CC2D1A, a DM14 and C2 Domain Protein, Activates NF-κB through the Canonical Pathway

Meng Zhao, Xiao-Dong Li, and Zhijian Chen

From the Department of Molecular Biology and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9148

CC2D1A is an evolutionarily conserved protein that contains four DM14 domains at the N terminus and a C2 domain at the C terminus. Loss-of-function mutations in CC2D1A have been linked to mental retardation in human, but the biochemical function of this protein is largely unknown. Here, we show that CC2D1A is a potent activator of NF-κB. The activation of NF-κB by CC2D1A requires its C2 domain. CC2D1A activates NF-κB in a manner that depends on the ubiquitin-conjugating enzyme Ubc13, TNF receptor-associated factor TRAF2, the protein kinase TAK1, and the IkB kinase (IKK) complex. In addition, the deubiquitination enzyme Cylindromatosis (CYLD) negatively regulates the activity of CC2D1A. These results suggest that CC2D1A activates NF-κB through the canonical IKK pathway.

NF-κB (nuclear factor κ enhancer binding protein) is a family of transcription factors that regulate immunity, inflammation, and cell survival (1, 2). Under basal conditions, NF-κB dimers are sequestered in the cytoplasm by the inhibitors of NF-κB (IκB) proteins. Therefore, a prerequisite of NF-κB activation is to degrade IκB proteins or to remove the inhibitory domains of NF-κB precursors.

The NF-κB activation pathways can be categorized into canonical and noncanonical pathways. In canonical pathways, IκB proteins are degraded, whereas in noncanonical pathways, the NF-κB precursors p100 and p105 are processed into mature forms. Most NF-κB activation stimuli such as proinflammatory cytokines and microbial ligands go through canonical pathways. Upon binding of ligands to their cognate receptors, the signal is transduced through adaptors such as tumor necrosis factor receptor-associated factor (TRAF)2 proteins to the protein kinase TGFβ-activated kinase 1 (TAK1). TAK1 then phosphorylates and activates the IκB kinase complex (IKK), which consists of IKKα, IKKβ, and an essential regulatory subunit NF-κB essential modulator (NEMO). Phosphorylation of IκB at serines 32 and 36 by IKK leads to its ubiquitination and eventually degradation by the proteasome. The liberated NF-κB dimers translocate into the nucleus and turn on gene expression. The noncanonical pathway is activated by a subset of receptors in B cells, such as CD40 and B cell-activating factor receptor. The activation of these receptors results in the activation of IKKα, which phosphorylates p100 (3). In both pathways, IKK activation is pivotal and is regulated by ubiquitination.

Ubiquitin is a highly conserved protein in eukaryotes consisting of 76 amino acids (4). The enzymatic reactions of ubiquitination are carried out by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin protein ligases (E3s) in an ATP-dependent manner (5, 6). Ubiquitin is conjugated to the target via an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine of the target. Ubiquitin itself has seven lysines; therefore, it can be further assembled into polymeric chains (7). Polyubiquitin chains linked through different lysines have different topology and functions. Lys-48 linked polyubiquitination precedes degradation of IκB proteins and processing of NF-κB precursors through a proteasome-dependent mechanism. By contrast, Lys-63 linked polyubiquitination regulates TAK1 and IKK activation independent of the proteasome. Deubiquitination is carried out by a large family of deubiquitination enzymes (8, 9). Several deubiquitination enzymes such as CYLD and A20 function as inhibitors of IKK in the NF-κB pathways, reaffirming that ubiquitination is an important regulator for protein kinase activation.

