A Novel Enhancer of the Apaf1 Apoptosome Involved in Cytochrome c-dependent Caspase Activation and Apoptosis*

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Apaf1/CED4 family members play central roles in apoptosis regulation as activators of caspase family cell death proteases. These proteins contain a nucleotide-binding (NB) self-oligomerization domain and a caspase recruitment domain (CARD). A novel human protein was identified, NAC, that contains an NB domain and CARD. The CARD of NAC interacts selectively with the CARD domain of Apaf1, a caspase-activating protein that couples mitochondrion-released cytochrome c (cyt-c) to activation of cytosolic caspases. Cyt-c-mediated activation of caspases in cytosolic extracts and in cells is enhanced by overexpressing NAC and inhibited by reducing NAC using antisense/DNAzymes. Furthermore, association of NAC with Apaf1 is cyt-c-inducible, resulting in a mega-complex (>1 MDa) containing both NAC and Apaf1 and correlating with enhanced recruitment and proteolytic processing of pro-caspase-9. NAC also collaborates with Apaf1 in inducing caspase activation and apoptosis in intact cells, whereas fragments of NAC representing only the CARD or NB domain suppress Apaf1-dependent apoptosis induction. NAC expression in vivo is associated with terminal differentiation of short lived cells in epithelia and some other tissues. The ability of NAC to enhance Apaf1-apoptosome function reveals a novel paradigm for apoptosis regulation.

CED4 family proteins constitute a unique family of caspase-activating molecules. The founding member of this family, CED4, was discovered in the nematode Caenorhabditis elegans in screens for genes that are essential for developmental programmed cell death (1). CED4 contains an N-terminal CARD1 followed by an NB domain, the latter containing classical Walker A and B box motifs recognized as important in binding nucleotide triphosphates. CED4 functions as an activator of the caspase, CED3, in vitro and in vivo (2, 3). The NB domain of CED4 oligomerizes in an ATP-dependent manner (4, 5), whereas the CARD binds a complementary N-terminal CARD found in the zymogen proform of CED3 (6). Protease activation is thought to result from the induced proximity of CED3 zymogens bound to oligomerized CED4, where the weak intrinsic protease activity of the proenzymes is sufficient for trans-proteolysis of closely juxtaposed pro-caspases (4, 7). Proteolytic cleavage of pro-CED3 then produces the large and small subunits of the heterotetrameric, autonomously active enzyme.

The closest homologue of CED4 identified in humans and other mammals thus far is Apaf1 (apoptosis protease-activating factor-1) (8). Similar to CED4, the Apaf1 protein contains a CARD, followed by an NB domain that shares significant amino acid sequence identity with the NB domains of CED4 and a family of ATPases associated with pathogen resistance (R genes) in plants (3, 5, 9), thus constituting the NB-ARC (Apaf-1/R gene/CED4) domain family (also known as NACHT domain). Unlike CED4, however, the NB-ARC domain of Apaf1 is followed by multiple WD repeats. These WD domains participate in auto-repression of Apaf1, locking it into an inactive, unoligomerized state until bound by cyt-c. In response to multiple cell death stimuli, changes in mitochondrial membrane permeability result in release of cyt-c into the cytosol, where it binds and activates Apaf-1, thus coupling mitochondrial damage to a mechanism for caspase activation (10). Cyt-c, in conjunction with dATP or ATP, induces formation of a large Apaf1 oligomer (estimated to be an octamer), via its NB-ARC domain, and exposes the CARD of Apaf1 for interactions with a complementary CARD found in the N-terminal prodomain of pro-caspase-9 (11–14). By the induced proximity method, juxtaposed pro-caspase-9 (pro-Casp9) zymogens bind to oligomerized CED4, whereas the CARD binds a complementary N-terminal CARD found in the zymogen proform of CED3 (6). Protease activation is thought to result from the induced proximity of CED3 zymogens bound to oligomerized CED4, where the weak intrinsic protease activity of the proenzymes is sufficient for trans-proteolysis of closely juxtaposed pro-caspases (4, 7). Proteolytic cleavage of pro-CED3 then produces the large and small subunits of the heterotetrameric, autonomously active enzyme.

