CADD, a Chlamydia Protein That Interacts with Death Receptors*

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We report here the identification of a bacterial protein capable of interacting with mammalian death receptors in vitro and in vivo. The protein is encoded in the genome of Chlamydia trachomatis and has homologues in other Chlamydia species. This protein, which we refer to as “Chlamydia protein associating with death domains” (CADD), induces apoptosis in a variety of mammalian cell lines when expressed by transient gene transfection. Apoptosis induction can be blocked by Caspase inhibitors, indicating that CADD triggers cell death by engaging the host apoptotic machinery. CADD interacts with death domains of tumor necrosis factor (TNF) family receptors TNFR1, Fas, DR4, and DR5 but not with the respective downstream adaptors. In infected epithelial cells, CADD is expressed late in the infectious cycle of C. trachomatis and co-localizes with Fas in the proximity of the inclusion body. The results suggest a role for CADD modulating the apoptosis pathways of cells infected, revealing a new mechanism of host-pathogen interaction.

Chlamydia trachomatis is a eubacterial pathogen accounting for the major cause of blindness in Asia and Africa and is the most common sexually transmitted disease in the United States. Chronic Chlamydia infections have also been linked to cancer (1, 2). Chlamydiae are obligate intracellular bacteria. Cytotoxicity associated with Chlamydia infection is well recognized and has been linked to induction of apoptosis (3–5). However, controversy exists as to the nature of the apoptotic mechanisms, and more than one route to cell death may be utilized depending on the host cell type involved (epithelial cell versus macrophage), examination of infected versus neighboring cells, and other issues (3, 5).

Apoptosis induction by Chlamydiae appears to be independent of host cell protein synthesis under conditions where bacterial protein synthesis remains intact (4, 6). This observation has led to the postulation that Chlamydiae encode factors capable of regulating programmed cell death of host cells (4). Using bioinformatics approaches to study Chlamydiae genomes, we identified hypothetical proteins that share significant amino acid sequence homology to the death domains (DDs) of members of the mammalian TNF receptor family. In this report, the corresponding gene from C. trachomatis was cloned from bacterial genomic DNA, expressed, and functionally characterized.

MATERIALS AND METHODS

Cloning of CADD—Chlamydia proteins with significant similarity to mammalian DDs were identified using Saturated Blast searches. A representative set of DDs was used as queries, and a cascade of TBLASTN and PSI-BLAST searches was performed on nucleotide data bases at NCBI (htgs, gss, and dbest) and the NR protein data base. The candidate DDs were confirmed by running a FAS sequence comparison (7) against a data base of proteins of known structure (PDB) enriched for apoptotic domains. The C. trachomatis hypothetical protein CT610 (GI accession no. 3329055) had 26% identity and a FAS Z-score of 9.3 (similarity measure) with human DR5 and was chosen for further characterization. Genomic DNA from C. trachomatis, LGV-II, strain 434 (AB/Maryland) served as a template for cloning CT610 using specific primers (forward primer, 5′-ATGATGGAGGTGTTTATG-3′; reverse primer, 5′-ATAGATTGTAGCACAATTAC-3′).

DNA sequencing revealed three deviations from the published sequence, including two silent and one nonsilent nucleotide exchange (bp 75 G → A; bp 615 G → A; bp 664 C → G changing amino acid 222 Arg → Gly). A homologous gene from Klebsiella (PQQ synthesis protein C (PQQC), NCBI accession no. F27505) was cloned from genomic DNA (a gift of M. McClelland) and subcloned into pEGFP-C2, serving as a control.

Bacterial Strains and Infections—C. trachomatis L2/434/Bu cells were obtained from ATCC. Preparation of EBs and determination of infectivity were performed as described previously (8, 9). Briefly, HeLa 229 cells were grown in 9-cm Petri dishes to 70% confluency and then infected at a multiplicity of infection (m.o.i.) of 1. To remove unabsorbed EBs, plates were washed three times with PBS, then supplied with fresh medium, and incubated at 37 °C in a 5% CO2 humidified atmosphere. At various times postinfection supernatants and adherent cells were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C.