In this study, we characterize a new NF-κB activator, CC2D1A, also known as Freud-1/Aki-1 (10, 11). We show that CC2D1A activates NF-κB in a TRAF2, TAK1, and IKK-dependent manner. In addition, NF-κB activation by CC2D1A requires the ubiquitin E2 Ubc13 and is inhibited by CYLD. These results suggest that CC2D1A activates NF-κB through the canonical IKK pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Proteins**—Human CC2D1A sequence was amplified by PCR using the IMAGE clone 6585236 (ATCC) as a template and then subcloned into the mammalian expression vector pcDNA3 (Invitrogen) in-frame with an N-terminal FLAG tag. The truncated forms of CC2D1A were cloned using similar strategy. C2 domain and DM14 domain deletion mutants were generated by overlap extension PCR. pcDNA3-FLAG-CYLD was described previously (12). All constructs were...
verified by automated DNA sequencing. Plasmids for p-κB3-TK-Luc and pCMV-LacZ were described previously (13).

**Antibody**—To generate polyclonal antibody against human CC2D1A, a recombinant protein containing residues 263–512 of human CC2D1A was expressed in *Escherichia coli* as a His-tagged protein and affinity-purified. The CC2D1A protein was used to immunize rabbits (Rockland), and the resulting antibody was affinity-purified using an antigen column. The antibodies for TRAF2, TRAF6, IKKα, NEMO, and TAK1 were from Santa Cruz Biotechnology. The antibodies for IKKβ, Ubc13, GST (4C10), and FLAG (M2) are from BD Biosciences, Zymed Laboratories, Inc., Covance, and Sigma, respectively.

**Cell Culture, Transfection, and Reporter Gene Assay**—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (penicillin G (100 μg/ml) and streptomycin (10 μg/ml)). Transfection of HEK293 cells was carried out by calcium phosphate precipitation. For luciferase reporter assays, cells were seeded in 12-well plates at a density of 2 × 10^5 cells per well. On the second day, cells were co-transfected with 50 ng of p-κB3-TK-Luc reporter gene, 25 ng of pCMV-LacZ, and 350 ng of the indicated expression vectors. Each experiment was carried out in duplicate. Cells were harvested 48 h after transfection and lysed in the passive lysis buffer (Promega). Luciferase activity was measured with a luminometer (Roys Anthos Lucy2) using luciferin as a substrate, and β-galactosidase activity was measured with a Thermo Labsystems microplate reader (Thermo Fisher Scientific) at the wavelength of 420 nm using o-nitrophenyl-β-D-galactopyranoside as a substrate.

U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. The U2OS cell line was stably incorporated with short hairpin RNA expression vector or rescue vector under the control of tetracycline-inducible promoter (14). After tetracycline treatment for 5 days, the cells were seeded in 12-well plates at a density of 2 × 10^5 cells per well. On the sixth day, cells were transfected with 100 ng of the p-κB3-TK-Luc reporter gene, 50 ng of pCMV-LacZ, and 350 ng of the indicated expression vectors. On the eighth day, luciferase assay was carried out as mentioned above.

**IKK Kinase Assay**—To measure IKK activity following expression of CC2D1A, HEK293T cells were transfected with either pcDNA3-CC2D1A or control pcDNA3 using Lipofectamine 2000 (Invitrogen). After 14 h, cells were resuspended in a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 20 mM 2-glycerol-phosphate, 1 mM Na_3VO_4, and protease inhibitor mixture from Roche Applied Science). The IKK complex was immunoprecipitated with anti-NEMO antibody conjugated to protein A/G-Sepharose. The beads were washed with lysis buffer and then kinase buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 20 mM 2-glycerol-phosphate, 1 mM Na_3VO_4, 0.1 mM ATP, 10 mM MgCl_2, and protease inhibitor mixture from Roche Applied Science). 90% of the washed beads were incubated with 1 μg of GST-1κBαNT and γ-32P-ATP in 10 μl of the kinase buffer at 30 °C for 30 min. The reaction mixtures were subjected to 12% SDS-PAGE and analyzed using a PhosphorImager. 10% of the washed beads were used to examine IKKβ on Western blot.

