A Novel Caspase-2 Complex Containing TRAF2 and RIP1*

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The enzymatic activity of caspases is implicated in the execution of apoptosis and inflammation. Here we demonstrate a novel nonenzymatic function for caspase-2 other than its reported proteolytic role in apoptosis. Caspase-2, unlike caspase-3, -6, -7, -9, -11, -12, and -14, is a potent inducer of NF-κB and p38 MAPK activation in a TRAF2-mediated way. Caspase-2 interacts with TRAF1, TRAF2, and RIP1. Furthermore, we demonstrate that endogenous caspase-2 is recruited into a large and inducible protein complex, together with TRAF2 and RIP1. Structure-function analysis shows that NF-κB activation occurs independent of enzymatic activity of the protease and that the caspase recruitment domain of caspase-2 is sufficient for the activation of NF-κB and p38 MAPK. These results demonstrate the inducible assembly of a novel protein complex consisting of caspase-2, TRAF2, and RIP1 that activates NF-κB and p38 MAPK through the caspase recruitment domain of caspase-2 independently of its proteolytic activity.

NF-κB and p38 MAPK activation are involved in the transcriptional activation of numerous inflammation-related and antiapoptotic genes in response to cytokines, bacterial products, and cellular stress conditions (1, 2). Pathogen-derived products such as peptidoglycan, double-stranded RNA, and lipopolysaccharide lead to NF-κB activation through stimulation of surface-exposed TLR-2, -3, and -4, respectively (3). In addition to pathogen-derived products, the proinflammatory cytokines TNFα and interleukin-1 also induce NF-κB activation by binding to their respective cell surface receptors (4, 5). These receptors signal through distinct pathways (3, 4, 6). For example, activation of NF-κB through TNF-R1 stimulation occurs through assembly of an intracellular signaling complex containing TRADD, TRAF2, and RIP1 (7–9). Whereas RIP1 is essential for the TNF pathway (10), RIP2 has recently been identified as the central downstream mediator of NF-κB activation arising from TLR-2, -3, and -4 (11, 12).

MAPKs are an evolutionarily conserved family of cytosolic serine/threonine kinases that modulate the activity of other intracellular proteins by phosphorylation of specific serine/threonine residues. Extracellular stimuli of the p38 MAPK pathway include a variety of cytokines and a number of pathogens that activate p38 through the different TLR receptors (13). Moreover, MAPK is induced by several growth factors (14), as well as by environmental factors such as heat shock, changes in osmolarity, and ultraviolet irradiation (13). The downstream targets of p38 MAPK are other kinases or transcription factors such as AP-1. The main biological response to p38 MAPK activation involves the production and activation of inflammatory mediators to initiate leukocyte recruitment and activation.

Caspase-2 is one of the most conserved caspases across species, and it clusters phylogenetically with CED-3 and the inflammatory caspases, caspase-1, -4, -5, -11, and -12 (15). Initially caspase-2 was described as a neuronally expressed caspase that is down-regulated during the course of brain development (16). Caspase-2 recently was demonstrated to be required for mitochondrial membrane permeabilization and the release of apoptogenic factors, such as cytochrome c and Diablo, in response to several DNA-damaging agents (17–20). Interestingly, it seems that full maturation of the protease, rather than its enzymatic activity, is required for the release of cytochrome c and Diablo from mitochondria (18).

The death domain superfamily includes the death domain (DD), the death effector domain, the caspase recruitment domain (CARD), and the recently identified PYRIN domain (21, 22). Members of this superfamily of homotypic oligomerization domains are involved in both the recruitment of adaptor proteins and the assembly of protein complexes, hereby promoting proteolytic activation of the recruited caspases in the context of apoptosis and inflammation. At its N terminus, caspase-2 contains a CARD that is involved in the formation of filamentous and dotlike structures (23, 24). Besides caspase-2, several other CARD- or death effector domain-containing proteins, including caspase-8, FADD, RAIDD, and Bcl-10, have been shown to oligomerize and to form filamentous structures (25–27). In the case of caspase-8, FADD, and RAIDD, such structures are associated with the induction of apoptosis (25, 26), whereas Bcl-10-containing filaments correlate with the activation of NF-κB (27). Via its CARD domain, caspase-2 interacts with the CARD-containing adaptor proteins RAIDD/CRADD (24, 28, 29), ARC (30, 31), and PACAP (32). It can therefore be argued that caspase-2, through its CARD, may be recruited to large...
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multiquitin complexes in response to death or inflammatory signals. Indeed, upon incubation of cell lysates at 37°C, endogenous caspase-2 elutes in high molecular weight fractions ($M_r > 670,000$), suggesting that it can be recruited in vivo to a large protein complex similar to the caspase-9-containing apoptosome ($M_r > 700,000$) (33) and caspase-1-containing inflammasomes ($M_r > 700,000$) (34, 35). The adaptor proteins RAIDD and PIDD have recently been identified as members of this large caspase-2 complex designated the “PIDDosome” (36).

