Activation of the c-Jun N-terminal Kinase/Stress-activated Protein Kinase Pathway by Overexpression of Caspase-8 and Its Homologs*

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Caspase-8 and its homologs play an essential role in the mediation of apoptosis by several DD receptors such as tumor necrosis factor receptor 1 (TNFR1), Fas/Apo-1, death receptor 3 (DR3, or Ws1, APO-3, or TRAMP), death receptor 4 (DR4, or TNF-related apoptosis-inducing ligand receptor 1), and death receptor 5 (DR5, or TNF-related apoptosis-inducing ligand receptor 1) (1–4). Caspase-8 is recruited to these receptors via the interaction of its prodomain with Fas-associated death domain (FADD), or MORT1), which leads to formation of the death-inducing signaling complex (DISC) (5–7). Upon its recruitment to the DISC, caspase-8 is activated by an autoproteolytic mechanism involving the removal of the prodomain and the release of its activated protease subunits into the cytosol (8). Activated caspase-8 acts as the initiator caspase in the caspase cascade, activation of which eventually results in cell death (9). The DISC-containing prodomains are also found in two additional cellular proteins; caspase-10 (Mch4, FLICE2), a proteolytically active caspase-8 homolog (3, 10), and Mach-related inducer of toxicity (MRIT; also called c-FLIP, Casper, I-FLICE, FLAME, CASH, and CLARP), a caspase-8 homolog devoid of protease activity (11–17).

In addition to its ability to activate the caspase cascade, TNFR1 is also known to activate a kinase cascade involving activation of the nuclear factor-κB (NF-κB) and the JNK pathways via recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP) (18). On the other hand, Fas/Apo-1 was recently shown to activate the JNK pathway via the adaptor protein Daxx (19). Activation of the JNK pathway leads to the phosphorylation-induced activation of transcription factors c-Jun, activating transcription factor-2, and Elk-1 (20, 21). While JNK activation has been implicated in oncogenic transformation and immune response in mammalian cells, its role in cell death is still controversial. For example, overexpression of several kinases of this pathway has been shown to induce cell death, whereas their corresponding dominant negative mutants can block stress-induced and DD receptor-induced apoptosis in several cell lines (22–25). JNK activation has also been implicated in nerve growth factor withdrawal-induced apoptosis of PC12 cells, which can be blocked by JNK-interacting protein-1 (JIP-1), a recently identified inhibitor of the JNK pathway (26, 27). Daxx was recently shown to activate JNK via apoptosis signal-regulating kinase 1 (ASK1) and act cooperatively with the FADD-induced caspase cascade in the mediation of Fas-induced apoptosis (19, 28). Finally, gene knock-out of JNK3, a terminal kinase of the JNK pathway leads to resistance against neuronal cell death (29). On the contrary, JNK activation was found to be dispensable for the TNFα- and/or Fas-induced apoptosis in HeLa and Jurkat cells, suggesting that the requirement for the JNK pathway in apoptosis is cell type- and stimulus-dependent (18, 30).

The role of caspase cascade in JNK activation is also controversial. Fas/Apo-1-induced JNK activation in a Fas-sensitive neuroblastoma cell line was shown to be resistant to the caspase inhibitor z-VAD-fmk (23). Similarly, Fas-induced JNK activation in 293 cells was resistant to caspase inhibitors CrmA, z-VAD, and z-DEVd (19). On the contrary, Fas-induced JNK activation was found to be sensitive to caspase inhibitors in Jurkat (T cell leukemia) and SKW6.4 (B lymphoblastoid) cells (31, 32). Finally, a recent study analyzed Fas-mediated JNK activation in Jurkat cells and discovered that while the JNK activity induced by low levels of Fas cross-linking could be blocked by z-VAD, that activated at higher levels of Fas cross-linking was z-VAD-resistant (30). Collectively, these studies suggest that, depending on the cell type and the magnitude of the stimulus, Fas can activate the JNK pathway by caspase-dependent and -independent pathways.