**RT-PCR**—HEK 293 cells were transfected with CC2D1A expression vector or empty vector using Lipofectamine 2000 (Invitrogen). After 18 h, total RNA was isolated by using the TRIzol LS reagent (Invitrogen) and treated with TURBO DNA-free (Ambion) to clear residual genomic DNA contamination. Same amount of total RNA was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad). The sequences of the primers are as follows: MCP-1, GCCCGAATTCTACCTGCTGTAT (forward) and TTGGCCACAAATGTTCTTGAA (reverse); TNFα, AGGCCGTGCTTTGCTTCTCA (forward) and TGGGCCAGGGCTGTATTA (reverse); and glyceraldehyde-3-phosphate dehydrogenase, AAAATCAAGTGGGGCGATGCT (forward) and GGGCGAGATGATGACCCCTTT (reverse). RT-PCR products were separated by 2% agarose gel electrophoresis.

**RNA Interference**—siRNA oligonucleotides at a final concentration of 20 nM were transfected into HEK293 cells using the calcium phosphate precipitation method. On the second day, cells also were transfected with expression plasmids together with siRNA. Cells were harvested on the fourth day for analysis. The sequences of the siRNA oligonucleotides are as follows (only the sense strands are shown): green fluorescent protein (471–489), GCAGAGAAGCAGCAUCAUG; TRAF2 (1167–1185), GAUGUGUCUGUGAUCAUG; IKKα (1901–1919), GCUGACAAUACUGUCAGU; IKKβ (507–525), UGCCEAAGGAGUGUGAUGGUGAU; NEMO (832–850), ACAGGAGGUGUCGUACGUAG; and CYLD (1744–1762), GAAGGCUUGGAAGUAUGATGA. These RNA oligonucleotides were synthesized at the University of Texas Southwestern Center for Biomedical Invention facility. TAK1 and NF-κB Inducing Kinase (NIK) siRNA were from Dharmacon: TAK1 (280–298), GAGGAAA-GCCUGUAAUGUAAG; and NIK (1161–1179), GCCAGUGGAGUUAUGUAC.

**GST Pulldown Assay**—GST-tagged Ubc13 and Ubc5 were expressed in bacteria, pulled down by glutathione-Sepharose 4B (GE Healthcare) and eluted with glutathione. FLAG-tagged CC2D1A and mutants were overexpressed in HEK293T cells, pulled down using anti-FLAG-M2-agarose (Sigma), and eluted using FLAG peptides. The final GST and FLAG-tagged proteins were in dilution buffer (50 mM Tris-Cl (pH 7.50), 50 mM NaCl, 0.1% CHAPS, and protease inhibitor from Roche Applied Science). The mixture of GST- and FLAG-tagged proteins was rotated at 4 °C for an hour before addition of glutathione beads. After an additional 0.5 h, the beads were washed with dilution buffer three times, spun down by centrifugation, and boiled in SDS sample buffer. The proteins were resolved by SDS-PAGE.

**Yeast Two-hybrid Screen**—Matchmaker™ GAL4 two-hybrid system (Clontech) was used to do the screen following instructions from Clontech. The bait encoding amino acids 215–405 of CC2D1A was cloned into pGBK7 and expressed as a fusion to the GAL4 DNA binding domain, whereas a HeLa cDNA library was in pGADT7 vector and expressed as a fusion to the GAL4 activation domain. The AH109 yeast strain was used as the host strain, and Tyr-187 was used as a mating partner to verify protein interactions.
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**Multiple Alignments**—The multiple alignment file was generated by ClustalW2. The shaded alignment was carried out by BoxShade.