Here, we demonstrate that caspase-2, like caspase-1 and -8, but unlike caspase-3, -6, -7, -9, -11, -12, and -14, potently induces p38 MAPK and NF-κB activation. This NF-κB-activating function is independent of its enzymatic activity and resides in the N-terminal CARD domain. Caspase-2 interacts with TRAF1, TRAF2, and RIP1. Furthermore, endogenous caspase-2, along with TRAF2 and RIP1, is recruited into a large and inducible protein complex ($M_r > 670,000$). RIP1, a known substrate of caspase-8 (37, 38), is not cleaved by caspase-2. These results suggest that, in addition to its role in nuclear stress-induced apoptosis, caspase-2 may be involved in proinflammatory pathways through the TRAF2- and RIP1-mediated activation of p38 MAPK and NF-κB.

Experimental Procedures

Plasmids—The cloning of cDNAs encoding murine caspase-2, -3, -6, -7, -9, -11, -12, and -14 have been described (39, 40). pCAGGS-caspase-2 C230A, coding for the enzymatically inactive mutant of murine caspase-2, was constructed by site-directed mutagenesis PCR. Caspase-2 was cloned into pCMV-3-lacZ. pCAGGS-E vector. The cDNA encoding human caspase-2 CARD was amplified by reverse transcription-PCR from a THP-1 cDNA library and cloned into pCAGGS-E vector in frame with the N-terminal E-tag. Full-length RIP1 was cloned into the pEF1 vector (Invitrogen) in frame with a C-terminal V5-HIS epitope tag. All PCR products were checked by sequencing to ensure that no errors had been introduced by PCR. The following expression plasmids were obtained from the indicated sources: pNLS-Egfp-conLac, encoding the luciferase reporter gene driven by a minimal NF-κB-responsive promoter, was a gift from Dr. A. Israel (Institut Pasteur, Paris, France). The plasmid pUT651, encoding β-galactosidase, was obtained from Eurogentec (Seraing, Belgium). pNLS-EgFP is a modified pEeGFP-N1 plasmid (Clontech, Palo Alto, CA), encoding enhanced green fluorescent protein with a nuclear localization signal. Plasmids encoding dominant negative forms of IKK-α and IKK-β were generous gifts from Dr. J. Schmid (University of Vienna, Austria) and Dr. D. V. Goeddel (Genentech, South San Francisco, CA), respectively. pcR3-RIP2 and pcR3-RIP2-CARD (RIP2 DN) were kindly provided by Dr. J. Tschopp (University of Lausanne, Epalinges, Switzerland). The plasmid encoding murine A20 was kindly provided by Dr. K. Heynink (Ghent University, Ghent, Belgium). Plasmids encoding TRAF1 and TRAF2 were gifts of Dr. I. Carpentier (Ghent University, Ghent, Belgium).

