

CLAN, a Novel Human CED-4-like Gene

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Proteins governing cell death form the basis of many normal processes and contribute to the pathogenesis of many diseases when dysregulated. Here we report the cloning of a novel human CED-4-like gene, CLAN, and several of its alternatively spliced isoforms. These caspase-associated recruitment domain (CARD)-containing proteins are expressed at varying degrees in normal human tissues and may contribute to a number of intracellular processes including apoptosis, cytokine processing, and NF- κ B activation. The CARD of the CLAN proteins binds a number of other CARD-containing proteins including caspase-1, BCL10, NOD2, and NAC. Once their physiological functions are uncovered, CLAN proteins may prove to be valuable therapeutic targets.

Key Words: apoptosis, CARD, CED-4, NACHT, LRR, cell death, caspases

INTRODUCTION

Apoptosis is a highly regulated program of cell suicide that has important roles in the normal development of multicellular organisms, maintenance of tissue homeostasis, and elimination of potentially harmful cells from the body. During mammalian embryonic development, apoptosis governs the proper formation of limbs and digits through the control of mesodermal tissue loss [1], the development of the nervous system [2], and the elimination of self-recognizing B cells and T cells [3]. Alterations in the regulatory components of the apoptotic machinery contribute to many diseases, including autoimmunity, AIDS, stroke, heart failure, neurodegenerative disorders, and cancer [4].

The core components of the apoptotic machinery are evolutionarily conserved and many were originally discovered in the nematode *Caenorhabditis elegans*. One of these required components, CED-4, is critical in coupling cell death stimuli to the activation of dormant proteases known as "caspases" [5]. Proteins within the CED-4 family are characterized by a nucleotide-binding oligomerization domain (NB-ARC, or NACHT domain) and a caspase-associated recruitment domain (CARD), which binds the pro-forms of certain caspases.

We discovered the coding sequence for a novel CED-4-like protein through a bioinformatics approach using public databases. Homology screening found a genomic region on chromosome 2 that was predicted to contain both a NACHT domain and a CARD domain. Corresponding cDNAs were cloned, verifying that this gene is expressed. We have designated this gene *CLAN* (for CARD, LRR, and

NACHT-containing protein) because at least one of the proteins it encodes contains CARD, leucine-rich repeat (LRR), and NACHT domains. Here we report the molecular cloning of cDNAs encoding *CLAN* and several smaller isoforms of this protein that are differentially expressed in human tissues. All four isoforms contain a CARD motif capable of associating with several other CARD-containing proteins, including pro-caspase-1.