Until recently, DISC-containing proteins were mainly known for their interaction with FADD and the resultant activation of...
The caspase cascade. However, a recent study, using yeast two-hybrid interaction and coimmunoprecipitation assays, demonstrated that MRIT/Casper can also interact with various TRAF family members (12). This study, however, did not test the functional significance of the above interaction. In the present study, we explore the ability of MRIT and other caspase family members to interact with the TRAFs and activate the JNK pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—293T and MCF7 cells were obtained from Dr. David Han (University of Washington, Seattle). 293 EBNA cells were obtained from Invitrogen. All cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 5% carbon dioxide. Rabbit polyclonal antibodies against FLAG, hemagglutinin (HA), and Myc tags were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Murine antibody against human FADD was obtained from Transduction Laboratories. Flag beads were obtained from Kodak Scientific Imaging Systems. A pull-down kinase assay kit for stress-activated protein kinase/JNK was obtained from New England Biolabs, and the constructs for PathDetect luciferase reporter assay were purchased from Stratagene.

Expression Constructs—The expression constructs for the death receptors, FADD, CrmA, MRIT isoforms, and the various caspases have been described previously (11, 33). Constructs encoding caspase-1, -2, -3, -6, -7, and -9 were gifts from Michael Wright (University of Washington). Constructs encoding caspase-10 (Mch4 isoform) and its C358A mutant (3) have been described previously and were obtained from the indicated source. A construct encoding JBD-JIP1 was a gift from Dr. Roger Davis, and dominant negative rMEKK1 was obtained from Dr. Richard Gaynor. Constructs encoding mTRAF1, mTRAF2, TRAF3, mTRAF5, and I-TRAF were prepared using the corresponding IMAGE consortium expressed sequence tag clones (Genome Systems) as templates. The various point mutants of caspase-8, -7, and -9 were generated using the Quickchange site-directed mutagenesis kit (Stratagene). The various deletion and fusion constructs were made by polymerase chain reaction using custom primers. Epitope-tagged expression constructs for caspase-3, -8, and -10; MRIT; TRAFs; and their deletion and point mutants were tagged at the C termini. The sequence of all constructs was confirmed by automated fluorescent dye-terminator sequencing on an ABI 373 sequencing machine.

Experiments were confirmed by Western analysis on total cell extracts.

C-Jun Transcriptional Activation Assay—293 EBNA cells (1.2 x 10⁵) were transfected in duplicate with various expression constructs (500 ng) along with a fusion transactivator plasmid containing yeast GAL4 DNA-binding domain fused to transcription factor c-Jun (pFA-cJun) (50 ng), a reporter plasmid encoding the luciferase gene downstream of the GAL4 upstream activating Sequence (pFR-luc) (500 ng), and a Rous sarcoma virus/LacZ (β-galactosidase) reporter construct (75 ng). Transfection was performed using the calcium phosphate coprecipitation method. 32–40 h later, cell extracts were prepared using the Luciferase assay were purchased from Stratagene.

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precoated with 2% bovine serum albumin. Beads were washed twice with lysis buffer, twice with a wash buffer containing 0.1% Triton X-100, 20 mM sodium phosphate (pH 7.4), 500 mM NaCl and again with lysis buffer. Bound proteins were eluted by boiling, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by Western blot.

RESULTS
Activation of the JNK Pathway by Caspase-8 and Homologs—As a measure of JNK activation, we tested the ability of caspase-8 and its homologs to induce phosphorylation of transcription factor c-Jun in a “pull-down” kinase assay. As shown in Fig. 1A, expression of active site mutants of caspase-8 and -10 and MRIT led to significant JNK activation in 293 EBNA cells, which was comparable in magnitude with that observed with CD40, a known activator of this pathway. All of the above DED-containing proteins are missing the critical cysteine residue at the putative caspase active site and are proteolytically inactive. Therefore, JNK activation by them is not related to the activation of the caspase cascade and the resultant activation of the stress-response genes. Activation of the JNK pathway by the above DED-containing proteins was confirmed using a reporter assay in which luciferase expression was driven by JNK-mediated phosphorylation of the activation domain of transcription factor c-Jun fused to the GAL4 DNA binding domain (Fig. 1B). This assay further demonstrated that the DED-containing prodomains of caspase-8 and MRIT were even more effective than the full-length proteins in c-Jun transcriptional activation (Fig. 1B). Additional experiments localized the JNK-activating ability of caspase-8 to its prodomain, while no significant JNK activation was observed with the construct expressing its protease domain (Fig. 1C). Coexpression of the caspase-8 prodomain with either a GAL4-cFos fusion construct or a GAL4 DNA binding domain construct failed to activate the luciferase reporter construct, thereby demonstrating the specificity of the assay (Fig. 1C). The observed activation of the JNK pathway was not limited to 293 EBNA cells, since the caspase-8 prodomain activated this pathway in COS (African green monkey kidney) and 293 (human embryonic kidney) cells as well (data not shown). However, we have failed to observe significant activation of the JNK pathway by the above proteins in the baby hamster kidney cells (data not shown), which indicates tissue/species specificity of this response.