Transfection, Co-immunoprecipitation, and Immunoblotting—Asays—293T cells were transfected using the calcium phosphate precipitation method (41). Cells were seeded the day before transfection in 6-well plates (BD Biosciences) at 2 × 10⁶ cells/well. Cells were transfected for 4 h, washed, and incubated for another 24 h before lysates were prepared. Lysates were prepared by harvesting the cells and lysing them in ice-cold Nonidet P-40 lysis buffer (10 mM HEPES, pH 7.4, 142 mM KCl, 0.2% Nonidet P-40, 5 mM EDTA) or using three cycles of freeze-thawing in TNE buffer (30 mM Tris, pH 7.5, 120 mM NaCl, 5 mM EDTA) supplemented with 1 mM dithiothreitol, 12.5 mM β-mercaptoethanol, 1 mM NaVO₃, 1 mM phenylmethylsulfon fluoride, and 1× protease inhibitor mix (Roche Applied Science). Cell lysates (0.5 ml) were clarified by centrifugation at 14,000 × g for 5 min and immunoprecipitated with specific antibodies, including anti-E (Amersham Biosciences), anti-RIP1 (BD Pharmingen), and anti-HA antibodies (Babeo, Richmond, CA), in combination with 15 μl of Protein G-Sepharose (Amersham Biosciences). Immune complexes were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were subsequently incubated with various antibodies, including the 11B4 rat monoclonal anti-caspase-2 antibody (kindly provided by Dr. S. Kumar, Hanon Institute and Adelaide University, Adelaide, Australia), anti-TRAP2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-E (Amersham Biosciences), and anti-V5 antibodies (Invitrogen), followed by horseradish peroxidase-conjugated secondary antibodies, and detected by an ECL method. Alternatively, lysates were precipitated directly by the immunoprecipitation/Radiochemical blotting. Rabbit polyclonal antibodies against recombinant murine caspase-3, -6, -7, -11, -12, and -14 were prepared at the Centre d’Economie Rurale (Laboratoire d’Hormonologie Animale, Marloie, Belgium). Anti-p38 MAPK antibodies were from Cell Signaling (Beverly, MA). Anti-green fluorescent protein antibody was from Clontech. Anti-human caspase-3 antibody was purchased from BIOSOURCE (Camarillo, CA).

Quantification of NF-κB Activity—293T cells were transfected with the indicated expression vectors in combination with 100 ng of NF-κB-luciferase and pUT651-β-galactosidase reporter plasmids. In some experiments, cells were treated for 6 h with 500 IU/ml TNF-α prior to harvesting. Twenty-four hours after transfection, the cells were collected, washed in phosphate-buffered saline, and lysed in Tris phosphate (25 mM, pH 7.8), 2 mM dithiothreitol, 2 mM cyclohexane-trans-1,2-diaminotetraacetic, 10% glycerol, and 1% Triton X-100. After the addition of 50 μl of substrate buffer (658 μM luciferin, 378 mM co- enzyme A, and 742 μM ATP) to 20 μl of cell lysates, luciferase activity was assayed in a TopCount NXT microplate scintillation reader (Packard Instrument Co.). To normalize transfection efficiency, cell lysates were also subjected to a β-galactosidase colorimetric assay. In brief, 20 μl of cell lysates were incubated for 5 min at room temperature with 200 μl of a solution containing 0.9 mg/ml of nitrophenyl-β-D-galactopyranoside, 1 mM MgCl₂, 45 mM β-mercaptoethanol, and 100 mM sodium phosphate, pH 7.5. The optical density was read at a wavelength of 455 nm. Results are expressed as relative luciferase units/optical density for β-galactosidase activity. The data represent the average ± S.E. of at least three independent experiments.

In Vitro Transcription/Translation—pGEM-caspase-2 (40) and pEF1-RIP1 plasmids (1 μg each) were used as a template for in vitro coupled transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). Translation reactions (2 μl each) were incubated with 200 nm purified recombinant caspase-2 and -8 (42) in a total volume of 25 μl of “cell-free system” buffer (0.1% CHAPS, 50 mM HEPES, pH 7.5 (adjusted with KOH), 1 mM EDTA, 10 mM dithiothreitol, 1 mM phenylmethylsulfony fluoride, 50 μg/ml leupeptin, 20 μg/ml aprotinin) for 45 min at 37°C. The resulting cleavage products were analyzed by autoradiography of SDS-polyacrylamide gels.

Two-hybrid Analysis—The yeast shuttle vectors pAS2 (GAL4 DNA binding domain), pGAD424 (GAL4 activation domain), pY3A (p53), and pTD1 (SV40 large T antigen) were from Clontech. The cDNA encoding enzymatically inactive murine caspase-2 C230A was cloned in frame with the 4 DNA binding domain of pAS2. In a similar way, murine TRAF1 was inserted into the pGAD424 vector in frame with the GAL4 activation domain. The yeast strain YRG-2 was obtained from Stratagene (La Jolla, CA). his3 and lacZ were used as reporter genes, and the auxotrophic markers trp1 and leu2 were used for plasmid selection following transformation. Yeast cells were grown overnight at 30°C in complete yeast-peptone-dextrose medium (1% yeast extract, 2% peptone, and 2% glucose). Transformation of yeast was done by the lithium acetate procedure (43) using YEASTMAKER Carrier DNA (Clontech). PGAD424-mTRAF1 was transformed into the YRG-2 strain with either empty pAS2 vector or pAS2-caspase-2 C230A. In another setup, the YRG-2 strain was cotransformed with pAS2-caspase-2 C230A and the empty pGAD424 vector. Three days after transformation, β-galactosidase activity was determined using the colony lift filter assay (77) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate. Interaction between the oncoprotein p53 and SV40 large T antigen was used as a positive control.