MATERIALS AND METHODS

cDNA Cloning and Plasmid Construction—The NAC cDNA sequence was found using PSI-BLAST and the CARD sequence of CARD/No1

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were incubated with 1 mg/ml bovine serum albumin in 100 times in 500 and affinity-purified using GSH-Sepharose. Purified GST fusion protein antibodies and protein G or A beads. Immune complexes were identified by centrifugation and subjected to immunoprecipitation using specific inhibitor mix (Roche Molecular Biochemicals). Cell lysates were clarified from pGEX-4T1 in XL-1-blue with cyt-c (10 μg) processed using buffer B (above) as described (21) and incubated (1.5 mg) for 30 min at 25 °C. The beads were then incubated overnight at 4 °C. The resultant cDNA fragments were ligated into mammalian expression vector pcDNA3-Myc. From these overlapping cDNA fragments, full-length NAC cDNA was assembled in pcDNA3-Myc and pcDNA3-HA at EcoRI and XhoI cloning sites. The nucleotide sequence of the assembled full-length NAC was confirmed by DNA sequencing analysis. The regions encoding the CARD or the NB domain (amino acids 329–547) were polymerase chain reaction-amplified from Jurkat cDNA using primer set 1 and the primers 5'-CCCGAATTCACGACCTGCAATGCGTACTGC-3' (forward) and CCGCTCGAGTCAACAGAGGGTTGTGGTGGTCTT-3' (reverse). The resultant polymerase chain reaction fragments were digested with EcoRI and XhoI and ligated into pcDNA3-Myc and into vector pEX4T1 for GST fusion protein production.

**Antibodies**—Polyclonal antisera were generated in rabbits using keyhole limpet hemocyanin- and ovalbumin-conjugated (Pierce) synthetic peptides with sequences corresponding to residues aa 161–180 (Bur241) or aa 1058–1077 (Bur242) of NAC. Mouse monoclonal antibody recognizing human APAF1 was purchased from R&D Systems (Minneapolis, MN). Epitope-specific antibodies for FLAG, HA, or Myc tag were obtained from Sigma, Roche Molecular Biochemicals, and Santa Cruz Biotechnology, respectively.

**DNAzymes**—Anti-NAC DNAzyme oligonucleotide was designed by the method of Joyce (19), targeting the translation initiation region of NAC mRNA and containing 2-O-methyluracil at the 5'-end and an inverted thymidine at the 3'-end for nuclease resistance. The sequences of the catalytic (AS) and control (C) noncatalytic oligonucleotides are as follows: AS, 5'-2-O-MeC2-C20-O-MeCAGCCAAGGGTTTGTTGGTC-TTG-3' (reverse), respectively. The resultant polymerase chain reaction fragments were digested with EcoRI and XhoI and ligated into pcDNA3-Myc and into vector pEX4T1 for GST fusion protein production.

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**Immunohistochemistry**—Normal human tissues for immunohistochemical analysis were obtained from biopsy and autopsy specimens, fixed in Bouin’s solution (Sigma), and embedded in paraffin. Tissue sections were immunostained using a diaminobenzidine-based detection method employing the Envision-Plus-horseradish peroxidase system (Dako). Nuclei were counterstained with hematoxylin.

**Coimmunoprecipitation and Immunoblotting Assays**—For immunoprecipitation and immunoblotting analyses, cells were lysed in either buffer A (5 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 0.4% Nonidet P-40, for whole cell extracts), all EDTA, for hypotonic lysis), or ELB (50 mM HEPES (pH 7.4), 250 mM NaCl, 5 mM EDTA, and 0.4% Nonidet P-40, for whole cell extracts), all supplemented with 1 mM dithiothreitol, 12.5 mM β-glycerol phosphate, 1 μg/ml Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml protease inhibitor mix (Roche Molecular Biochemicals). Cell lysates were clarified by centrifugation and subjected to immunoprecipitation using specific antibodies and protein G or A beads. Immune complexes were resolved in SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with antibodies followed by detection using ECL (Amer sham Pharmacia Biotech) (20).