**RESULTS**

**CC2D1A Is a Potent NF-κB Activator**—To identify NF-κB activators, Matsuda *et al.* carried out a large scale overexpression screen of human genes (15). In total, 58 “named” genes and 28 “novel” genes activated the NF-κB luciferase reporter gene in the screen. Among “novel genes,” clone 031N was later identified as an essential antiviral adaptor MAVS, which mediates the activation of NF-κB and IRF3 in response to viral infection (16). MAVS is a membrane protein on the mitochondria, and its localization is critical for its function, implying the involvement of mitochondria in innate immunity. Interestingly, there are five “novel genes” annotated with potential mitochondrial localization. We cloned each of these genes in mammalian expression vector pcDNA3-FLAG and tested their ability to activate interferon-β and NF-κB in HEK293T cells. Although none of them induced interferon-β (data not shown), clone 023N potently induced the NF-κB luciferase reporter (Fig. 1B).

Clone 023N is known as CC2D1A/Freud-1/Aki-1, which contains four DM14 domains at the N terminus and a C2 (CalB) domain at the C terminus (Fig. 1A). DM14 domain (*Drosophila melanogaster* 14 domain) is unique to this protein family, and its function has not been characterized. Protein kinase C conserved region 2 (CalB) is the Ca\(^{2+}\)-binding motif present in phospholipases, protein kinase C, and various synaptic proteins. Five conserved aspartic acids of C2 domains are critical for calcium binding (17) but are absent from the C2 domain of CC2D1A, indicating it may not bind Ca\(^{2+}\). However, the *Drosophila* ortholog of CC2D1A, Lgd (Lethal (2) giant discs), has been shown to bind phospholipids present on early endosomes (18). Therefore, it is possible that CC2D1A binds phospholipids independently of Ca\(^{2+}\). CC2D1A is a highly conserved protein from worm to human (see supplemental Fig. 1), especially at the C2 domain, suggesting its functional importance.

To determine whether CC2D1A activates IKK, we transfected pcDNA3-FLAG—CC2D1A into HEK293T cells and then immunoprecipitated the IKK complex from cell lysates using NEMO antibody. Indeed, IKK from CC2D1A—transfected cells was able to phosphorylate IκBα (Fig. 1C). Wild type or mutant (S32A/S36A) IκBα was able to block the activation of NF-κB by CC2D1A (Fig. 1D), indicating that CC2D1A activates the canonical NF-κB pathway in which IκBα is degraded. RT-PCR analysis showed that CC2D1A expression induced the NF-κB target genes tumor necrosis factor α (TNFα) and MCP-1 (monocyte chemoattractant protein 1; Fig. 1E). These results show that CC2D1A activates IKK and induces NF-κB target genes.

To test whether autocrine factors such as TNFα are responsible for the NF-κB luciferase activity induced by CC2D1A, we collected the medium 24 h after transfection and carried out NF-κB luciferase assay. The conditioned medium from CC2D1A—transfected cells failed to turn on the reporter (supplemental Fig. 2), suggesting that CC2D1A activates NF-κB independently of autocrine factors.

**Conserved Domains of CC2D1A Are Important for NF-κB Activation**—Although NF-κB can be activated by a variety of proteins in many different signaling pathways, CC2D1A is unique in that it contains conserved DM14 and C2 domains that have not been reported to mediate NF-κB activation. To determine whether these conserved domains are important for NF-κB activation, we constructed a series of CC2D1A mutants with progressive truncations from the N and C termini and tested the ability of each construct to activate NF-κB (Fig. 2A). Deletion of N-terminal 138 amino acids decreased the activity to ~50% (ΔN1), as did deletion of the first two DM14 domains (ΔN5). Further deletions (ΔN6–ΔN10) resulted in <20% activity. Therefore, the N-terminal DM14 domains are important for NF-κB activation. However, deleting any DM14 domain (ΔDM14 I, II, III, and IV; Fig. 2B) alone did not have much effect, suggesting that four DM14 domains may compensate for each other. Deletions from C terminus were more detrimental. Removal of the C-terminal 181 amino acids almost completely abrogated the ability of CC2D1A to induce NF-κB (ΔC1, Fig. 2A); Deletion of the C2 domain alone (ΔC2 only; Fig. 2B) also dramatically reduced its activity. Therefore, the C2 domain and the C terminus are crucial for NF-κB activation.