RNA Extraction, cDNA Synthesis, and RT-PCR—RNA from infected HeLa cells was extracted using a modified chloroform/phenol procedure (TRIZOL, Invitrogen). RNA (3 μg) from each sample was treated with DNase (Roche Molecular Biochemicals), and cDNA was generated using reverse transcriptase (RTase) (Superscript, Invitrogen) following the manufacturer’s protocol. Aliquots (5% (v/v)) of the cDNAs and no-RTase control samples were subsequently amplified by PCR using Taq DNA polymerase (Qiagen) and the following primer sets: CADD forward and reverse (see above), groEL forward (5′-GGATGGCATGGGGGAGGGCATA-3′) and reverse (5′-CAGGAAACCGGACATAACTG-3′), and human β-actin forward (5′-TGATATCGCCGCGCTCGTCGTC-3′) and reverse (5′-GGATGGCATGGGGGAGGGCATA-3′). Amplified fragments were analyzed by agarose gel electrophoresis and stained with ethidium bromide, and their identity was confirmed by DNA sequencing.

Protein Expression and Purification—The plasmid pGEX4T-CADD was introduced into Escherichia coli XLI-Blue. Glutathione S-transferase (GST) fusion proteins were obtained by induction with 0.1 mM isopropyl β-thiogalactoside at 25 °C for 8 h and then purified using

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1 The abbreviations used are: DD, death domain; EB, elementary body; m.o.i., multiplicity of infection; CADD, Chlamydia protein associating with death domains; zVAD-fmk, benzoyl-Val-Ala-Asp-fluoromethylketone; TNF, tumor necrosis factor; ORF, open reading frame; PBS, phosphate-buffered saline; RT, reverse transcription; RTase, reverse transcriptase; HA, hemagglutinin; AFC, 7-amino-4-trifluoromethylcoumarin; PQQC, PQQ synthesis protein C; GFP, green fluorescent protein; GST, glutathione S-transferase; synthesis protein C; GFP, green fluorescent protein; GST, glutathione S-transferase.
glutathione-Sepharose (Amersham Biosciences, Inc.).

**Protein Binding Assays—Plasmids containing various DD-containing proteins were in vitro transcribed and translated in the presence of L-[35S]methionine using the TNT kit from Promega. GST-CADD and control GST-CD40 (cytosolic domain) fusion proteins were immobilized on glutathione-Sepharose at 1 µg and incubated with in vitro translates target proteins for 1 h at 4 °C. Beads were then washed in three times in 1 ml of 140 mM KCl, 20 mM Hepes, pH 7.5, 5 mM MgCl2, 2 mM EGTA, 0.5% Nonidet P-40 and analyzed by SDS-PAGE/fluorography.

**Co-immunoprecipitation and Immunoblotting—HEK293 cells (5 × 106) cultured in the presence of 50 µM Z-VAD-fmk (Enzyme Systems Products) were co-transfected with 1 µg of plasmid DNA using a lipofection reagent (Geneporter, Gene Therapy Systems). At 24 h posttransfection, cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P40, 10% glycerol, and complete protease inhibitor mixture (Roche Molecular Biochemicals)) for 15 min on ice. After extensive preclearing immunoprecipitations with 2 µg of anti-Myc (Santa Cruz Biotechnology), polyclonal rabbit anti-Trail R-2 (Alexis), monoclonal anti-FLAG (Sigma), or control mouse IgG (DAKO)-conjugated Sepharose beads were performed for 4 h at 4 °C. Beads were then washed four times in 1 ml of lysis buffer and boiled in Laemmli solution before performing SDS-PAGE/immunoblotting using monoclonal mouse anti-Myc (Zymed Laboratories Inc.), polyclonal rabbit anti-Trail R-2 (Alexis), monoclonal anti-FLAG (Sigma), or control mouse IgG (DAKO)-conjugated Sepharose beads for 48 h at 4 °C. The relative location of these genes in the C. trachomatis genome is indicated below (base pairs).