**In Vitro Protein Interaction Assays**—GST fusion proteins were expressed from pGEX4T1 in XL-1 blue Escherichia coli cells (Stratagen e) and affinity-purified using GSH-Sepharose. Purified GST fusion proteins (0.1–0.5 μM) immobilized on 10–15 μM of GSH-Sepharose beads were incubated with 1 mg/ml bovine serum albumin in 100 μM of buffer A for 30 min at 25 °C. The beads were then incubated overnight at 4 °C with 1 μg/ml of rabbit reticulocyte lysozyme (Promega) containing 5% IVT mix. IVT proteins in 100 μl of buffer A supplemented with 0.5 mg/ml bovine serum albumin. Proteins on beads were washed four times in 500 μl of buffer A, followed by boiling in 20 μl of Laemmli-SDS sample buffer, SDS-PAGE and detection by fluorography.

**Gel-sieve Chromatography Analysis**—Cytosolic extracts were prepared using buffer B (above) as described (21) and incubated (1.5 mg) with cyt-c (10 μM) and dATP (1 mM) for 10 min at 30 °C, and then 100 μM ZVAD-fmk was added. The treated protein lysates were immediately fractionated by using a Superose-6 HR 10/30 gel filtration column in elution buffer containing 50 mM Tris (pH 7.4), 100 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol. Column fractions (0.5 ml) were analyzed for NAC and Apaf1 by SDS-PAGE, followed by immunoblotting.

**Caspase Assays**—Cytosolic extracts were prepared in hypotonic buffer B as described (21) and incubated (10 μg) with various concentrations of cyt-c and 1 mM dATP in Caspase buffer (21) for 30 min at 30 °C. Caspase substrate Ac-DEVD-AFC (100 μM) (Calbiochem) was then added, and protease activity was measured continuously by monitoring release of Fluorogenic AFC (21). Alternatively, transfected cells were directly lysed in Caspase Lysis buffer (10 mM HEPES (pH 7.4), 25 mM NaCl, 0.25% Triton X-100, and 1 mM EDTA), normalized for protein content, and monitored for cleavage of Ac-DEVD-AFC as described (21). Processing of IVT 35S-labeled pro-caspase-9 in cytosolic extracts was monitored by SDS-PAGE (20).

**Apoptosis Assays**—Cells were transfected with pEGFP (CLO-TECH) and effecter plasmids using SuperFect transfection reagents (Qiagen) as indicated. After culturing 1.5 days in media containing reduced serum (0.1% fetal bovine serum), floating and adherent cells (recovered by trypsinization) were pooled, and cells were fixed in 3.7% formaldehyde/PBS, stained with 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI), and the percentage of GFP-positive cells with apoptotic morphology (nuclear fragmentation, chromatin condensation) was determined by fluorescence microscopy (20, 21).

**Molecular Modeling**—A three-dimensional model of the CARD domain of NAC was generated using the MODELLER program, essentially as described (22), based on the structures of the CARDs of Apaf1, pro-Casp9, and Radd3 (23, 24).