**IKK Complex, TRAF2, and TAK1 Are Required for CC2D1A to Activate NF-κB**—IKK complex consists of catalytic subunits IKKα and IKKβ and an essential regulatory subunit NEMO. Upstream of IKK, TRAF proteins and the transforming growth factor (TGF)-β-activated kinase (TAK1) play key roles in the canonical pathway of NF-κB activation. Protein kinase NIK is critical in the noncanonical pathway of NF-κB activation. To determine whether IKK, TRAF2, TRAF6, TAK1, and NIK are important for NF-κB activation by CC2D1A, we used siRNA to knock down the expression of each protein. RNA interference of IKKα, IKKβ, NEMO, TRAF2, and TAK1, but not TRAF6 and NIK inhibited NF-κB activation by CC2D1A (Fig. 3). These results suggest that CC2D1A activates NF-κB and IKK through a TRAF2- and TAK1-dependent pathway.

**Ubc13 Is Required for CC2D1A to Activate NF-κB**—To identify interacting partners of CC2D1A, we carried out a yeast two-hybrid screen using the second and third DM14 domains as bait and identified Ubc13 as a CC2D1A binding protein. Ubc13 forms an E2 ubiquitin-conjugating enzyme complex with Uev1A. This E2 complex catalyzes the synthesis of K63-linked polyubiquitin chains, which are important for TAK1 and IKK activation (13, 19). To test the functional involvement of Ubc13, we used tetracycline-inducible short hairpin RNA to deplete endogenous Ubc13 in the human osteosarcoma cell line U2OS (Fig. 4A). We also generated another U2OS cell line in which depletion of endogenous Ubc13 was accompanied by the expression of wild-type Ubc13, whose expression was also controlled by a tetracycline-inducible promoter (14). After treatment in the presence or absence of tetracycline, these cells were transfected with NF-κB-luciferase reporter together with expression vectors for CC2D1A, TRAF6, or NIK. NF-κB activation by CC2D1A and TRAF6 was reduced markedly when cells were depleted of Ubc13 and TAK1, but not TRAF6 and NIK inhibited NF-κB activation by CC2D1A (Fig. 3). These results suggest that CC2D1A activates NF-κB and IKK through a TRAF2- and TAK1-dependent pathway.
down Ubc13 did not affect NIK-induced NF-κB activity. These results indicate that CC2D1A requires Ubc13 to activate NF-κB.

We also confirmed the interaction between Ubc13 and CC2D1A by GST pulldown assay. CC2D1A has higher binding affinity for GST-Ubc13 than GST-Ubc5 or GST alone (Fig. 4E).

We went on to test the interaction between Ubc13 and several CC2D1A mutants—ΔDM14 I, ΔC2-only, and ΔN5. Fig. 4F shows the FLAG proteins used in the pulldown assay. Like the full-length CC2D1A, ΔDM14 I, and ΔN5 also preferably bind to Ubc13, whereas ΔC2-only does not (Fig. 4E). Interestingly, ΔDM14 I and ΔN5 activate NF-κB luciferase reporter compa-
rably to CC2D1A, whereas ΔC2-only barely turns on the reporter (Fig. 2). Given that Ubc13 is required for CC2D1A to activate NF-κB, the C2 domain may be critical in maintaining the proper protein structure, which is necessary for binding to Ubc13.

Deubiquitination Enzyme CYLD Negatively Regulates CC2D1A—CYLD is a ubiquitin protease that specifically cleaves Lys-63-linked polyubiquitin chains (12, 20–22). To determine whether Lys-63 polyubiquitination is involved in NF-κB activation by CC2D1A, we co-transfected CC2D1A and wild-type or catalytically inactive mutant of CYLD into HEK293T cells and measured NF-κB activation (Fig. 5, A and B). Wild-type but not the mutant CYLD strongly inhibited NF-κB activation by CC2D1A, whereas RNA interference of CYLD enhanced NF-κB activation by CC2D1A (Fig. 5C). These results, together with our finding that Ubc13 is required for NF-κB activation by CC2D1A, suggest that Lys-63 polyubiquitination plays an important role in NF-κB activation in this pathway.