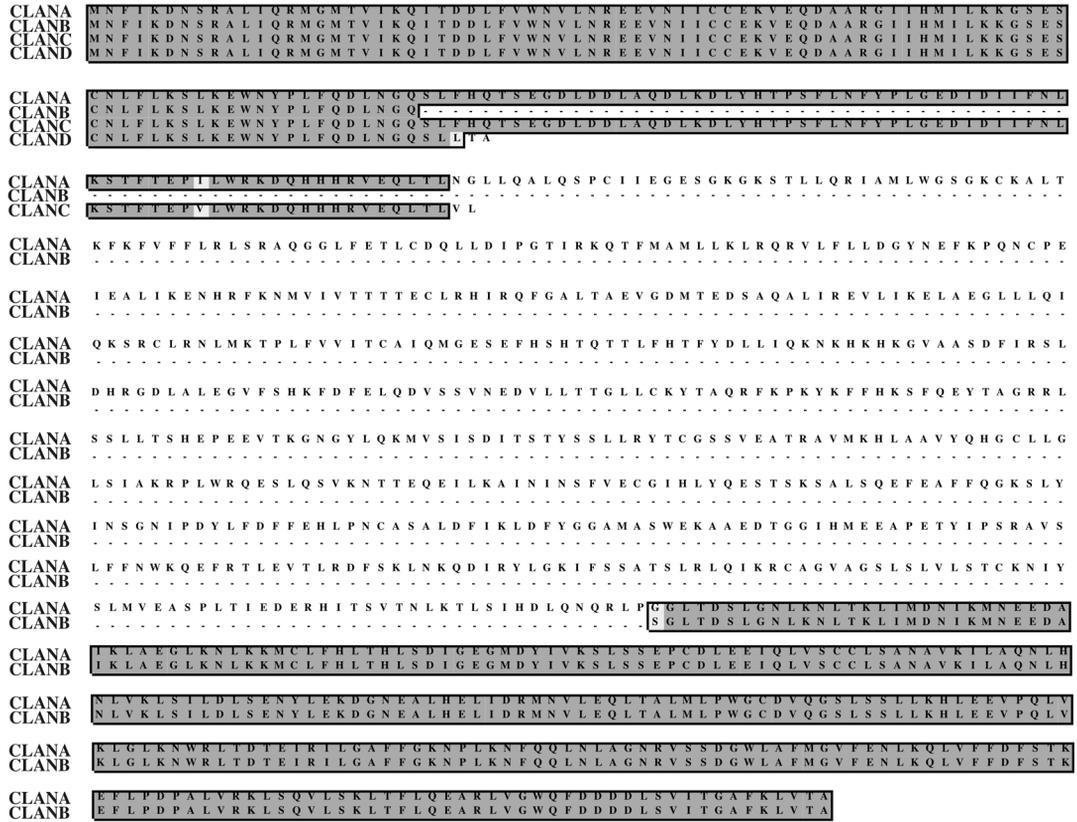
RESULTS

Cloning of *CLAN* cDNAs

We searched the HTSG database of human genomic DNA sequence data for regions capable of encoding CARDs using the amino acid sequence of the cellular inhibitor of apoptosis protein-1 (cIAP1) as a query with the TBLASTn method. A genomic locus on human chromosome 2p21-p22 was thus identified. This locus was not recognized in the human genomic database and was not previously annotated. Using GENESCAN for exon prediction, additional regions potentially encoding a NACHT domain and regions corresponding to LRR domains were also recognized 3' to the potential CARD-encoding sequences, suggesting the presence of a CED-4-like gene. Several partial EST clones have since appeared in the NCBI database (acc. nos. AV719179, AI263294, AV656315, AW337918, BF207840, AW418826, BK903662, AI023795, H25984), none of which contain the *CLAN* CARD region.

Gene-specific primers based on the genomic sequence data were used in conjunction with two universal primers to

FIG. 1. Clustal-W alignment of the amino acid sequence of the four CLAN splice variants. Identical residues are shaded darker; conserved amino acid changes are shaded lighter.



isolate several cDNAs from human liver and lung RNA sources (Fig. 1). The longest transcript, termed *CLAN-A*, is 3.370 kb with an open reading frame (ORF) encoding a 1024 amino-acid protein containing CARD, NACHT, and LRR domains, as well as a predicted sterile α -motif (SAM) domain. A second transcript, termed *CLAN-B*, is 1.374 kb, with an ORF encoding a 359 amino-acid protein containing an identical CARD directly spliced to the LRRs. *CLAN-C*,

the third transcript isolated, is 0.768 kb and encodes a 156 amino-acid protein containing just the CARD and an additional region lacking homology to recognizable domains. Finally, the shortest transcript found, *CLAN-D*, is 0.578 kb and contains an ORF encoding a 92 amino-acid protein encompassing only the CARD followed by 9 amino acids.

Comparisons of these cDNA sequence data with the genomic DNA sequence data found in the HTSG database indicated that *CLAN* consists of 12 exons, spanning 41.3 kb on chromosome 2p21-p22 (Fig. 2A). Three differences were found between the sequence of the *CLAN* cDNA and the sequence within the public database. Also, the region encompassing the first 12 nucleotides of *CLAN* (5' UTR) does not have an equivalent fragment in the public database. Two different transcriptional start sites are used (corresponding to the beginning of either exon 1 or 2), but both are spliced to exon 3 at the beginning of the CARD. Exons 6 and 7 contain additional internal splice donor sites used to generate *CLAN-C*. mRNA splicing events are predicted to give rise to the *CLAN-A*, *CLAN-B*, *CLAN-C*, and *CLAN-D*

A chromosome 2p21-22 (41.3kb)