**RESULTS**

The genomes of C. trachomatis, Chlamydia pneumoniae, and Chlamydia muridarum contain ORFs encoding hypothetical proteins sharing sequence similarity and predicted structural homology to DDs. In C. trachomatis, the predicted CADD protein is encoded in a continuous ORF representing a protein of 231 amino acids (Fig. 1). This protein has been annotated in the NCBI Chlamydia genome data base as CT610, a hypothetical protein of hitherto unknown function. CT610 is located on the complementary DNA strand close to the Chlamydia rpoD gene encoding the major sigma factor (σ70) (Fig. 1C). Amino acids 75–154 from CADD are strikingly similar to the DDs of human DR5 (26% identity, 37% similarity) and human DR4 (29%, 37%) followed by human Fas (25%, 33%). This degree of sequence identity is comparable to the homology shared among the DDs of the human TNF receptor family members.

The CADD protein also shares overall sequence homology with coenzyme PQQC family members, which have been shown to be necessary for PQQ synthesis in Klebsiella (12).

**Cell Culture, Transfections, Apoptosis Measurements, and Caspase Assays—HEK293, HeLa, HepB3, and COS7 cells (106) were transfected as described above. Both floating and adherent cells were recovered 1 day later and pooled, and the percentage of transfected (green fluorescent) cells with nuclear apoptotic morphology was determined by staining with 0.1 µM 4′,6-diamidino-2-phenylindole (3). Cytosolic extracts from HeLa and HepB3 cells were assayed for Caspase activity, measuring release of 7-amino-4-trifluoromethylcoumarin (AFC) from Ac-DEVD-AFC (Calbiochem) as described previously (10).

**Generation of CADD Antibody—BALB/c mice were injected intraperitoneally with 20 µg of recombinant CADD with Freund’s complete adjuvant. This was repeated after 2 weeks followed by two additional boosters with CADD in incomplete adjuvant at day 28 and day 42. Serum was collected at day 60. Prior to immunization the GST tag of CADD was removed by thrombin cleavage.

**Immunofluorescence Confocal Microscopy—HeLa cells were cultured on four-well, covered chamber slides (Naîge Nunc) until reaching 70% confluence. At various times postinfection (m.o.i. = 0.5), cells were washed with PBS and then fixed with methanol/acetone (50%, v/v) for 5 min on ice. After washing with PBS, specimens were incubated with either 0.1% (v/v) polyclonal rabbit anti-Fas (Santa Cruz Biotechnology sc-715, directed against a C-terminal peptide), monoclonal mouse anti-Fas (Transduction Laboratories F-22120, directed against the N terminus of Fas), polyclonal rabbit anti-EB (BiosPacific), 0.5% (v/v) polyclonal mouse anti-CADD, preimmune mouse serum, or combinations of these antibodies. Secondary antibodies conjugated with fluorescein or rhodamine (Molecular Probes) were then applied, and confocal microscopy was performed using a two-photon system (MRC 1024) (Bio-Rad).

**To determine whether CADD is actually expressed in infected cells, RT-PCR analysis was performed, monitoring CADD gene expression as a function of time during the course of Chlamydia infection (13).** For these experiments, HeLa cell cultures were inoculated with C. trachomatis, and RNA was recovered at various times thereafter for RT-PCR analysis. As shown in Fig. 2, mRNA corresponding to CADD became detectable at ~36 h after infection, reaching maximum levels at 48–72 h. In contrast, expression of bacterial groEL was detectable within 4 h after inoculation of HeLa cell cultures with Chlamydia (Fig. 2C). Thus, CADD is expressed late in the Chlamydia infectious cycle. Not only was the CADD mRNA expressed in bacteria-infected cells, but the CADD protein was also demonstrated by immunoblotting using a CADD-specific antisera (Fig. 2E).

