Nod1, an Apaf-1-like Activator of Caspase-9 and Nuclear Factor-κB*

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Ced-4 and Apaf-1 belong to a major class of apoptosis regulators that contain caspase-recruitment (CARD) and nucleotide-binding oligomerization domains. Nod1, a protein with an NH2-terminal CARD-linked to a nucleotide-binding domain and a COOH-terminal segment with multiple leucine-rich repeats, was identified. Nod-1 was found to bind to multiple caspases with long prodomains, but specifically activated caspase-9 and promoted caspase-9-induced apoptosis. As reported for Apaf-1, Nod1 required both the CARD and P-loop for function. Unlike Apaf-1, Nod1 induced activation of nuclear factor-kappa-B (NF-κB) and bound RICK, a CARD-containing kinase that also induces NF-κB activation. Nod1 mutants inhibited NF-κB activity induced by RICK, but not that resulting from tumor necrosis factor-α stimulation. Thus, Nod1 is a leucine-rich repeat-containing Apaf-1-like molecule that can regulate both apoptosis and NF-κB activation pathways.

Apoptosis, or programmed cell death, is a process that is essential for normal development and homeostasis of multicellular organisms (1–3). Genetic studies in the nematode Caenorhabditis elegans have identified core components of the death machinery, which are conserved in vertebrates, including humans (1–3). One of these, Ced-4, is required for developmental cell death in the worm (1, 2). Ced-4 physically interacts with Ced-3 and promotes the proteolytic activation of the immature Ced-3 caspase into enzymatically active subunits (4–7). Apaf-1, a mammalian homologue of Ced-4, has been identified (8). Both Apaf-1 and Ced-4 are composed of an NH2-terminal caspase recruitment domain (CARD)1 linked to a nucleotide-binding domain (NBD), also known as the NB-ARC or NOD domain (3, 8–10). Ced-4 and Apaf-1 self-associate via the NBD, a process that mediates proximity and activation of immature Ced-3/caspase-9 molecules (11–13). The COOH-terminal region of Apaf-1 lacks homology with Ced-4 and is composed of 12 WD-40 repeats (8). In response to certain apoptotic stimuli, cytochrome c is released from the mitochondria and binds to Apaf-1 (8, 14), and in the presence of dATP or ATP, Apaf-1 associates with and activates procaspase-9 (8, 14).

Recent analyses of Apaf-1-deficient mice suggest a central role of Apaf-1 in apoptosis induced by chemotherapeutic drugs, ultraviolet radiation, and signals associated with neuronal development (15, 16). Mutant mice deficient in caspase-9 exhibit abnormalities similar, but not identical, to those observed in mice lacking Apaf-1 (17, 18). Unlike C. elegans, mice and humans contain multiple initiator caspases, suggesting that mammalian genomes may contain caspase activators other than Apaf-1. Significantly, Apaf-1 knockout mice lack apparent abnormalities in tissues, such as the thymus, whose appropriate cellular development depends on apoptosis (15, 16). The latter observation suggests the existence of additional Apaf-1-like molecules or apoptosis pathways that are Apaf-1-independent. In this study, we identified and characterized Nod1, an Apaf-1-like protein that associates with and regulates procaspase-9. Unlike Apaf-1, however, Nod1 contains leucine-rich repeats (LRRs) and induces NF-κB activation.