Failure of Non-DED-containing Caspases to Activate the JNK Pathway—We also tested the JNK activating ability of several non-DED-containing caspases, such as caspase-1, -2, -3, -7, and -9 and active site mutants of caspase-7 and -9. However, none of these caspases were able to activate the JNK pathway in 293 EBNA cells (Fig. 2A and data not shown), supporting the requirement of the DEDs for this process.

Effect of CrmA and p35 on the JNK Activation by Caspase-8 and Its Homologs—The ability of MRIT and the active site mutants of caspase-8 and -10 to activate the JNK pathway suggests that this ability is independent of their role in the activation of the caspase cascade and the resultant activation of the stress response genes. However, it is conceivable that the overexpression of these proteins leads to their oligomerization with the endogenous caspases and the resultant activation of the caspase cascade. Similarly, the prodomain of caspase-8 could activate the JNK pathway by oligomerizing and activating the endogenously expressed caspase-8. To rule out this possibility, we tested the ability of CrmA and p35 to block JNK activation by the caspase-8 prodomain and full-length MRIT (MRIT-a1 isoform). CrmA is a cowpox virus-encoded inhibitor of the protease activity of the proximal caspases, whereas p35 is a baculovirus-encoded inhibitor of both proximal and distal caspases. As demonstrated in Fig. 2B, both CrmA and p35 failed to block JNK activation by the caspase-8 prodomain, thereby confirming that it activates the JNK pathway independent of the activation of the caspase cascade. Surprisingly, both CrmA and p35 failed to block JNK activation by MRIT (Fig. 2C).
which suggests a difference in the mechanism of JNK activation between MRIT and caspase-8.

Virally Encoded DED-containing Proteins Fail to Activate the JNK Pathway—Several viruses were recently shown to encode proteins that contain DEDs, and these proteins were found to block apoptosis mediated by death domain receptors by binding to FADD or caspase-8 protease domain plasmid. Values shown are mean ± S.E. of a representative of two independent experiments performed in duplicate. B, caspase-8 D73A mutant blocks JNK activation by various DD receptors. The experiment was performed as described above using 150 ng/well of empty vector or various receptors and 750 ng/well of empty vector or the D73A plasmid. C, inhibition of caspase-8 predomin-induced JNK activation by a dominant-negative mutant of TRAF2 (DN-TRAF2). 293 EBNA cells were transfected with an empty vector or an expression vector for caspase-8 predomin (4 μg each) with or without an expression vector for DN-TRAF2 (4 μg), and the "pull-down" kinase assay performed as described for Fig. 1A. The total amount of DNA was kept constant by adding empty vector.

**Fig. 3. Modulation of caspase-8-mediated JNK.**

A, deletion and point mutants of caspase-8 block c-Jun transcriptional activation in a dominant negative fashion. The experiment was performed as described in the legend to Fig. 1B using 150 ng/well of empty vector, the caspase-8 prodomain, or caspase-10 C358A plasmid, and 750 ng/well of control vector, caspase-8 D73A, or the caspase-8 protease domain plasmid. Values shown are mean ± S.E. of a representative of two independent experiments performed in duplicate. B, caspase-8 D73A mutant blocks JNK activation by various DD receptors. The experiment was performed as described above using 150 ng/well of empty vector or various receptors and 750 ng/well of empty vector or the D73A plasmid. C, inhibition of caspase-8 prodomain-induced JNK activation by a dominant-negative mutant of TRAF2 (DN-TRAF2). 293 EBNA cells were transfected with an empty vector or an expression vector for caspase-8 prodomain (4 μg each) with or without an expression vector for DN-TRAF2 (4 μg), and the "pull-down" kinase assay performed as described for Fig. 1A. The total amount of DNA was kept constant by adding empty vector.

D, inhibition of the caspase-8 prodomain-induced JNK activation by dominant-negative full-length rat MEKK1. 293 EBNA cells were transfected with the indicated plasmids, and a "pull-down" kinase assay was performed as described for Fig. 3C. Lack of JNK inhibition with wild-type MEKK1 demonstrates the specificity of the assay. A representative of two independent experiments is shown.