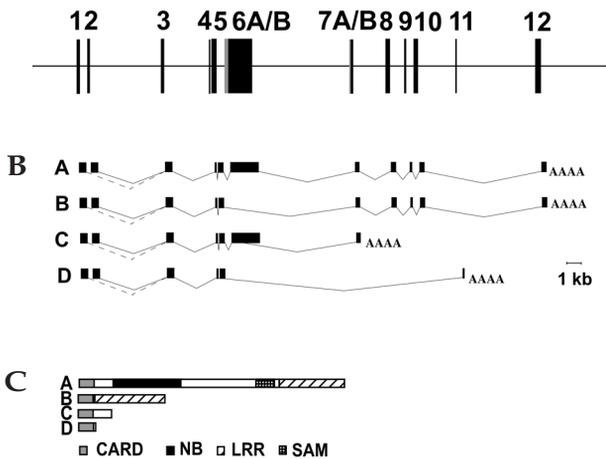


FIG. 2. Intron/exon organization of *CLAN*. (A) Genomic organization of *CLAN* on chromosome 2 deduced from the BAC 164M19 sequence from the SPG4 candidate region at 2p21-2p22 (acc. no. AL121653) and *Homo sapiens* chromosome 2 working draft sequence (acc. no. NT_005194.1). (B) mRNA splicing generates *CLAN-A*, *CLAN-B*, *CLAN-C*, and *CLAN-D*. (C) Deduced domain structure for the splice variants.