MATERIALS AND METHODS

Isolation of the Nod1 cDNA—The partial nucleotide sequences of cDNAs encoding peptides with homology to a consensus CARD motif (10) were found in the EST data base of Human Genome Sciences using the TBLASTN and HMM programs. Overlapping cDNA clones that encode the 5’ end of the Nod1 mRNA were isolated from a B-cell human lymphoma cDNA library by the polymerase chain reaction (PCR) using two Nod1-specific primers and two universal primers for the vector. The entire open reading frame was verified by sequence analysis of several Nod1 cDNAs isolated from a primary mammary tumor, normal mammary tissue, and BJAB and 293 cells. Northern Blot and in Situ Hybridization Analysis—A 2-kilobase pair HindIII fragment of Nod1 cDNA was radiolabeled by random priming using a commercial kit (Boeringer Mannheim) and applied for Northern blot analysis of human poly(A)* RNA blots from various tissues (CLONTECH) according to the manufacturer’s instructions. Slides containing mouse embryo tissues were prepared as described (19). Each specimen was hybridized with a digoxigenin-labeled antisense RNA probe synthesized from a mouse Nod1 cDNA (EST clone 944836) using an in vitro transcription kit (Promega). Hybridization, development, and mounting of slides were performed as described (19).

Construction of Expression Plasmid—The entire open reading frame of Nod1 was amplified by PCR and was cloned into pcDNA3-Myc and pcDNA3-HA (20) to generate pcDNA3-Nod1-Myc and pcDNA3-Nod1-HA. Deletion and site-directed mutants of Nod1 (1–648, 649–953, pressed sequence tag; PCR, polymerase chain reaction; HA, hemagglutinin; WT, wild-type; DED, death effector domain.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF113925.

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The abbreviations used are: CARD, caspase-recruitment domain; NF-κB, nuclear factor-κB; I-κB, inhibitor of NF-κB; IKK, I-κB kinase; NBD, nucleotide-binding domain; LRRs, leucine-rich repeats; EST, expressed sequence tag; PCR, polymerase chain reaction; HA, hemagglutinin; WT, wild-type; DED, death effector domain.

1 The entire open reading frame of Nod1 was amplified by PCR and was cloned into pcDNA3-Myc and pcDNA3-HA (20) to generate pcDNA3-Nod1-Myc and pcDNA3-Nod1-HA. Deletion and site-directed mutants of Nod1 (1–648, 649–953,
Visualization of HA-tagged Nod1 in transfected 293T cells was analyzed by anti-FLAG antibody and detection with anti-Myc antibody (21). Local binding was confirmed by reciprocal experiments using immunoprecipitation with anti-HA or anti-FLAG antibody were detected with anti-FLAG or anti-HA monoclonal antibody. As control, immunoprecipitated proteins co-immunoprecipitated with anti-Myc polyclonal antibody were detected with anti-FLAG antibody. As control, proteins co-immunoprecipitated with anti-HA antibody were detected with anti-FLAG antibody. As control, RICK and Nod1 proteins in total lysate were detected by anti-FLAG and anti-HA monoclonal antibody, respectively. For mutant analysis, 293T cells was co-transfected with 50 ng of pcDNA3-FLAG-TRAF2, or pcDNA3-HA. 24 h post-transfection, the percentage of apoptotic cells was determined in triplicate cultures as described above. The proform and mature form of tagged caspase-9 were detected with anti-FLAG antibody in total lysate from an aliquot of the same cultures. To test the interaction between caspase-9 and Nod1 mutants, 293T cells were co-transfected with pcDNA3-caspase-9-C287S-Myc and WT or mutant Nod1 expression plasmids. Proteins co-immunoprecipitated with anti-Myc antibody were detected with anti-HA antibody. As control, immunoprecipitated caspase-9 was detected by re-blotting with anti-Myc monoclonal antibody. Nod1 proteins were detected with anti-HA monoclonal antibody and were detected by anti-HA monoclonal antibody. 293T cells were co-transfected with 200 ng of pBIX-Luc (32) or control plasmid plf-Luc plus each expression plasmid and 100 ng of pcDNA3-β-gal in triplicate in the presence of 2 μg of pcDNA3-p35 to prevent caspase activation and cell death. 24 h post-transfection, cell extract was prepared, and its relative luciferase activity was measured as described (36). To test the interaction between caspase-8 and Nod1 mutants, 293T cells were co-transfected with pcDNA3-FLAG-RICK and WT or mutant Nod1 expression plasmids. Proteins co-immunoprecipitated with anti-HA antibody were detected with anti-FLAG antibody. As control, RICK and Nod1 proteins in total lysate were detected by anti-FLAG and anti-HA monoclonal antibody, respectively. To test the interaction between WT Nod1 and RICK mutants, 293T cells were co-transfected with pcDNA3-FLAG-RICK (1–374), or pcDNA3-FLAG-RICK (374–540) (21). Proteins co-immunoprecipitated with anti-HA antibody were detected with anti-Myc monoclonal antibody. As control, proteins co-immunoprecipitated with anti-FLAG antibody and detection with anti-HA monoclonal antibody, respectively.