**RESULTS AND DISCUSSION**

NAC-encoding cDNAs were obtained by reverse transcriptase-polymerase chain reaction, revealing a continuous open reading frame encoding a 1473-amino acid protein (Fig. 1). The predicted NAC protein contains an NB domain, followed by leucine-rich repeats (LRR), and a CARD domain. Thus, unlike Apaf1/CED4 family proteins that also contain CARD and NB domains, the CARD domain of NAC is located at its C rather than N terminus. The NB domain of NAC contains classical Walker A and B boxes indicative of ATP-binding proteins and is most similar in amino acid sequence to the NB domain of Nod1/CARD4 (29%) (25, 26), followed by human APAF1 (17%), the Drosophila Apaf1 homologue (12%), and the C. elegans CED4 protein (12%) (Fig. 1C). Moreover, recombinant NAC NB domain was observed to bind ATP and to self-associate in an ATP-dependent manner in vitro (not shown). The CARD domain of NAC shares 21, 19, and 8% amino acid identity with the CARD domains of Nod1/CARD4, huApaf1, and CED4, respectively (Fig. 1D). The NAC CARD sequence was readily threaded onto the structures of other CARDs using the MODELLER program (Fig. 1E), suggesting conservation of the 6-helical fold typical of these domains (23). The LRRs of NAC are reminiscent of Nod1/CARD4 and plant stress-response (R) proteins, which also contain LRRs. In NB-containing plant R proteins, the LRRs function as interaction motifs for pathogen responses (9), suggesting a possible role of these structures in linking NAC to specific signaling pathways. Additional NAC cDNAs were obtained that presumably represent alternative mRNA splicing products that encode shorter proteins lacking 31–45-amino acid segments (or both) located between the LRR and CARD (Fig. 1).

NAC mRNAs were widely expressed in human tissues, with highest levels found in blood leukocytes, thymus, spleen, and heart (Fig. 1A). A similar expression pattern is seen in the testis (Fig. 1B). A smaller anti-NAC immunoreactive band was detected in thymus lysates, which...
FIG. 1. Sequence analysis of NAC. A, schematic representation showing domain structure of human NAC. The NB domain (aa 329–547, filled box), the leucine-rich repeats (aa 808–947, filled bars), and the CARD (aa 1373–1473, dotted box) are depicted. Hatched boxes indicate sequences derived from two alternatively spliced exons. B, predicted amino acid sequences of human NAC. The positions for the P-loop (Walker A) and Walker B of NB domain are indicated. The amino acids sequences of LRR repeats and CARD are underlined and in bold letters, respectively. Italic letters indicate sequences for the alternatively spliced exons. C, alignment of the NB domain of NAC (aa 329–547) with NB domains of Nod1/CARD4 (aa 197–408), Apaf1 (aa 138–355), and C. elegans CED4 (aa 154–374). Alignment was conducted using ClustalW. Identical and similar residues are shown in black and gray shades, respectively. Positions of P-loop and Walker B sequences are indicated. D, alignment of CARDs of NAC (aa 1373–1465), Nod1/CARD4 (aa 15–104), Apaf1 (aa 1–89), and CED4 (aa 2–89). Identical and similar residues are shown in black and gray shading, respectively. E, three-dimensional model of NAC CARD domain, showing predicted 6 α-helices (labeled H1–H6).