Gel Filtration Chromatography—293T cells were washed twice with phosphate-buffered saline and resuspended in buffer A (20 mM HEPES-KOH, 1 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, pH 7.5) supplemented with protease inhibitors (Complete™, Roche Applied Science). Resuspended cells were subjected to four rounds of freeze/thawing using liquid nitrogen and ice water. Cellular debris was removed by centrifuging twice at 11,000 × g for 20 min at 4°C. Cleared lysates were fractionated at 4°C on a Superdex 200 column (Amersham Biosciences). The column was equilibrated with buffer A, the lysates were applied, and the proteins were eluted in 400-μl fractions with buffer A at a flow rate of 0.4 ml/min.

Results

Caspase-2 Induces NF-κB Independently of Its Enzymatic Activity—Since CARD domains have been implicated in NF-κB modulation, we investigated the NF-κB activating capability of...
the CARD-containing caspase-2. In accordance with published results, we found that wild type caspase-2 induces apoptotic cell death upon overexpression in 293T cells (Fig. 1A). The induction of apoptosis is lost when the catalytic Cys320 in the active site of caspase-2 is mutated to Ala, demonstrating that caspase-2 maturation is required for its proapoptotic function (Fig. 1A). Interestingly, wild type cells overexpressing caspase-2 show significant dose-dependent levels of NF-κB activation, when measured before any morphological signs of apoptosis are visible (Fig. 1B). The mutant expressing enzymatically inactive caspase-2 C320A was similarly potent in inducing NF-κB activity (Fig. 1C), demonstrating that NF-κB activation occurs independently of the proteolytic activity of caspase-2.

**Specificity of Caspase-2-induced NF-κB Activation**—We next asked whether caspases other than caspase-2 can activate NF-κB. Western blot analysis of lysates of 293T cells overexpressing caspase-1, -2, -3, -6, -7, -8, -9, -11, -12, or -14 confirmed the appropriate expression of all of these caspases and the presence of some characteristic proteolytic fragments (Fig. 2A). In addition to the 50-kDa band characteristic of full-length caspase-2, anti-caspase-2 antibodies recognized an additional 34-kDa band, as reported before (23, 44). Besides detecting the full-length protein, the anti-caspase-11 antibody also detected an additional 27-kDa fragment characteristic of caspase-11 activation (45). Nevertheless, of all caspases in this panel, only wild type caspase-2 is able to activate NF-κB. These results demonstrate that caspase-2-derived activation of NF-κB is not provoked by the transient expression of proteins in general. Because overexpression of pro-caspase-1, -8, and -9 is highly cytotoxic, as demonstrated by their barely detectable expression levels on Western blots (Fig. 2B), we included the enzy-
matically inactive procaspase-1 C284A and procaspase-8 C362A mutants and the naturally occurring procaspase-9S variant to study their potential NF-κB-inducing activity. In accordance with previous reports (46–48), we observed significant NF-κB activation with the enzymatically inactive mutants of procaspase-1 and -8, suggesting that the NF-κB-inducing activity of their wild type counterparts is masked by their high cytotoxic activities. In contrast to procaspase-1 and -8, both procaspase-9 and -9S are incapable of activating NF-κB (Fig. 2B).

Caspase-2 CARD Is Sufficient for Activation of NF-κB and p38 MAPK—To determine which part of caspase-2 is responsible for NF-κB activation, we generated plasmids encoding different domains of caspase-2 and compared their NF-κB-inducing abilities with that of full-length caspase-2 C320A (Fig. 3A). Western blotting analysis confirmed the appropriate expression of all domains and their combinations. The CARD was the only domain capable of inducing NF-κB activity on its own. Moreover, the level of NF-κB activity induced by the CARD was comparable with that observed with caspase-2 C320A, suggesting that the CARD is sufficient for NF-κB activation. We next analyzed the NF-κB-activating ability of the
human caspase-2 CARD. As could be expected, a potent dose-dependent induction of NF-κB activation was observed upon overexpression of human caspase-2 CARD in 293T cells (Fig. 3C), demonstrating that the NF-κB-inducing ability of caspase-2 CARD is conserved in mice and humans. We further analyzed whether overexpression of caspase-2 CARD in cells is sufficient for the activation of endogenous p38 MAPK, another important mediator of inflammatory signaling pathways (2). Although both caspase-2 CARD- and green fluorescent protein-overexpressing cells contain similar basal levels of p38 MAPK, a strong induction of p38 MAPK phosphorylation was observed only in cells expressing caspase-2 CARD (Fig. 3D). All together, these results demonstrate that caspase-2 CARD is capable of activating both NF-κB and p38 MAPK.