RESULTS AND DISCUSSION

Identification of Nod1—To identify novel Apaf-1-like molecules, we searched EST data bases for cDNAs encoding proteins with homology to a consensus CARD motif and found a novel EST cDNA that encodes a CARD protein (10). Sequence analysis of multiple cDNAs encoding the same protein revealed an open reading frame of 953 amino acids (Fig. 1). We termed

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**Fig. 1.** Deduced amino acid sequence, domain structure of human nod1. A, amino acid sequence of Nod1. Sequences homologous to the CARD, NBD, and LRRs are indicated by reverse highlighting, underlining, and arrows, respectively. The consensus sequence of the P-loop (Walker A box) is indicated by boxes. B, schematic representation of Nod1. Numbers corresponds to amino acid residues shown in A. The region homologous to the CARD, NBD, and LRRs are indicated by a black closed box, a closed shaded box, and hatched boxes, respectively.

**Immunodetection of Mature and Precursor Forms of Caspase-8 and Caspase-9—**Total lysate from 293T cells transfected with 50 ng of pcDNA3-caspase-8-FLAG or pcDNA3-caspase-8-HA in the presence of 100 ng of pcDNA3-HA or pcDNA3-Nod1-HA was prepared 24 h post-transfection and was subjected to 15% SDS-polyacrylamide gel electrophoresis. Tagged caspase-9 and caspase-8 were detected by anti-FLAG and anti-HA antibodies, respectively. For mutant analysis, 293T cells was co-transfected with 50 ng of pcDNA3-FLAG-TRAF2, or pcDNA3-HA. 24 h post-transfection, the percentage of apoptotic cells was determined in triplicate cultures as described above. The proform and mature form of tagged caspase-9 were detected with anti-FLAG antibody in total lysate from an aliquot of the same cultures. To test the interaction between caspase-8 and Nod1 mutants, 293T cells were co-transfected with pcDNA3-caspase-9-FLAG, or pcDNA3-caspase-9-HA and pcDNA3-HA or pcDNA3-Nod1-HA, and 200 ng of pcDNA3-β-gal. As control, proteins co-immunoprecipitated with anti-HA antibody were detected with anti-Myc monoclonal antibody. As control, proteins co-immunoprecipitated with anti-FLAG antibody and detection with anti-HA monoclonal antibody, respectively.
this novel protein Nod1. Data base search and alignment analyses revealed that Nod1 is composed of an NH2-terminal CARD fused to a region of 319 amino acids (residues 171–490) with homology to an ATP/GTPase domain that includes consensus nucleotide-binding motifs (Walker A box, P-loop and Walker B box, Mg2+ binding site, Ref. 37) (Figs. 1 and 2B). The COOH-terminal region of Nod1 (residues 649–953) contained 10 LRRs that function as a protein-protein interaction domain in many proteins (38), most significantly in several plant resistance proteins that contain NBDs with significant amino acid homology to those of Apaf-1 and Ced-4 (9). The NBD of Nod1 is most homologous to that of CIITA (Fig. 2B), a transcription activator with LRRs (39). The CARD of Nod1 was most similar to the CARD of RICK, also called RIP2/CARDIAK, a serine-threonine kinase that regulates apoptosis and NF-kB activation (21, 40, 41). Alignment analysis revealed that each of the 10 LRRs of Nod1 contained a putative a helix and b sheet (Fig. 2C). This LRR alignment of Nod1 is consistent with LRRs in several proteins that form a horseshoe-shaped structure with a parallel b sheet lining the inner circumference of the horseshoe and a helices flanking its outer circumference (38, 42).