**DISCUSSION**

CC2D1A was first identified by Matsuda et al. (15) as an NF-κB activator through a large scale screen of human genes. Although CC2D1A was annotated as a mitochondrial protein, our analyses using subcellular fractionation and immunofluorescent microscopy indicate that it is predominantly in the cytoplasm (data not shown). Nevertheless, CC2D1A is one of the most potent activators of NF-κB when it is overexpressed in cells. Furthermore, it has unique DM14 and C2 domains that have never been observed in other NF-κB activators. It is therefore interesting to understand how CC2D1A activates NF-κB.

In this study, we show that CC2D1A activates NF-κB through a canonical signaling cascade involving TRAF2, Ubc13, TAK1, and IKK. The involvement of Ubc13 in this pathway suggests that Lys-63 polyubiquitination is involved in IKK activation by CC2D1A. This is further supported by our data that the Lys-63-specific deubiquitinase CYLD inhibits NF-κB activation by CC2D1A.
FIGURE 3. **CC2D1A requires IKK, TRAF2, and TAK1 to activate NF-κB.** A, siRNAs targeting green fluorescent protein (GFP), TRAF2, TRAF6, IKKα, IKKβ, and NEMO were transfected into HEK293T cells followed by transfection of CC2D1A expression plasmid and NF-κB luciferase reporters. The endogenous proteins were examined for RNA interference efficiency. The overexpression of CC2D1A was examined by Western blotting. IB, immunoblot; MW, molecular weight.

B, siRNAs targeting TAK1 and NIK were transfected into HEK293T cells. These cells were then transfected with CC2D1A expression plasmid and NF-κB luciferase reporters as in A. The endogenous TAK1 was examined to test the RNA interference (RNAi) efficiency, whereas FLAG-NIK was used to test the efficiency of siRNA-targeting NIK.
NF-κB is activated by a large variety of agents in different cell types. An important question that remains to be resolved is the specific signaling pathway in which CC2D1A plays a role. We have examined the potential role of CC2D1A in NF-κB activation by different agents, including TNFα, interleukin-1β, conditions that cause endoplasmic reticulum stress, as well as various agonists of Toll-like receptors, epidermal growth factor receptor and T cell receptor. So far, we have no evidence in support of a role of CC2D1A in signaling by any of these agents. Given the unusual domain structure of CC2D1A, it is possible that this protein is involved in a novel pathway of NF-κB activation. It also is possible that CC2D1A plays a role in NF-κB activation in specific cell types (e.g. neuronal cells; see below).

Mutations in the gene encoding CC2D1A have been associated with nonsyndromic mental retardation in human (23). The mutation is a deletion of 3567 nucleotides from introns 13 to 16, abolishing the fourth DM14 domain and C2 domain. We have tested a similar mutation in NF-κB luciferase assay (Fig. 2C). It retains ~20% activity of the full-length protein, suggesting that this is a hypomorphic mutation.

NF-κB activity is prominent in the central nervous system throughout development. Several brain-specific factors such as the neurotransmitter glutamate and neurotrophins (24) have been shown to activate NF-κB. Genetic evidence indicates that NF-κB is involved in learning and memory (25, 26). Future studies should determine if and how CC2D1A regulates NF-κB in the brain.
It is likely that the functions of CC2D1A are not limited to NF-κB activation. Indeed, the Drosophila ortholog of CC2D1A, known as Lgd (Lethal (2) giant discs), is a negative regulator of Notch signaling and involved in endosomal trafficking (18, 27, 28). Further studies of CC2D1A in an animal model should help delineate the physiological functions of this unusual protein and provide insights into human diseases such as mental retardation.
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