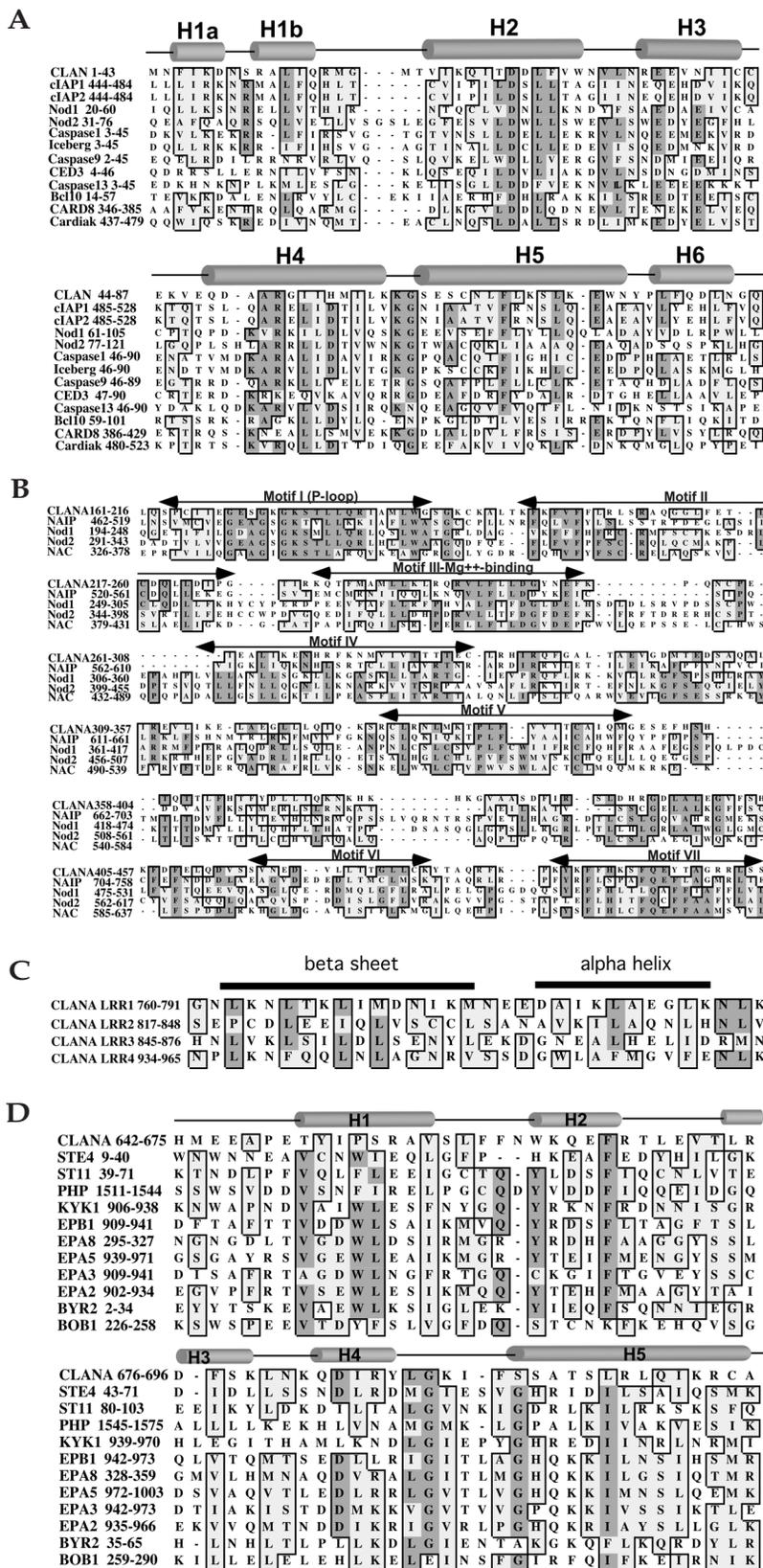


FIG. 3. Domain alignments in CLAN. (A) Predicted amino-acid sequence of CLAN CARD and alignment with the most similar CARDs of other proteins. Top, the predicted secondary structure for the CLAN CARD, as predicted from the solved structure of ICEBERG. Accession numbers are as follows: cIAP1, Q9UP46; cIAP2, Q13489; NOD1, Q9Y239; NOD2, AAG33677; caspase-1, Q9TV13; ICEBERG, P57730; caspase-9, Q9IB63; CED3, P45436; caspase-13, O75601; BCL10, CAA06955; CARD8, AAG50014; CARDIAK, AAC25668. (B) Alignment of the NACHT domain of CLAN with most homologous NACHT domains from NAIP, NOD1, NOD2, and NAC. Shown are the seven conserved boxes including the Walker A and B box. Accession numbers are as follows: NAIP, AAC62261; NOD1, Q9Y239; NOD2, AAG33677; NAC, AAK00748. (C) Alignment of the four LRRs found in CLAN. Top, the secondary structure as predicted from the known structure of the human placental ribonuclease/angiogenin inhibitor. (D) Alignment of the SAM domain found in CLAN with the most similar known SAM domains. At top is shown the secondary structure as predicted from the solved structure of the Eph receptor SAM domain. Identical residues are shaded darker, conserved amino acid changes are shaded lighter. Accession numbers are STE4 (X61924), ST11 (P23561), PHP (P39769), KYK1 (P18160), EPB1 (P54762), EPA8 (P29321), EPA5 (P54755), EPA 3 (P29318), EPA 2 (P29317), BYR2 (P28829), and BOB1 (P38041), respectively.

transcripts and encoded proteins (Fig. 2B). All the exon/intron splice junctions follow the conserved GT/AG consensus rule.

As predicted by SMART (EMBL, Heidelberg, Germany), CLAN contains a CARD. A ψ -BLAST search of the non-redundant database using the CLAN CARD as query identified several homologous CARDs, including those from cIAP1 and -2 (58%), caspase-1 and ICEBERG (50%), NOD1, NOD2, and CARD8 (~38%) and caspase-13, CED3, caspase-9, BCL10 (CIPER) and CARKIAK/RIP2 (~30%; Fig. 3A). Following the CARD, we observed a domain containing a consensus sequences for Walker A (GXXXGKT/S) and B boxes (V/LI/V/LV/IL/VDS) [6] and additional characteristics of the newly founded family of NTPases termed the NACHT family [7]. By ψ -BLAST search, the NACHT domain of CLAN shows highest similarity to the NACHT domain of NAIP (60%), followed by NOD1 (49%) and NOD2 (47%; Fig. 3B). LRR domains are also found near the carboxy terminus of the A and B isoforms of the protein (Fig. 3C). The C-terminal end consists of four repeated LRRs, each containing a predicted β -sheet and α -helical structure, which is in agreement with the prototypical horseshoe-shaped structure of LRRs [8]. LRR 1 represents a non-Kobe and Deisenhofer (non-K/D) LRR of the structure $XaXXaXaX(N/C/T/Q)\pm Xa$ (where "X" is any amino acid and "a" represents an aliphatic amino acid), whereas LRRs 2, 3, and 4 are in accordance with Kobe and Deisenhofer (K/D) LRR with the consensus sequence $XaXXaXaXX(N/C/T/Q)\pm Xa$. LRR 2 also shares sequence homology to a prototypical ribonuclease inhibitor type A (RI type A). By ψ -BLAST searches the LRRs show 49% sequence identity to the placental ribonuclease/angiogenin inhibitor (RAI; Fig. 3C). Sequences between the NACHT and LRR domains show some similarity to the SAM, a domain consisting of five α -helices, originally found in proteins involved in developmental processes. The SAM domain has been shown to function as a protein-protein interaction domain, with ability to homooligomerize as well as hetero-oligomerize with other SAMs [9] (Fig. 3D).