Nod1 Is Expressed in Multiple Tissues and Exhibits Restricted Distribution in Embryonic Tissues—Northern blot analysis showed Nod1 to be expressed as a 4.4-kilobase transcript in various human adult tissues including heart, placenta, lung, skeletal muscle, liver, kidney, spleen, thymus, and ovary (Fig. 3A). We evaluated the expression of Nod1 mRNA in the mouse embryo by in situ hybridization. At stage E15.5 of embryonic development, Nod1 labeling was detected in the liver, thymus, cortical region of the kidney, lung, gut epithelium, and in certain regions of the central nervous system (Fig. 3B, panels a–f). Therefore, expression of Nod1 appears to be regulated differentially in embryonic and adult tissues. In the developing brain, Nod1 labeling was detected in the inferior tectal neuroepithelium of the developing inferior colliculus, germinal layer of the neocortex, olfactory epithelium, and choroid plexus (Fig. 3B, panel a). The expression of Nod1 in the developing brain is different and more restricted to that observed for Apaf-1 (15).

Genomic Organization and Chromosomal Localization of the Human Nod1 Gene—Search of the human genomic data base with the Nod1 cDNA mapped the Nod1 locus to chromosome 7 at 7p14-15 within human genomic PAC clone DJ0777023 and BAC clone GS114109 (GenBank™ accession numbers.

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**FIG. 2. Alignment of human Nod1 and related proteins.** A, alignment of CARDs of Nod1, RICK (GenBank™ accession number AF027706), ARC (GenBank™ accession number AF043244), RAIDD (GenBank™ accession number U79115), caspase-2 (GenBank™ accession number U13021), Ced-3 (GenBank™ accession number L29052), Ced-4 (GenBank™ accession number X69016), caspase-9 (GenBank™ accession number U56590), Apaf-1 (GenBank™ accession number AF013263), and c-IAP-1 (GenBank™ accession number L49431). The residues identical and similar to those of Nod1 are shown by reverse and dark highlighting, respectively. The putative a helices, H1 to H6, are shown according to the three-dimensional structure of the CARD of RAIDD (23). B, alignment of NBDs of Nod1, CIITA (GenBank™ accession number X74301), Apaf-1 (GenBank™ accession number AF013263), and Ced-4 (GenBank™ accession number X69016). The residues identical and similar to those of Nod1 are shown by reverse and dark highlights, respectively. The consensus sequence of the P-loop (residues 280–284, Walker A box) and the Mg2+ binding site (residues 280–284, Walker B box) are indicated by boxes. C, alignment of LRRs in Nod1. The conserved positions with leucine and other hydrophobic residues are indicated by highlights. The putative a helix and b sheet are shown according to the three-dimensional structure of the ribonuclease inhibitor (42).
AC005154 and AC006027, respectively). Comparison of genomic and cDNA sequences revealed that the Nod1 gene is composed of 14 exons, including 7 coding and 7 noncoding exons.\(^2\)