Caspase-2 Activates NF-κB through TRAF2 and the IKK Complex—To investigate the signaling pathway downstream of caspase-2, we made use of several inhibitors and dominant negative (DN) molecules of key proteins involved in known NF-κB signaling pathways. Because most NF-κB signaling pathways converge at the IKK complex, we started with a kinase-dead mutant (K44A) of IKK-β that functions as a dominant negative inhibitor (IKK-β DN) to analyze whether caspase-2 signals through this central NF-κB-activating complex. Indeed, low levels of IKK-β DN abolish both TNF- and caspase-2-induced NF-κB activation, suggesting that the IKK complex is a central downstream mediator of caspase-2-induced NF-κB activation (Fig. 4A). A20 is an inhibitor of several NF-κB-activating pathways, including those induced by lipopolysaccharide (49, 50), TNF (49,
Caspase-2 activates NF-κB through TRAF2 and the IKK complex. A and B, 293T cells were transiently cotransfected with an NF-κB-dependent luciferase reporter together with plasmids encoding caspase-2 C320A in the presence or absence of A20 or dominant negative inhibitors of IKK-β or TRAF2 (A) or RIP2 DN (B). As a control, cells were transfected with RIP2 or treated with 500 IU/ml human TNF to induce NF-κB activation. 24 h after transfection, lysates were analyzed for luciferase activity. C and D, co-immunoprecipitation assays were performed using lysates of 293T cells that had been transiently co-transfected with plasmids encoding caspase-2 C320A and E-tagged TRAF2 (C) or Myc-tagged TRAF1 (D). Immunoprecipitates were prepared using the indicated antibodies, adsorbed to protein G-Sepharose, and analyzed by SDS-PAGE/immunoblotting using an anti-caspase-2 antibody. Aliquots of the same lysates were also analyzed directly by SDS-PAGE/immunoblotting as indicated. IP, immunoprecipitation; WB, Western blotting. E, yeast two-hybrid assays were performed with TRAF1 in the activating domain vector pGAD424 and caspase-2 C320A in the DNA binding domain vector pAS2. Transformants were checked for interaction by survival and β-galactosidase activity.
Caspase-2-Induced NF-κB—To further analyze the involvement of TRAF2 in the caspase-2 signaling pathway, we performed co-immunoprecipitation experiments with enzymatically inactive caspase-2 C320A and E-tagged TRAF2. We demonstrate that caspase-2 interacts with TRAF2 when co-expressed in cells (Fig. 4C). Taken together, these results suggest a downstream role for TRAF2 in caspase-2-activated NF-κB activation. Although TRAF1 and TRAF2 share a conserved C-terminal TRAF domain (59), TRAF1 differs from TRAF2 and other TRAF proteins, since it contains only one zinc finger structure and lacks the N-terminal RING finger domain required for TRAF2- and TRAF6-mediated activities (60). However, since TRAF1 and -2 form heterotrimeric (61) and many TRAF2-interacting proteins have been found to bind TRAF1, we studied whether caspase-2 could be immunoprecipitated with Myc-tagged TRAF1. Indeed, co-immunoprecipitation analysis demonstrated that caspase-2 C320A clearly interacts with Myc-tagged TRAF1 in 293T cells (Fig. 4D). To rule out the possibility that TRAF1 interacts with caspase-2 via its binding to TRAF2 (59), we used a GAL4-based yeast two-hybrid approach, since no TRAF2 homologue is encoded in the yeast genome (data not shown). Negative controls demonstrated that the pGAD424-TRAF1 and the pAS2-caspase-2 plasmids are not autoactivating in yeast (Fig. 4E). However, significant β-galactosidase activity was detectable upon co-transformation of the yeast with the caspase-2- and TRAF1-expressing constructs, confirming that the two proteins interact. Further studies have shown that the C-terminal TRAF domain is sufficient for interaction with caspase-2 in yeast (data not shown). These results suggest that caspase-2 and TRAF1 interact both in 293T cells and in yeast via a direct physical association involving the C-terminal TRAF domain of TRAF1.