In vivo expression of CLAN

We examined which of the splice variants of CLAN are expressed in adult human tissues. Northern blot analysis using the CARD of CLAN as a probe revealed expression of an approximately 1.5-kb transcript corresponding to CLANB in nearly all tissues examined with highest expression in lung and spleen (Fig. 4A). The CLAN-A, CLAN-C, and CLAN-D splice variants are not detected on this blot, probably due to lower expression levels. Northern blot analysis using the NACHT and LRR of CLAN-A as a probe revealed expression of an approximately 3.5-kb mRNA corresponding to CLAN-A, primarily in the lung (Fig. 4B).

To further explore the tissue-specific patterns of expression of CLAN splicing variants, RT-PCR assays were devised

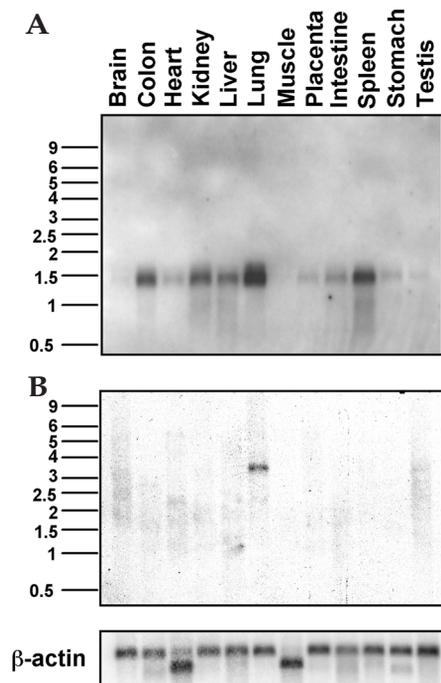


FIG. 4. Expression of CLAN mRNA in normal human tissues. Northern membranes composed of normalized poly(A)⁺ RNA from human tissues were hybridized with a labeled probe corresponding to the CARD of CLAN (A) or to the NACHT and LRR of CLAN (B). As a loading control, blots were re-probed with a cDNA corresponding to β -actin. Molecular weight markers are indicated in kilobase-pairs (kbp).

which are specific for the CLAN-A, CLAN-B, CLAN-C, and CLAN-D isoforms. RT-PCR analysis showed that CLAN-B was present throughout human tissues, consistent with the northern blot analysis (Fig. 5). In contrast, CLAN-A was restricted to lung, colon, brain, prostate, spleen, and leukocytes. Further analysis of leukocyte sub-populations revealed expression of the CLAN-A isoform predominantly in the monocyte cell fraction, with lower expression found in granulocytes and no expression in lymphocytes. Though these PCR assays were not carried out in a quantitative fashion, PCR amplification of CLAN-A was at the highest levels in the lung, which is in agreement with the northern blot results. Expression of CLAN-C was absent from all normal tissues tested, but expression was evident in the cell line HEK293T (data not shown), indicating this transcript can be produced under some circumstances. CLAN-D transcripts were detected only in brain by RT-PCR.