**Nod1 Interacts with Caspases with Long Prodomains and RICK**—The CARD and its structurally related death effector domain (DED) are peptide motifs that mediate interactions between apoptosis-regulatory proteins.\(^3\) To identify the binding partners of Nod1, we tested the ability of Nod1 to associate with a panel of CARD and DED-containing proteins in 293T cells (Fig. 4). We also examined whether Nod1 interacts with additional proteins that regulate apoptosis or NF-\(\kappa\)B signaling, as Nod1 was found to induce activation of NF-\(\kappa\)B (see below). In these experiments, Nod1 was tagged at the COOH terminus with a Myc- or HA-epitope and transiently expressed in 293T cells. Overexpressed Nod1 was a cytosolic protein as determined by confocal microscopy.\(^2\) Co-immunoprecipitation assays with Nod1 and tagged proteins revealed that Nod1 preferentially interacted with several procaspases containing long prodomains with CARDs or DEDs, including caspase-1, caspase-2, caspase-4, caspase-8, and caspase-9, but not those with short prodomains like caspase-3 or caspase-7 (Fig. 4). In addition, Nod1 interacted with Nod1 itself, with RICK and CLARP, but not with other CARD or DED-containing proteins including RAIDD, Apaf-1, Ced-4, Bcl-10, FADD, PEA15, or DEDD (Fig. 4). In addition, Nod1 failed to associate with multiple regulators of NF-\(\kappa\)B activation, including several TRAFs, IKK\(\alpha\), IKK\(\beta\), NIK, or A20 (Fig. 4).

**Nod1 Enhances Apoptosis Induced by Caspase-9 but Not That Mediated by Caspase-4, Caspase-8, or CLARP**—In order to determine the functional relevance of these interactions, we transiently expressed HA-tagged Nod1 in 293T cells and tested the ability of Nod1 to modulate the apoptosis regulatory function of binding proteins in 293T cells. Although Nod1 did not activate apoptosis by itself, it significantly enhanced apoptosis induced by caspase-9, but not that induced by caspase-4, caspase-8, or CLARP (Fig. 5, A and B). Consistent with its inability to modulate caspase-8-mediated apoptosis, Nod1 did not affect apoptosis induced by FADD, CLARP, TRAMP, or TNFR1 (Fig. 5B), which induce apoptosis through caspase-8 activation.\(^3\) In addition, expression of Nod1 did not change the apoptosis regulatory function of caspase-1, caspase-2, or

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\(^{2}\)N. Inohara and G. Nuñez, unpublished data.
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Fig. 5. Enhancement of caspase-9-induced apoptosis and caspase-9 maturation by Nod1. A, regulation of caspase-9, but not caspase-8, induced cell death by Nod1. 293T cells were transfected with 50 ng of caspase-9 (panels a and b) or caspase-8 (panels c and d) expression plasmids plus β-galactosidase plasmid in the presence of vector control (panels a and c) or Nod1 expression plasmid (panels b and d). 24 h post-transfection, the transfected cells were fixed and visualized by staining. Arrowheads show rounded apoptotic cells with membrane blebbing. B, regulation of caspase-9-induced cell death by Nod1. 293T cells were transfected with caspase-9, caspase-8, FADD, CLARP, TRAMP, or TNFR1 expression plasmids plus β-galactosidase plasmid in the presence of vector control or Nod1 expression plasmids. 24 h post-transfection, the percent of apoptotic cells ± S.D. was calculated in triplicate cultures. C, induction of caspase-9 maturation by Nod1. 293T cells were co-transfected with caspase-9 or caspase-8 expression plasmids in the presence of vector control or Nod1 plasmids. Tagged caspase-9 and caspase-8 in total lysate were detected by anti-FLAG- and anti-HA antibodies, respectively. The asterisk and double asterisks indicate nonspecific proteins cross-reacting with antibodies and truncated products from HA-Nod1, respectively.

These results indicate that the ability of Nod1 to regulate apoptosis is highly specific and limited thus far to that activated by caspase-9. The effect of Nod1 on caspase-9-induced apoptosis was not due to increased expression of caspase-9, as there was no alteration in the levels of this procaspase-9 in cells transfected with Nod1 plasmids compared with control plasmids (Fig. 5C).

