ICE at levels comparable to the constitutive expression of endogenous procaspase-1 in THP-1 cells (Fig. 8A). As expected, neither COP/Pseudo-ICE- nor INCA-expressing cells released mature IL-1β in unstimulated cells (Fig. 8, B and C). INCA was as effective as COP/Pseudo-ICE in inhibiting IL-1β generation at both doses of LPS used in this experiment (Fig. 8, B and C). Taken together, our results show that INCA significantly reduces the release of mature IL-1β in monocyctic THP-1 cells and suggest that the binding of INCA to procaspase-1 prevents the CARD-mediated activation of the enzyme (13, 29).
DISCUSSION

We have identified and characterized the functions of a new human protein and designated it INCA. The protein shows striking similarity to the prodomain of procaspase-1, and thus represents a new member of the human CARD-containing family of proteins. Structurally, INCA is comparable to COP/Pseudo-ICE and ICEBERG, because they all consist mainly of a CARD domain that shares high homology with the prodomain of procaspase-1 (16–18). INCA significantly reduces the release of mature IL-1β from the monocytic cell line THP-1 following LPS stimulation of TLR-4. This effect is probably mediated by its ability to interfere with the CARD-mediated activation of procaspase-1 (13, 29). These results demonstrate that INCA can regulate at least one important signaling pathway in anti-pathogen responses. INCA shares this feature with COP/Pseudo-ICE and ICEBERG, which were also shown to down-modulate the release of IL-1β in response to LPS in monocyes (16, 17). Therefore, COP/Pseudo-ICE, ICEBERG, and INCA are functionally similar to the caspase-8 inhibitor FLICE (Fas receptor-associated death domain (FADD)-like interleukin-1β converting enzyme)-inhibitory protein, short isoform. This death effector domain-containing protein prevents the activation of procaspase-8 by impairing the association of this caspase with its death effector domain-containing adaptor Fas receptor-associated death domain (33). In this respect, COP/ Pseudo-ICE, ICEBERG, and INCA may act as decoys or dominant negative molecules of caspase-1.

Interestingly, the genes encoding COP/Pseudo-ICE, ICEBERG, and INCA all map to human chromosome 11q22, where the genes for the inflammatory caspases-1, -4, and -5 also reside. 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. These gene duplications must have occurred recently, because the mouse and invertebrate genomes do not encode such CARD-only proteins (data not shown). It would be interesting to assess the presence of such decoy molecules in the genomes of other mammals that are more related to humans, such as the pig and the chimpanzee. However, at this moment the data in the draft versions of these genomes is too limited to answer this question.

RT-PCR assays revealed that INCA is co-expressed with procaspase-1 in many normal human tissues. Therefore, INCA may function as one of the countermeasures preventing the accidental activation of this inflammatory caspase. The expression levels of both INCA and procaspase-1 increase in IFN-γ-stimulated monocytes. Indeed, a computational analysis of the INCA promoter sequence using MatInspector (34) revealed putative binding sites for the interferon-regulated transcription factors STAT1 and IRF1 and -2 (data not shown). Similar sites have been identified in the promoter region of procaspase-1 (data not shown) and were shown to be essential for the up-regulation of this protease in response to IFN-γ (25, 26, 35). Interestingly, a number of tissues, such as salivary gland, express high constitutive levels of INCA, while procaspase-1 is merely detectable. This suggests that INCA may have additional roles beside the modulation of procaspase-1 activation.

Several CARD-containing proteins have been shown to either positively or negatively regulate NF-κB activation (15). Unlike COP/Pseudo-ICE and the prodomain of procaspase-1, ICEBERG and INCA are not capable of interacting with RIP2 or inducing NF-κB activation. Therefore, procaspase-1 CARD and COP/Pseudo-ICE may share on their surfaces a common RIP2-binding and NF-κB-activating interface that is not present in INCA and ICEBERG. Hence, identification of critical amino acids, involved in RIP2 binding and NF-κB activation on the surfaces of procaspase-1 CARD and COP/Pseudo-ICE, may represent a first step in the design of drugs that specifically interfere with RIP2-dependent signaling pathways leading to NF-κB activation. Based on previous reports and the results we have described here, one now can formulate a number of “rules and tools” that can be useful in the search for these critical residues. (a) Based on the NMR structure of ICEBERG (17) and the high degree of sequence homology of procaspase-1 CARD, COP/Pseudo-ICE, and INCA with ICEBERG, reliable three-dimensional models of these CARD domains can be produced. (b) CARD domains are characterized by large basic and acidic patches on opposite ends of their surfaces. (c) Hydrogen bonds between oppositely charged residues play a crucial role in homotypic CARD/CARD interactions (36, 37). (d) Surface residues from helices 1 and 4 from one CARD domain interact with residues from helices 2, 3, and 5 from the other CARD motif (36, 37). (e) The majority of differential amino acids between the RIP2-interacting CARD domains (procaspase-1 CARD and COP/Pseudo-ICE) on one hand and INCA and ICEBERG on the other hand is located in helices 2 and 3. Based on these assumptions and data, we suggest that a number of negatively charged amino acids in the acidic patch (helices 2, 3, and 5) of procaspase-1 CARD and COP/Pseudo-ICE, which are absent in ICEBERG and INCA, may be crucial for RIP2-binding and consecutive NF-κB activation.

Although INCA binds to COP/Pseudo-ICE and the prodomain of procaspase-1, it is not capable of inhibiting the induction of NF-κB activation induced by the latter two molecules. Similarly, although several CARD-containing proteins have been shown to induce apoptosis, we failed to find a pro-apoptotic role for INCA (data not shown). All together, these observations argue that the predominant role of INCA is mainly to function as a negative regulator of procaspase-1 activation and IL-1β release. At low concentrations, IL-1β primarily mediates local inflammation by stimulating the production of chemokines and IL-6, which recruit and activate various leukocyte populations at the site of inflammation (10). However, at higher doses, it can have potentially lethal systemic effects, generally known as septic shock (38, 39). Sepsis currently represents the most common cause of mortality in patients treated in the intensive care setting (40, 41). Therefore, maintenance of IL-1β homeostasis to prevent systemic inflammatory reactions is important for survival following exposure to a minor pro-inflammatory insult. It is therefore not surprising that several endogenous inhibitors of IL-1β have been identified. IL-1 receptor antagonist, for example, is a biologically inactive IL-1-like molecule that binds to IL-1 receptors and functions as a competitive inhibitor of IL-1 (42). The recently discovered molecules COP/Pseudo-ICE, ICEBERG, and now INCA represent another level of regulation allowing maintenance of appropriate levels of IL-1β by preventing procaspase-1 activation in the inflammasome complex (12, 16–18). Therefore, further understanding of the mechanisms that control the expression and function of caspase-1 and its antagonists may be an important first step in the design and development of new anti-inflammatory strategies.

Acknowledgment—We thank W. Burms for IL-1β bio-assays.

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J. Biol. Chem. 2004, 279:51729-51738.
doi: 10.1074/jbc.M407891200 originally published online September 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407891200

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