NAC expression in human tissues. A, Northern analysis. A 32P-labeled partial NAC cDNA was hybridized to filter-immobilized poly(A)⁺ RNA from various tissues (1 µg/lane) (CLONTECH) and visualized by x-ray autoradiography (upper panel). The same RNA blot was subsequently hybridized with a human β-actin cDNA probe, controlling for RNA loading (bottom panel). PBL, peripheral blood leukocytes. B, validation of anti-NAC antibody. 293T cells were transfected with plasmids encoding either Myc-tagged full-length (FL) NAC (which contains the sequence (aa 161–180) recognized by anti-NAC peptide antiserum) or Myc-CARD (containing only the C-terminal CARD domain of NAC). Lysates from transfected cells were analyzed by immunoblotting 2 days later, probing blots with anti-Myc (left panel) or anti-NAC (Bur241) (right panel) antibodies. C, NAC protein expression in adult human tissues. Whole cell lysates (50 µg) from the indicated human cells were analyzed by SDS-PAGE/immunoblotting using anti-NAC (Bur241) antisemur with ECL-based detection. Dark and open arrowheads indicate full-length NAC protein and a smaller NAC fragment (seen only in thymus) possibly arising by protease cleavage or alternative mRNA splicing. D, immunohistochemistry was used to analyze NAC expression in human tissues. Examples provided illustrate induction of NAC expression during epithelial differentiation in skin (epidermis) stained with anti-NAC antisera (Bur241) in the absence (a) and presence (b) of the competing immunogenic peptide, and in esophagus (c) and uterine cervix (d). Similar results were obtained using two different anti-peptide antisera.
some types of cells, suggesting that other proteins may associate with the Apaf1-Casp9 complex (29). In cyt-c-stimulated extracts from HEK293 cells, which contain relatively little endogenous NAC, as determined by immunoblotting (not shown), a single Apaf1-containing apoptosome of ∼700 kDa was observed by gel-sieve chromatography (Fig. 2F). However, in extracts from NAC-transfected HEK293 cells, Apaf1-containing apoptosomes were evident, including a large >1 Mda complex that contained both Apaf1 and NAC (as determined by coimmunoprecipitation analysis of the column fractions in which Apaf1 and NAC coeluted) (Fig. 2F). Similarly, in cells that contain relatively high levels of endogenous NAC, such as Jurkat T-cells, the endogenous NAC and Apaf1 molecules coeluted in gel-sieve experiments, revealing two apoptosomes where greater amounts of NAC were present in the larger of these multiprotein complexes (Fig. 2F, top). In extracts lacking cyt-c treatment, Apaf1 eluted as a monomer, whereas NAC was spread over multiple fractions without a clear elution peak (not shown). Also, NAC did not coelute in gel-sieve chromatography experiments with Nod1 using untreated or cyt-c-stimulated extracts, confirming that NAC associates selectively with the Apaf1 apoptosome.