Nod1 Promotes the Activation of Procaspase-9 but Not That of Procaspase-8—Production of enzymatically active caspases including caspase-9 requires proteolytic processing of the immature form of the enzyme (3). To determine if Nod1 enhances caspase-9 processing, 293T cells were transiently expressed in the presence or absence of HA-tagged Nod1. Expression of Nod1 induced the proteolytic activation of procaspase-9, but not that of procaspase-8 (Fig. 5C). Thus, the ability of Nod1 to enhance caspase-9, but not caspase-8-mediated, apoptosis correlates with its ability to induce proteolytic processing of procaspases. By analogy with Nod1, Apaf-1 also binds to several caspases with long prodomains, but only promotes the activation of procaspase-9 (24), suggesting that binding of Nod1 or Apaf-1 alone is not sufficient for activation of target proteins.

The CARD and NBD Are Essential for Nod1 to Activate Procaspase-9—We engineered mutant forms of Nod1 to determine the regions of Nod1 that are required for caspase-9 activation. Expression of a mutant containing the CARD and NBD (residues 1–648) retained its ability to enhance caspase-9-induced apoptosis, but the mutant containing the LRRs (residues 649–953) alone did not (Fig. 6B). This indicates that Nod1 promotes caspase-9 apoptosis through the CARD and/or NBD, as it was reported previously for Apaf-1 and Ced-4 (38–42). The conserved lysine residue in the P-loop of Ced-4 and Apaf-1 is critical for both caspase activation and apoptosis enhancement (13, 43, 44) as are the conserved residues in the CARD of RAIDD (41). We therefore introduced point mutations in highly conserved residues of the CARD (V41Q) and the P-loop of Nod1 (K208R), the corresponding mutations of which results in loss-of-function of RAIDD and Apaf-1/Ced-4, respectively (13, 43–45). Both V41Q and K208R mutations inhibited the ability of Nod1 to enhance caspase-9-induced apoptosis and caspase-9 maturation (Fig. 6B). Thus, the CARD and NBD appear essential for Nod1 to activate procaspase-9 and to promote apoptosis. These results suggest that Nod1 and Apaf-1 activate procaspase-9 by a similar mechanism, which may involve conformational changes and NBD oligomerization of these caspase activators, bringing several molecules of procaspase-9 into close proximity (3, 11, 13).

Nod1 Requires Its CARD to Bind Procaspase-9—Apaf-1 associates with caspase-9 via the CARD (8, 11, 46). We therefore determined the regions of Nod1 that are required for association with procaspase-9. We transiently co-transfected 293T cells with expression plasmids producing caspase-9 and wild-type or mutant forms of Nod1. Immunoblotting analysis of protein complexes revealed that residues 1–648 of Nod1 containing the CARD and NBD co-immunoprecipitated with caspase-9, but the LRR domain did not (Fig. 6C). The K208R mutant still bound to caspase-9, although its binding was reduced when compared with wild-type Nod1 (Fig. 4C). Nod1 with a mutation in a highly conserved residue of the CARD (V41Q) failed to associate with caspase-9 (Fig. 6C). Another mutant lacking the CARD (residues 126–953) also failed to interact with and activate procaspase-9. Thus, the CARD is essential for Nod1 to bind and activate procaspase-9, as well as to promote apoptosis.

Nod1 Induces NF-κB Activation and Synergizes with RICK—
The results presented in Fig. 4 showed that Nod1 also interacts with RICK, a CARD-containing serine-threonine kinase that promotes apoptosis and NF-κB activation (21, 40, 41). Because RICK induces NF-κB activation, we asked if Nod-1 could activate NF-κB. To test if Nod1 activates NF-κB, a Nod1 expression plasmid was co-transfected into 293T cells with pBIIx-Luc, a luciferase NF-κB reporter plasmid or pLuc-Luc control plasmid lacking NF-κB binding sites (36). Nod1 induced activation of NF-κB in a dose-dependent manner, whereas Apaf-1 did not (Fig. 7A). We also confirmed that Nod1 induced NF-κB activation in parental 293 cells and HeLa cells.2 RICK alone induced NF-κB activation as reported (40, 41). Importantly, co-expression of RICK and Nod1 resulted in synergistic NF-κB activation (Fig. 7A).