CLAN protein interactions

As the CARD is a homophilic interaction domain [10], we investigated which of the known CARD proteins can bind the CARD of CLAN, thus providing hints about possible functions of the CLAN proteins. The CARD of CLAN was expressed as an epitope-tagged protein in HEK293T cells in co-transfections with a variety of other epitope-tagged CARD-containing proteins, and lysates derived from these cells were used for co-immunoprecipitation assays (Fig. 6). The CARD of CLAN bound readily to full-length pro-caspase-1, but did not bind another CARD-containing caspase, caspase-9. Among the other CED-4 family members which contain a CARD in conjunction with a nucleotide-binding

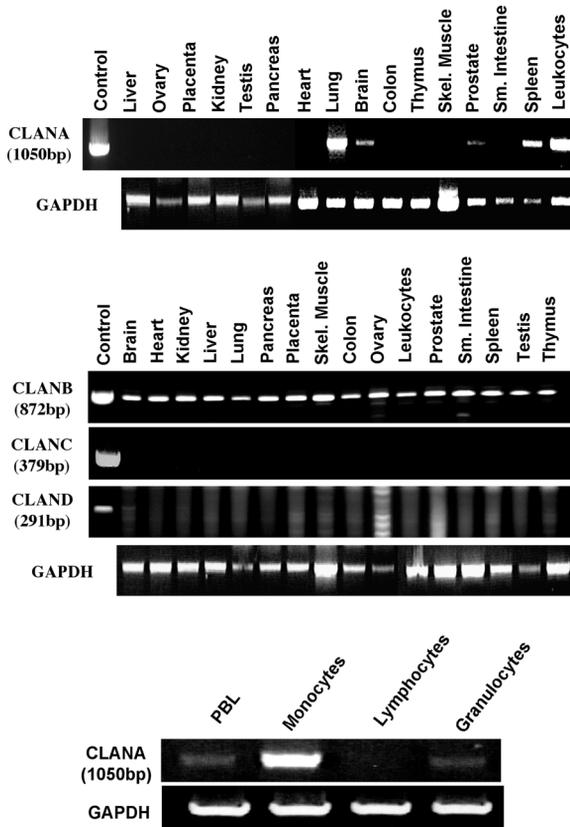


FIG. 5. RT-PCR analysis of *CLAN* expression. cDNAs derived from multiple human tissues were subjected to PCR using *CLAN* isoform-specific primers. In addition, circulating blood leukocytes were fractionated and cDNA was prepared for PCR analysis. As a control for message integrity, PCR for GAPDH was also carried out. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining with UV illumination.

domain, CLAN interacted with the CARDs of NOD2 and NAC, but not with Apaf-1 or NOD1. Finally, the CLAN CARD was found to associate with BCL10, but not with another adapter protein, RAIDD.

DISCUSSION

Here we have described a novel human gene, *CLAN*, capable of producing transcripts encoding several CARD-containing proteins. The predicted CLAN protein contains three domains ordered in a manner similar to that seen in NOD1 and NOD2 [11,12]: a CARD domain located near the amino terminus, followed by a central NACHT domain and then a span of LRR near the C terminus. Unlike NOD1 and NOD2, however, a putative SAM domain is also found in this protein. The NACHT domain contains a P-loop region that has previously been shown to be important for nucleotide-dependent self-oligomerization. This structural feature is also shared with plant disease resistance "R"

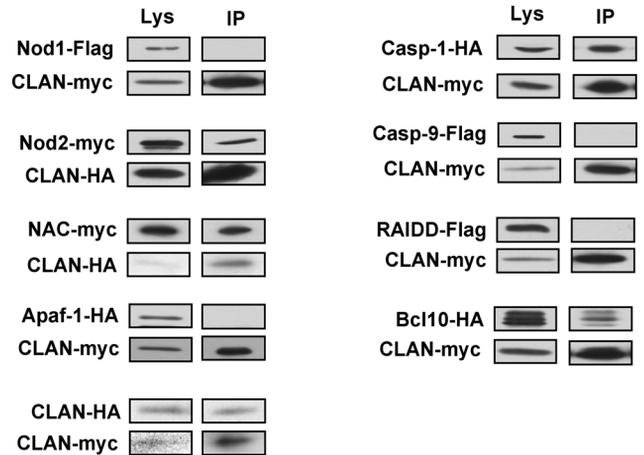


FIG. 6. Identification of CLAN interaction partners by co-immunoprecipitation. The CARD common to all CLAN isoforms was co-expressed as an epitope-tagged protein in HEK293T cells together with other epitope-tagged CARD-containing proteins by transient transfection. Immune complexes (IP) were separated by 12–15% SDS-PAGE and subjected to immunoblotting with antibodies corresponding to each epitope. As a control, 10% of the input cell lysate (Lys) was loaded directly in gels.

proteins. Similar to the Ced-4 homologues of animals, these plant proteins contain N-terminal effector domains linked to a NACHT domain followed by multiple LRR repeats. These proteins mediate defense responses in plants to pathogens [13]. The expression of the longest isoform, CLAN-A, seems to be limited primarily to lung and also to monocytes, where another CED-4 homologue, NOD2, has been reported to be exclusively expressed [12].