To explore whether the Apaf1-containing apoptosome with which NAC associates displays caspase activity, endogenous NAC was immunoprecipitated from control- and cyt-c-stimulated Jurkat cell extracts, and associated caspase activity was measured based on cleavage of the fluorogenic caspase-sub-
Encounter with Apaf1 Involved in Cyt c-dependent Caspase Activation

**Fig. 4. NAC modulates Apaf1 function.** A, NAC associates with active caspases after cyt-c stimulation. Jurkat cytoplasmic extracts were treated with cyt-c/dATP for 30 min at 30 °C (a, b and c) or left untreated (d), and then subjected to immunoprecipitation with anti-NAC antiserum (Bur241) (a, b, and d) in the presence (b) or absence (a) of competing immunogenic peptide or the corresponding preimmune sera (c). The resulting immune complexes were washed extensively and then monitored for DEVDase activity, expressing results as relative fluorescent units (RFU) released from Ac-DEVD-APC per μg of protein input for immunoprecipitation (data representative of three independent experiments). B, NAC enhances cyt-c-induced pro-Casp9 processing and activation of downstream caspases. 293T cells were transfected with empty pcDNA3 (CNTL) or pcDNA3 encoding NAC (10 μg of DNA) in 10-cm plates. Cytoplastic extracts were prepared from transfected cells after 24 h. Cell lysates (10 μg) were incubated with 35S-labeled pro-Casp9 in the presence or absence of cyt-c (10 μM) and dATP (1 mM) (cyt-c) at 30 °C for 1 h and then analyzed by SDS-PAGE/fluorography (top). Alternatively, various concentrations of cyt-c were added to extracts without IVT pro-Casp9 (bottom). Cytosolic activity of caspase was measured in 10-μg aliquots after incubation at 30 °C for 0.5 h by monitoring release of AFC from caspase substrate Ac-DEVD-AFC (expressed as relative fluorescent units (RFU) per μg protein per min). C, reductions in NAC decrease sensitivity to cyt-c. Jurkat T cells were subjected to two sequential lipofections (at 0 and 1 day) with either catalytically active anti-NAC DNAzyme (AS) or inactive control oligonucleotide (C) (20 μM final concentration). Whole cell extracts were prepared from an aliquot of the cells, normalized for protein content (50 μg), and analyzed by SDS-PAGE/immunoblotting using anti-NAC and anti-Apaf1 antibodies (top). Alternatively, detergent-free cytoplasmic extracts were prepared and treated either with 10 μM cyt-c and 1 mM dATP (cyt-c) or with 10 ng of granzyme B (Grz B) and DEVDase activity was measured at 0, 10, 20, and 30 min (mean ± S.E., n = 3) (bottom). D, NAC collaborates with Apaf1 and pro-Casp9 in inducing caspase activation. Cytoplastic extracts were prepared from 293T at 1 day following transfection with plasmids encoding the following: (a) pro-Casp9 (50 ng DNA), Apaf1 (50 ng), and NAC (2 μg); (b) pro-Casp9 and Apaf1; or (c) pro-Casp9 alone (all transfections normalized for 2.5 μg of total DNA using pcDNA3) and assayed for caspase activity by addition of 100 μM Ac-DEVD-APC to cell lysates and monitoring AFC release (RFU/μg lystate) over time. Although not shown, negligible caspase activity was detected in lysates of cells transfected individually with Apaf1 or NAC or with the combination of NAC and Apaf1 (in the absence of pro-Casp9). E, NAC enhances apoptosis induced by staurosporine and by Apaf1. 293T cells were transfected with pEGFP (0.1 μg) and plasmids encoding pro-Casp9 (0.05 μg), Apaf1 (0.05 or 2 μg), or NAC (0.5 or 2 μg), as indicated. Total DNA input was normalized with empty pcDNA3. Transfected cells were cultured in media containing 0.1% fetal bovine serum for 1.5 days (1st to 7th columns) or treated with staurosporine (STS) at the indicated concentration for 3 h (8th to 13th columns) prior to fixing and staining with DAPI (black bars) with or without NAC, white bars = without NAC. The % GFP-positive apoptotic cells (exhibiting nuclear fragmentation and chromatin condensation) was determined by fluorescence microscopy (mean ± S.E., n = 3). Immunoblot analysis of replicate transfections confirmed production of plasmid-encoded proteins and demonstrated that NAC and Apaf1 did not affect each other’s expression (not shown). F, dominant-negative mutants of NAC inhibit apoptosis induction by Apaf1 and staurosporine (STS) but not Fas. 293T cells were transfected with 0.1 μg of pEGFP DNA and either 0.5 or 2 μg of plasmids encoding the CARD or NB domain of NAC, together with plasmids encoding pro-Casp9 (50 ng) and Apaf1 (1 μg) (left) or Fas (0.5 μg) (right). Alternatively, cells were treated with 1 μM staurosporine for 5 h (middle). Cells were fixed and stained with DAPI, enumerating the percentage of apoptotic GFP-positive cells (mean ± S.E., n = 3). Immunoblot analysis confirmed production of the CARD and NB domains of NAC and demonstrated no affect on levels of Apaf1 or pro-Casp9 (not shown).

strate Ac-DEVD-APC (21). These experiments revealed that NAC is associated with active caspases after but not before cyt-c stimulation (Fig. 4A). Control immunoprecipitates lacked significant caspase activity, confirming the specificity of these results. Although activities of different size apoptosomes can vary depending on salt concentrations, use of detergents, and the presence of endogenous inhibitors (X-chromosome-linked inhibitor of apoptosis protein and second mitochondrial activator of caspase [29]), these findings argue that the larger apoptosome containing NAC is active.