Nod1 Interacts with RICK via a Homophilic CARD Association—Next we determined the regions of Nod1 involved in NF-κB activation. Expression of residues 1–648 of Nod1 induced NF-κB, while mutants containing the NBD plus LRRs (residues 126–953) or LRRs (residues 649–953) alone did not (Fig. 7C). Both V41Q and K208R mutations inhibited the ability of Nod1 to induce NF-κB activation (Fig. 7C). Thus, the CARD and the P-loop of Nod1 are essential for Nod-1 to activate NF-κB. To determine the regions of Nod1 and RICK involved in their interaction, 293T cells were transiently co-transfected with plasmids producing WT or mutant Nod1 and RICK. The analysis showed that the NH₂-terminal 1–648 amino acids, but not the LRRs of Nod1, mediate the interaction with RICK (Fig. 7D). The V41Q point mutant in the CARD, but not the K208R P-loop mutant, abolished the association with RICK (Fig. 7D). In addition, another Nod-1 mutant (residues 126–953) failed to associate with RICK. This result indicates that the CARD of Nod1 is critical for the Nod1-RICK interaction (Fig. 7D). Reciprocal experiments revealed that the CARD, but not the kinase domain of RICK, was required for the association with Nod1 (Fig. 7E). Thus, these results suggest that the Nod1/RICK interaction is mediated via their corresponding CARDs.

Mutants Forms of Nod1 Inhibit NF-κB Activation Induced by RICK, but Not That Resulting from Tumor Necrosis Factor-α Stimulation—Expression of Nod1 promotes both procaspase-9 activation and NF-κB activation, and the latter may involve the association of Nod1 with RICK. To determine if the NF-κB-inducing activity of RICK requires Nod1 or caspase-9 activity, we co-transfected 293T cells with plasmids expressing RICK and mutant forms of Nod1 or catalytically inactive caspase-9 or caspase-3 (as a control). Expression of Nod1 mutants lacking the CARD (residues 126–953) or containing only the LRRs (residues 649–953) inhibited RICK-mediated NF-κB activation, but not that induced by tumor necrosis factor α (TNFα) stimulation (Fig. 7F). Together with the results shown in Fig. 7B, the analysis suggest that both RICK and Nod1 activate a TNFα-independent pathway of NF-κB activation. In addition, the NF-κB-inducing activity of RICK was unaffected by mutant...
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Fig. 7. NF-κB Activation by Nod1. A, activation of NF-κB by Nod1. Induction of NF-κB activation was determined in triplicate cultures of 293T cells co-transfected with vector control, Nod1, RICK, TRAF2, or Apaf-1 expression plasmid and pBIIx-Luc or pEF-Luc in the presence of control plasmid pcDNA-3-β-gal. B, inhibition of Nod1-induced NF-κB activation by dominant negative mutant proteins of the NF-κB pathway. Induction of NF-κB activation was determined in triplicate culture of 293T cells co-transfected with Nod1 expression plasmid and IκBα S32A/S36A, IKKα K44A, IKKβ K44A, NIK KK429–430AA, TRAF2 (241–501), TRAF6 (289–522), RIP (558–671), or caspase-9 C287S expression plasmid in the presence of pBIIx-Luc and pcDNA-3-β-gal. C, Activation of NF-κB by Nod1 mutants. Induction of NF-κB activation was determined from triplicate culture of 293T cells co-transfected with WT or mutant Nod1 expression plasmid and reporter plasmids. D, interaction between RICK and mutant Nod1 proteins. 293T cells were co-transfected with vector control, WT, or mutant Nod1 expression plasmid and RICK expression plasmid. The co-immunoprecipitated RICK was detected by anti-FLAG antibody (upper panel). As control, total lysates were blotted with anti-FLAG (middle panel), or anti-HA (lower panel) antibody. E, interaction between Nod1 and mutant RICK proteins. 293T cells were co-transfected with WT (WT) or mutant RICK (N for the kinase domain and C for the CARD) expression plasmid and Nod1 expression plasmid or vector control. The co-immunoprecipitated RICK was detected by anti-FLAG antibody (upper panel). As control, total lysates were blotted with anti-FLAG (middle panel) or anti-HA (lower panel) antibody. F, suppression of RICK-induced, but not TNFα-induced, NF-κB activation by Nod1 mutants. Induction of NF-κB activation was determined from triplicate culture of 293T cells co-transfected with WT or mutant Nod1, caspase-9 C287S, caspase-3 C163S, IκBα S32A/S36A expression plasmids, and the reporter plasmids in the presence of RICK expression plasmid (closed bars). 293T cells were also transfected with WT or mutant Nod1 expression plasmid in the absence of RICK expression plasmid, and 22 h post-transfection the cells were treated with 10 ng/ml TNFα for 120 min (open bars).