RIP1 Is Recruited to the Caspase-2-TRAF2 Protein Complex—As mentioned above, the protein kinase RIP1 is a major regulator of NF-κB activation known to interact with TRAF2 (10). Therefore, we studied the interaction between HA-tagged caspase-2 and RIP1 by immunoprecipitating caspase-2 and comparing it with TRAF2 recruitment. In contrast to TRAF2, RIP1 was not present in HA immunoprecipitates (Fig. 5A, left and middle panels). However, when both TRAF2 and RIP1 were co-expressed with HA-tagged caspase-2, RIP1 was clearly detectable in the HA-immunoprecipitates (Fig. 5A, right panel).
These results demonstrate that RIP1 is recruited to caspase-2 only in the presence of TRAF2, suggesting the formation of a NF-κB signaling complex harboring caspase-2, TRAF2, and RIP1.

Several reports have demonstrated that caspase-8 cleaves RIP1 in vitro, in TNF-stimulated HeLa cells, and in FasL-stimulated 293T and Jurkat T cells (37, 38). This cleavage at residue Asp324 leads to the release of a C-terminal RIP1 fragment that functions as a dominant negative molecule on NF-κB activation (37, 38). In this respect, caspase-8 promotes cell death by shutting down the NF-κB survival pathway. To rule out a similar function for caspase-2, in vitro translated 35S-labeled RIP1 was incubated with purified recombinant caspase-2 and caspase-8 (Fig. 5B). In accordance with published results (37, 38), RIP1 was partially processed to fragments of 38 and 36 kDa by caspase-8. However, when incubated with caspase-2, RIP1 fragments were not detected, and no weakening of the band representing full-length RIP1 was observed. In contrast to RIP1, 35S-labeled caspase-2 was cleaved to a 38-kDa fragment by recombinant caspase-2, demonstrating that the enzyme was proteolytically active. These results suggest that RIP1 is not processed by caspase-2 to generate a dominant negative fragment when it is recruited into the caspase-2 complex.

Inducible Complex Formation between Endogenous Caspase-2, TRAF2, and RIP1—Taken together, the above results suggest that overexpressed caspase-2 initiates the activation of NF-κB through the recruitment of endogenous TRAF2 and RIP1. To confirm the formation of an endogenous caspase-2-TRAF2-RIP1 protein complex, stimulated and unstimulated cell extracts of 293T cells were analyzed by gel filtration in a manner similar to the approach used to characterize the caspase-containing apoptosome (62), inflammasome (34, 35), and PIDDosome (33, 36) complexes. When cell extracts incubated at 4 °C were analyzed by gel filtration chromatography, caspase-2 and RIP1 both eluted mainly in fractions corresponding to their theoretical masses (Fig. 6A). TRAF2, however, had an apparent molecular mass of ~300 kDa, indicating that even in resting conditions, TRAF2 (theoretical mass of 75 kDa) is a component of a multiprotein complex. Incubation of the cell extracts at 37 °C caused caspase-2 to elute in fractions corresponding to a large 670-kDa complex. The same shift to a large 670-kDa complex was observed for both TRAF2 and RIP1 (Fig. 6B). As a control, caspase-3 eluted at about 50 kDa in both stimulated and unstimulated cell extracts.

To confirm that caspase-2, TRAF2, and RIP1 were recruited in the same 670-kDa protein complex, we analyzed whether endogenous caspase-2 and TRAF2 could be retrieved in RIP1 immunoprecipitates of 293T cell lysates that have been incubated at 37 °C. Immunoprecipitates of 293T cell lysates that have been kept at 4 °C were analyzed as a negative control. Indeed, both caspase-2 and TRAF2 could be detected in the RIP1 immunoprecipitates of cell lysates that have been incubated at 37 °C (Fig. 7), whereas these proteins were absent in the immunoprecipitates of cell lysates that have been kept at 4 °C (Fig. 7). As a control, RIP1 itself was equally well immu-
neprecipitated in both cell lysates that have been incubated at 4 and 37 °C. These experiments confirm our previous results and point to the existence of an endogenous and inducible high molecular weight complex containing caspase-2, TRAF2, and RIP1.