The best characterized mammalian CED-4 homologue, APAF-1, recruits pro-caspase-9 (an initiator caspase) into a protein complex in response to mitochondrial cytochrome *c* release [14], thus initiating the proteolytic cascade and resulting in apoptosis. In addition to apoptosis, CARD-containing proteins have also been shown to have other effects on cellular functions including cytokine processing and NF- κ B induction. Some CED-4-like proteins, such as NOD1 (CARD4) and NOD2, activate NF- κ B through interactions with another CARD-containing protein, CARDIAK (RIP2/RICK), which in turn binds to NEMO (IKK γ), a component of the IKB-kinase complex [11,12,15–17]. The LRRs of NOD1 and NOD2 have recently been shown [18] to function in a manner analogous to plant "R" gene products in that they act as cytosolic receptors (either directly or indirectly) for lipopolysaccharides produced by invading bacteria, thus providing a trigger for NF- κ B activation and subsequent immune responses. In addition to these CED-4-like proteins, CARD-containing proteins without NACHT and LRR domains are also known to induce NF- κ B through direct binding to BCL10 or CARDIAK [19–21].

As evidenced by their interactions with other CARD proteins, the novel isoforms of CLAN described here may have an influence on apoptosis, cytokine processing, or NF- κ B activity. Interactions of CLAN with pro-caspase-1 can be indicative of a role for CLAN as an interleukin-1 β regulator. In this regard, different isoforms of CLAN would be predicted to have opposing effects on pro-caspase-1 activation. The longest isoform, CLAN-A, for example, might trigger pro-caspase-1 activation by the "induced proximity" mechanism as a result of oligomerization mediated by its NACHT domain. In contrast, the shorter isoforms of CLAN lacking this self-oligomerization would be expected to operate as competitive antagonists of pro-caspase-1 activation, analogous to ICEBERG, a CARD-containing protein that competes with CARDIAK (RIP2/RICK) for binding to pro-caspase-1 [21]. Tissue-specific differences in the expression of CLAN-A and shorter CLAN isoforms thus may have a profound effect on pathways involved in pro-caspase-1 activation and inflammatory responses. Interactions of CLAN with NAC also suggest this protein may have an influence on apoptosis mediated by APAF-1, as NAC binds APAF-1 and enhances its ability to activate caspase-9 in response to cytochrome *c* [22]. Finally, CLAN associations with NF- κ B regulators such as BCL10 and NOD2 suggest that at least some of the CLAN isoforms may influence the activity of this transcription factor. It is also notable that the CLAN locus lies in close proximity to the spastin gene (on chromosome 2p21-22), encoding a AAA protein, which is frequently mutated in autosomal dominant hereditary spastic paraplegia (AD-HSP) [23]. The CLAN locus is found on the strand opposite the SPG4 (SPAST) locus, but with no overlapping regions. It has yet to be determined whether mutations in CLAN occur in patients with this neurodegenerative disorder.

The physiological functions of the isoforms of CLAN remain to be delineated. But if it is found to have a regulatory role in the processes of inflammation or cell death, CLAN may prove to be a valuable pharmacological or genetic target for the treatment of a variety of illnesses.

MATERIALS AND METHODS

Bioinformatics. The CLAN cDNA was found using the HTGS database, performing searches with TBLASTn using the CARD sequence of cIAP1 as query. This search revealed strong homology with a human genomic clone (acc. no. AQ889169).

cDNA cloning. CLAN-specific primers corresponding to sequences within the putative CARD and NACHT regions (as determined from genomic DNA sequence data) were used in conjunction with two universal primers to isolate CLAN cDNAs from first-strand liver and lung cDNA by nested PCR according to the manufacturer's protocol (SMART RACE, Clontech). Primers used for amplification are 5' RACE primers (5'-CATGTGAATGATCCCTC-TAGCAG-3'; nested 5'-GGGCTCGCTATCGTGCTCTA-3') and 3' RACE primers (5'-ACGATAGCCGAGCCCTTATTC-3'; nested 5'-GTATGGAAT-GTCTGAATCGC-3'). Amplification products were purified from agarose gels, ligated into the TA cloning vector (Promega), and sequenced. Four ORFs were deduced and multiple clones of each isoform were sequenced to ensure fidelity of PCR products.