caspase-9 or caspase-3, but was inhibited by mutant IκBα (Fig. 7F). The latter results indicate that caspase-9 activity is not required for Nod1 or RICK to activate NF-κB activation and further suggest that both TNFα and the RICK/Nod-1 signaling pathways use common downstream components such as NIK, IKKs, and IκBα for activation of NF-κB.

These studies identify and characterize Nod1, a protein structurally related to Apaf-1. Like Apaf-1, Nod1 binds caspase-9 and promotes apoptosis induced by caspase-9. Both Apaf-1 and Nod1 share NH2-terminal CARDs followed by NBDs. The CARDs of both Nod1 and Apaf-1 appear to mediate, at least in part, the association of the proteins with the CARD of procaspase-9 (8, 11). The NBD is involved in Apaf-1 and Ced-4 self-association, a process that may result in procaspase aggregation and autoactivation (11–13). Nod1 can also self-associate, suggesting that Apaf-1 and Nod1 may share a common mechanism for procaspase-9 activation. Cytochrome c released from damaged mitochondria binds to Apaf-1, presumably via its WD-40 repeat region, and acts as a co-factor required for procaspase-9 activation (8, 13, 14). Nod1 lacks WD-40 repeat region and contains instead LRRs. The LRR domain is known to be involved in protein-protein interactions and in bacterial lipopolysaccharide binding to Toll-like proteins (38, 52). This suggests that different upstream signal molecules regulate the activation of Apaf-1 and Nod1. Apaf-1 exhibits homology to plant R resistance gene products and related proteins, which also contain NBDs linked to LRRs (9). These proteins act as intracellular receptors for products of invading pathogens and signal a plant response against pathogens that include activation of a form of programmed cell death at the site of pathogen invasion (53). The LRR domain of the R gene products is responsible for specificity for a particular pathogen (54). By analogy, the LRRs of Nod1 may bind to upstream activators that regulate its ability to activate procaspase-9 and/or NF-κB. The structural similarity between plant R resistance proteins and Ced-4-like molecules suggests that the basic regulation of cell death machinery is evolutionarily conserved in plants, nematodes, and vertebrates.
Several lines of evidence suggest that Nod1 and RICK act in the same pathway to activate NF-κB. First, Nod1 and RICK associate via their corresponding CARDs. Second, NF-κB activation induced by Nod1 and RICK is synergistic. Third, mutant forms of Nod1 that are deficient in function inhibit the NF-κB activity induced by RICK. Collectively, these results indicate that Nod1 and RICK form a protein complex that induce activation of NF-κB and suggest that Nod1 acts downstream of RICK to activate NF-κB. In some cells, NF-κB mediates anti-apoptotic signals (50, 55). Thus, the biological response mediated by Nod1 may depend on the cellular context, as is the case after stimulation of surface receptors that signal both NF-κB and apoptosis (50, 55). These studies were based on overexpression of Nod-1 and target proteins. Analysis of the endogenous proteins under physiological conditions will be important for understanding the function of Nod1 on caspase and NF-κB activation.

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