**Transfection experiments were performed to test the effects of CADD on apoptosis in mammalian cells. When transiently transfected into various cell lines, including HeLa (cervical), HepB3 (liver), COS7 (kidney), or Jurkat (T-lymphocyte) cells, plasmids producing CADD induced apoptosis to an extent comparable to prototypical apoptotic stimuli such as Bax, Fas, and DR5 (Fig. 3 and data not shown).** The morphology of the dying cells was typical of apoptosis with markedly condensed chromatin, fragmentation of the nucleus, membrane blebbing, cell rounding, and shrinkage. Similar results were obtained regardless of whether CADD was expressed with a Myc epitope tag or fused with GFP (Fig. 3). A fragment of CADD (amino acids 75–154) corresponding to the region sharing sequence similarity with DDs was sufficient for apoptosis induction (Fig. 3A), analogous to results obtained previously for many mammalian proteins that contain DDs. In contrast to CADD, ectopic expression of the Klebsiella PQQC protein that shares 27% amino...
FIG. 2. CADD expression in infected HeLa cells. Cultures of HeLa cells were inoculated with C. trachomatis EBs (m.o.i. = 1). At various times thereafter, cells were analyzed for CADD expression. A–D, RNA was isolated and used for RTase reactions using gene-specific primers. The resulting cDNAs were PCR-amplified with primers specific for the CADD-encoding gene (CT610) (A and B), bacterial groEL-1 (C), or human β-actin (D). Time (hours) after EB exposure to host cells is indicated above each lane. The control lane (c) refers to uninfected HeLa cells. The lanes labeled (–) and (+) indicate control PCRs supplied, respectively, with no template cDNA or with either genomic DNA purified from Chlamydia (A–C) or a plasmid containing human β-actin cDNA (D). Omitting RTase in B confirmed that no residual Chlamydia genomic DNA was present in the RNA preparations analyzed. GroEL-1 served as a marker of C. trachomatis infection (C), demonstrating that expression of this bacterial gene is present within 4 h after inoculation and persists late into the infection. E, detergent lysates were prepared from HeLa (lanes 1 and 2) and McCoy (lanes 3 and 4) cells after 48 h of culturing with (+) or without (−) Chlamydia exposure, normalized for total protein content (20 μg/lane) and analyzed by SDS-PAGE/immunoblotting using CADD-specific antisera. Right side: 1, 5, and 10 ng of recombinant CADD was loaded as a control. rec., recombinant; hu, human.

FIG. 3. Apoptosis induction and Caspase activation by CADD in human cells. HeLa (A and C) or Hep3B (B and D) cells were transfected with plasmids Myc-tagged CADD, GFP-CADD fusion protein, GFP-CADD (amino acids 75–154), GFP-DR5 (DD only), Bax, Fas, CrmA, or GFP alone or in combination, normalizing total DNA content. In some cases, 50 μM zVAD-fmk was added to cultures. A and B, the percentage of GFP-positive cells with apoptotic morphology was determined by UV microscopic analysis of 4,6-diamidino-2-phenylindole-stained cells (mean ± S.D., n = 3) at 1 day after transfection. C and D, Caspase activity was measured in cell lysates at 18 h after transfection using the fluorogenic substrate Ac-DEVD-AFC. Data are expressed as relative fluorescence units (RFU/μg of total protein (mean ± S.D., n = 3) after a 30-min reaction, which was empirically determined to be within the linear phase of the reactions. C, control; aa, amino acids.

CADD also induced activation of Caspasas as determined by enzyme assays measuring activity of proteases capable of cleaving Ac-DEVD-AFC (Fig. 3, C and D). CADD-induced apoptosis and Caspase activation were blocked by addition to cultures of zVAD-fmk, an irreversible broad spectrum Caspase inhibitor, and by co-expressing the cowpox protein CrmA, a selective inhibitor of Caspase-1 and -8 (14) (Fig. 3). Immunoblotting experiments confirmed that zVAD-fmk and CrmA did not interfere with CADD protein production (not shown). Thus, CADD induces apoptosis through a Caspase-dependent mechanism.

CADD was tested for interactions in vitro with a variety of human DD family proteins, including TNF family death receptors (TNFR1, DR4, DR5, Fas, adapter proteins (FADD, RIP, and RAIDD), and c-FLIP). For initial experiments, CADD was produced as a GST fusion protein and incubated with 35S-labeled protein. GST-CADD, but not GST-CD40 or a variety of other control proteins, bound TNF family death receptors Fas, DR4, DR5, and to some extent TNFR1 (Fig. 4A). CADD, however, neither interacted in vitro with a Fas mutant lacking its DD nor with FADD, RAIDD, RIP, c-FLIP, or itself (CADD). Co-immunoprecipitation experiments demonstrated that CADD is also capable of specifically interacting with TNF family death receptors such as DR5 in mam-