Plasmid construction. A cDNA encoding the CLAN CARD domain was amplified using PFU polymerase and specific primers (5'-CCCGATCCAT-

GAATTCATAAAGGACAATAGC-3', 5'-CCCTTCGAACAAGTCCT-GAAATAGAGGATA-3') containing *Bam*HI and *Hind*III sites. The resulting PCR product was ligated into pcDNA3.1(-)/Myc-His₆ A (Invitrogen) which places the myc-His₆ tag at the C terminus of expressed proteins. pcDNA3/HA-CLAN (CARD) was created using a similar strategy. Authenticity of all vectors was confirmed by DNA sequencing.

RT-PCR. Total RNA was isolated from cells using Trizol reagent (BRL) and 2 μ g was used to generate cDNA in a reverse transcription reaction with Superscript II (BRL). PCR was carried out in an Eppendorf thermal cycler using *Taq* polymerase (BRL) and the following isoform-specific primer pairs: CLAN-A, 5'-GGTGGAGCAGGATGCTGCTAGAGG-3', 5'-CACAGTG-GTCCAGGCTCCGAATGAAGTCA-3'; CLAN-B, 5'-CATCATTGCTCGGA-GAAGTGGAG-3', 5'-TTAACTTGGATAACACTTGGCTAAG-3'; CLAN-C, 5'-GTAACATCATTTGCTGCGAGAA-3', 5'-CCCAGGAGGAGTGAAGAT-GCTAT-3'; CLAN-D, 5'-AATTCATAAAGGACAATAGCCGAG-3', 5'-TGCTACTGACTTTCTAAGCTGT-3'. For determination of CLAN isoform expression in normal human tissues, we used a panel of cDNA specimens derived from various human tissues (Clontech). Peripheral blood leukocytes were obtained from heparinized venous blood by Ficoll-Paque (Amersham) density-gradient centrifugation. Red blood cells were removed from granulocytes by short incubation in hypotonic lysis buffer. Monocytes were separated from lymphocytes by adherence to plastic dishes. Cells were lysed in Trizol and RNA subjected to RT-PCR as described.

RNA blots. Hybridization probes corresponding to the common CARD domain of all 4 CLAN isoforms or the NACHT and LRR regions were radiolabeled by random priming with hexanucleotides (Roche) and [α -³²P]-dCTP, or digoxigenin-labeled with a commercially available kit (Roche), incubated with blots containing human poly(A)⁺ RNA derived from various human tissues (Origene), washed at high stringency, and exposed to X-ray film. Positive signals were detected by autoradiography or by immunoblotting with HRP-conjugated anti-DIG antibody and an enhanced chemiluminescence method (ECL; Amersham).

Co-immunoprecipitation assays. HEK293T cells were seeded onto six-well plates (35-mm wells) and transfected with 0.2-2 μ g plasmid DNA using Superfect (Qiagen) after 24 h. After culturing for 1 d, cells were collected and lysed in isotonic lysis buffer (142.4 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.2% NP-40, 12.5 mM β -glycerophosphate, 2 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and 1x protease inhibitor mix (Roche)). Lysates were clarified by centrifugation and subjected to immunoprecipitation using agarose-conjugated anti-c-myc antibodies (Santa Cruz), or non-specific control antibodies and Protein G-agarose for 2-24 h at 4°C. Immune complexes were washed 4 times with lysis buffer, boiled in Laemmli buffer, and separated by 12-15% PAGE. Immune complexes were then transferred to PVDF membranes and immunoblotted with anti-c-myc (Santa Cruz), anti-HA (Roche), or anti-Flag (Sigma) antibodies. Membranes were washed, incubated with HRP-conjugated secondary antibodies, and reactive proteins were detected using ECL.

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession numbers AY027787 (CLAN-A), AY027788 (CLAN-B), AY027789 (CLAN-C), and AY027790 (CLAN-D).