In HEK293 cells, which contain little endogenous NAC, overexpression of NAC by transient transfection promotes formation predominantly of the larger >1-MDa apoptosome upon cyt-c stimulation, with relatively little of the smaller ~700-kDa apoptosome present (Fig. 3F). To contrast the function of Apaf1 in the presence and absence of NAC, therefore, we compared cyt-c-induced proteolytic processing of pro-Casp9 and activation of downstream caspases (Ac-DEVD-APC cleavage) in extracts prepared from control- and NAC-transfected HEK293 cells (Fig. 4B). Extracts containing elevated NAC displayed increased processing of pro-Casp9 and greater cyt-c-induced activation of downstream caspases, suggesting that NAC enhances Apaf1 activity. Conversely, reducing NAC protein levels using antisense expression plasmids (not shown) or antisense/DNAzyme oligonucleotides decreased the ability of cyt-c to activate caspases in cell extracts in vitro. Fig. 4C, for example, shows experiments performed using Jurkat cells, which contain relatively higher levels of endogenous NAC, demonstrating DNAzyme-mediated ablation of NAC protein without concomitant changes in the levels of Apaf1. Extracts prepared from anti-NAC DNAzyme-treated Jurkat cells were less sensitive to cyt-c compared with control oligonucleotide-treated
cells, in terms of caspase activation. In contrast, sensitivity to granzyme B-mediated caspase activation was not affected, confirming a specific defect in the cyt-c pathway which depends on Apaf1 for caspase activation. Further evidence that NAC regulates the cyt-c/Apaf1-dependent activation of caspases in cell extracts was obtained by affinity preabsorption of NAC from extracts using a GST fusion protein containing the CARD of NAC and by expression in cells of a dominant-negative fragment of NAC consisting of only the CARD domain (not shown).

Consistent with experiments involving cell extracts, NAC also collaborated with Apaf1 in inducing caspase activation and apoptosis in intact cells. In transient transfection experiments using HEK293 (Fig. 4, D and E) or other cell lines (not shown), overexpression of NAC by itself (not shown) or in combination with pro-Casp9 had little effect on caspase activation or apoptosis. In contrast, overexpressing NAC together with Apaf1 and pro-Casp9 resulted in synergistic increases in activation of caspases and induction of apoptosis, as determined from co-transfections that employed suboptimal amounts of Apaf1-encoding plasmid (Fig. 4, D and E). Overexpression of NAC also sensitized cells to suboptimal concentrations of apoptosis inducers such as staurosporine, which triggers apoptosis through an Apaf1-dependent mechanism (30), but not by anti-Fas antibody which utilizes an Apaf1-independent pathway (Fig. 4E and not shown). Immunoblotting experiments demonstrated that the enhanced sensitivity of NAC-transfected cells to staurosporine was not due to differences in the levels of Apaf1 or pro-Casp9 proteins produced in cells (not shown).

Whereas full-length NAC enhanced apoptosis and caspase activation induced by overexpressing Apaf1 or by treatment of cells with Apaf1-dependent apoptotic stimuli (Fig. 4, D and E), fragments of NAC containing only the CARD or NB domain had the opposite effect, interfering with apoptosis induced by coexpression of Apaf1/pro-Casp9 and by Apaf1-dependent stimuli such as staurosporine without affecting Apaf1-independent pathways activated by Fas (Fig. 4F). Again, immunoblotting experiments demonstrated that these reductions in Apaf1-dependent apoptosis caused by these dominant-negative fragments of NAC were not secondary to effects on levels of the Apaf1 or pro-Casp9 proteins (not shown).

Although some CARD-containing proteins, including Nod1/CARD-4 (25, 26) and Bel10/mE10 (31, 32), reportedly induce NF-κB activation, NAC did not induce NF-κB, when overexpressed in cells (data not shown).

Cells of various tissues vary in their sensitivity to cyt-c-induced activation of caspases, a finding that cannot be accounted for by differences in the levels of Apaf1 protein or downstream caspases (33, 34). The discovery of NAC suggests a mechanism for fine-tuning Apaf1 function, based on whether NAC is expressed and perhaps on whether NAC interacts with unidentified proteins via its LRR or other domains. Apaf1/CED4 family proteins directly bind CARD-containing caspases, promoting protease activation upon oligomerization by bringing the suboptimally active pro-enzymes into close proximity, allowing them to trans-process each other (4, 12–14). Although NAC enhances cyt-c-mediated pro-Casp9 processing, it does not directly bind this caspase (nor caspases-1, -2, -6, -7, -8, -10, or -11).2 Rather, interactions of NAC with Apaf1 facilitate Apaf1-mediated activation of pro-Casp9, thus revealing a new paradigm for apoptosis regulation. The precise mechanism by which NAC enhances Apaf1 function remains to be elucidated.

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