acid sequence identity with CADD failed to cause apoptosis (Fig. 3A), demonstrating the specificity of CADD-induced cell death. Analysis of cells transfected with a plasmid encoding CADD protein revealed a cytosolic location for this protein (not shown).
Fig. 5. Immunofluorescence confocal microscopy. Analysis of endogenous CADD and Fas in HeLa cells (36–48 h postinfection) was performed. A, CADD antisera (green, anti-mouse IgG fluorescein isothiocyanate-labeled secondary antibody) and rabbit anti-Chlamydia (EB) (red, rhodamine-conjugated) were used. B, anti-Fas antibody (monoclonal antibody) (green) and rabbit anti-Chlamydia (EB) (red) were used. C, rabbit polyclonal Fas antibody (green) and CADD antisera (red) were used. Bars = 10 μm.

CADD Interacts with Death Receptors

Confocal microscopy was performed to determine the subcellular localization of CADD and death receptors in Chlamydia-infected cells. At 36–48 h postinfection, CADD was located at the periphery of the inclusion body (Fig. 5, top panels). Interestingly the control Chlamydia marker (anti-EB) only partially overlapped in distribution with CADD (see Fig. 5, merged images A), suggesting CADD is secreted from the bacteria. Furthermore, endogenous Fas accumulated in the vicinity of the Chlamydia inclusions. This result was confirmed by anti-Fas antibodies directed against two different nonoverlapping epitopes (Fig. 5, center and lower panels). Endogenous Fas and CADD were co-localized in these aggregations (Fig. 5, lower panels), whereas only partial overlap was seen for EBs and Fas (Fig. 5, center panels) or EBs and CADD (Fig. 5, top panels). These results suggest that Fas associates with CADD and is recruited to the vicinity of Chlamydia inclusions during infection.

DISCUSSION

It has long been recognized that viruses harbor genes that regulate apoptosis of host cells, making vital contributions to the virus life cycle (15). Similarly it seems likely that intracellular bacteria would also find it useful to regulate host cell apoptosis. Previous studies have established that infection of mammalian cells with Chlamydia species can either suppress or induce apoptosis, suggesting that these obligate intracellular bacteria possess genes capable of interfering with host cell apoptosis machinery (4, 16). Here we demonstrate that the C. trachomatis genome contains a gene encoding a protein capable of binding several DD-containing TNF family receptors. Ectopic expression of CADD induces Caspase activation and apoptosis of human cells. Closely related genes are also found in the genomes of other Chlamydia species, including C. pneumoniae, and C. muridarum, which create clinically significant infections in various species, suggesting evolutionary pressure for conservation of these genes.

The Chlamydia CADD protein is homologous to a coenzyme PQQC protein, which is necessary for PQQ synthesis in Klebsiella (12), but in Chlamydia species, most of the relevant PQQ synthetase genes are missing, and the remaining genes do not form an operon with the CADD gene. At this point it is unclear whether CADD possesses PQQ synthetase activity. However, a fragment of CADD containing only the region sharing sequence similarity with DDs was sufficient to induce apoptosis, suggesting that the enzymatic function is not critical for this function.

Our study demonstrates an association of CADD and Fas during Chlamydia infection in vivo. Fas was found to be recruited to the vicinity of the inclusion body where it co-localized with CADD, supporting a physiological role of this protein in regulating death receptor signaling. Intriguingly we found no evidence of apoptosis in Chlamydia-infected cells even after CADD expression and CADD-Fas co-localization. Recent reports (16, 17), indicate that Chlamydia infection efficiently blocks Fas-induced apoptosis. In this setting the role of CADD during infection may differ from the observed effects it exerts in an ectopic expression model. CADD would then bind Fas to prevent apoptosis by recruiting Fas to the Chlamydia inclusion. In contrast, during transient transfection where CADD is expressed from plasmids, the CADD protein localizes diffusely through the cytosol of cells. Since CADD binds the cytosolic domains of DD-containing TNF family death receptors, we presume that ectopic CADD expression triggers Caspase activation and apoptosis by activating these receptors in a ligand-independent fashion.

Thus, differences in subcellular localization may be of critical importance in dictating whether CADD has an inhibitory versus a stimulatory effect on host cell apoptosis. Future studies will determine whether Chlamydiae rely on their CADD-encoding genes as part of their virulence mechanisms.

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