DISCUSSION

Mounting evidence suggests an important role for caspases in processes beyond cell death, such as cell cycle regulation (63), cell proliferation (64–66), and differentiation (67, 68). Protease-independent signaling mechanisms initiated by caspases are beginning to be unraveled. In this respect, caspase-8 and -10 and the caspase-like inhibitor FLIP were shown to activate NF-κB signaling independently of their protease activity (47, 48). In a similar vein, we have recently demonstrated that caspase-1 is capable of activating p38 MAPK and NF-κB through its CARD motif, independently of cytokine maturation and without the need for its enzymatic activity (46). The crucial role of the CARD motif in NF-κB signaling is supported by recent in vivo data. Mice deficient in the CARD-containing Bel10 (69), the CARD of CARMA1 (70), or the CARD-containing kinase RIP2 (71) all display profound defects in T and B lymphocyte activation and proliferation, due to abrogated NF-κB activation following antigen receptor stimulation.

Although caspase-2 has been shown to be dispensable for cell death to proceed normally in almost all tissues (72, 73), several groups have determined that caspase-2 is essential for mitochondrial membrane permeabilization during apoptosis signaling and the consequent release of apoptogenic factors such as cytchrome c and Diablo (17, 19, 20). The release of these apoptogenic factors from mitochondria has recently been demonstrated to occur independently of caspase-2 enzymatic activity (18). In this study, we demonstrate that in addition to its role in the induction of apoptosis, caspase-2 is recruited to a large protein complex, which initiates inflammatory signaling pathways. The recruitment of caspase-2 into a large protein complex is in accordance with recent reports showing the elution of endogenous caspase-2 in high molecular weight fractions of cell lysates incubated at 37 °C (33, 36). The CARD-containing adaptor protein RAIDD and the death domain-containing protein PIDD have recently been identified as members of this large caspase-2 complex, named the "PIDDosome," which is associated with premitochondrial apoptosis signaling in response to DNA damage-inducing agents (36). Here, we demonstrate that caspase-2 also is implicated in signaling pathways leading to both NF-κB and p38 MAPK activation, through the recruitment of TRAF2 and RIP1 into a large and inducible 670-kDa complex (Fig. 8).

In contrast to apoptosis signaling, NF-κB and p38 MAPK activation are independent of the proteolytic activity of caspase-2, and require solely the N-terminal caspase-2 CARD domain. We recently identified a similar function for the CARD domain derived from caspase-1 (46). Although the CARD domains of both caspase-1 and -2 are capable of activating NF-κB, the signaling pathways involved are entirely different. Caspase-1-mediated activation of NF-κB occurs through the recruitment of RIP2 (46), whereas caspase-2-mediated NF-κB activation occurs through TRAF2. Moreover, we show that overexpressed RIP1, in the presence of TRAF2, forms a complex with procaspase-2. Finally, endogenous caspase-2 is recruited into a large and inducible protein complex containing TRAF2 and RIP1. All together these results suggest that, besides the proapoptotic function of caspase-2 that acts upstream of mitochondria, caspase-2 can also form a complex that initiates NF-κB and p38 MAPK activation through the recruitment of TRAF2 and RIP1, as depicted in Fig. 8. In view of the role of RIP1 in genotoxic stress-induced NF-κB activation (74) and caspase-2 in genotoxic stress-induced apoptosis (17), this complex may be implicated in the integration of these proin...
flamatory and proapoptotic signaling pathways. It is also conceivable that TRAF2 and RIP1 may initiate necrotic signaling (75, 26) through this complex.

Caspace-2 is known to form elaborate filamentous and dot-like structures through its CARD domain (23, 24). Besides caspase-2, other CARD proteins such as RAIDD and Bcl-10 have been demonstrated to form filamentous structures (25, 27). In the case of RAIDD, these structures correlate with the induction of apoptosis (25), whereas Bcl-10-containing filaments are essential for NF-κB activation through the recruitment of TRAF2 and RIP1 (27). The Bcl-10-dependent activation of NF-κB functions downstream of T-cell receptor engagement and is crucial for the maturation of thymocytes and the activation of peripheral T cells (69). Therefore, it will be interesting to examine whether the formation of such filaments is required for caspase-2-mediated activation of NF-κB and p38 MAPK.

This topic as well as the characterization of the cellular and physiological context in which caspase-2 mediates activation of NF-κB and p38 MAPK are subjects of ongoing research.

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