E, inhibition of the caspase-8 prodomain-induced JNK activation by JBD of JIP. The experiment was performed as described for Fig. 3C. A representative of two independent experiments.
The cellular DED-containing proteins. A Point Mutant of Caspase-8 DED Acts as Dominant Negative Inhibitor of JNK Activation by Death Receptors—To further define the role of DEDs in caspase-8-mediated JNK activation, we mutated several conserved residues in the DED1 of caspase-8 and tested their ability to activate JNK activation in the 293 EBNA cells. One of these mutants, containing an Asp to Ala substitution at amino acid residue 73 (caspase-8 D73A) was found to be incapable of JNK activation on its own and was selected for further studies (Fig. 3A and data not shown). As shown in Fig. 3A, this mutant effectively blocked JNK activation by caspase-8 prodomain and caspase-10 C358A in a dominant negative fashion (Fig. 3A). This mutant was also highly effective in blocking JNK activation induced by different DD receptors, including TNFR1, Fas/CD95, DR3, and DR4, consistent with the involvement of caspase-8 or its homologs in JNK activation by these receptors (Fig. 3B). Finally, the caspase homology domain of caspase-8 could partially block JNK activation induced by caspase-8 and -10 as well (Fig. 3A), thus explaining the stronger JNK activation seen with the construct encoding only the caspase-8 prodomain (Fig. 1B).

Inhibitors of Caspase-8-induced JNK Activation—TRAF2 has been shown to play an essential role in JNK activation by different members of the TNFR family. To determine its role in caspase-8-induced activation of the JNK pathway, we used a dominant negative mutant of TRAF2 (amino acids 87–501) that has been previously shown to block JNK activation by different TNFR family members. As shown in Fig. 3C, dominant negative TRAF2 could successfully block JNK activation by the caspase-8 prodomain. TRAF2 is believed to activate the JNK-stress-activated protein kinase pathway via mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1) (37, 38). Therefore, we next tested the involvement of MEKK1 in JNK activation by the caspase-8 prodomain. As shown in Fig. 3D, caspase-8-prodomain-induced JNK activation was effectively blocked by a kinase-defective mutant of full-length rat MEKK1.

JIP-1 is a recently identified cytoplasmatic protein that binds to JNK through its N-terminal JNK-binding domain (JBD) (27). JIP-1 and its JBD cause cytoplasmic retention of JNK and are specific inhibitors of the JNK signal transduction pathway in several cellular processes including cell death (27). As shown in Fig. 3E, JBD of JIP-1 was highly effective in blocking JNK activation mediated by the caspase-8 prodomain.

Interaction of Caspase-8 and Its Homologs with the TRAF Proteins—To delineate the mechanism of JNK activation by caspase-8 and its homologs, we tested their ability to physically interact with the TRAF family members using a coexpression–coimmunoprecipitation assay in 293T cells. Caspase-8 could communoprecipitate TRAF1 and TRAF2, but failed to communoprecipitate I-TRAF (Fig. 1A–C). This ability to communoprecipitate TRAF proteins was not limited to caspase-8, since caspase-10 could successfully communoprecipitate TRAF1 and TRAF2 as well (data not shown). We also confirmed the previously demonstrated interaction between MRIT/Casper and TRAFs (data not shown). However, caspase-3, a non-DED-containing caspase, failed to communoprecipitate TRAF1 in the above assay, thereby demonstrating the specificity of the interaction. The expression of caspase-8 is shown by Western analysis on the cell lysates. C, interaction of caspase-8 with TRAF5. 293T cells were transfected with the indicated plasmids. Flag-tagged murine TRAF5 was immunoprecipitated with Flag beads or control beads, and the communoprecipitated Myc-caspase-8 was detected by Western blot with a rabbit polyclonal antibody against the Myc tag. The expression of caspase-8 is shown by Western analysis on the cell lysates. D, interaction of caspase-8 and TRAF1 in a cell-free system. Myc-caspase-8 and HA-mTRAF1 were cloned in pET28 bacterial expression vector, and the proteins were expressed in the BL21(DE3)pLysS host cells following the manufacturer’s instructions (Novagen, Madison, WI). Cells were lysed by three cycles of freeze-thaw and subsequently sonicated. The lysed samples were centrifuged, and equal volumes of supernatant from cultures expressing Myc-caspase-8 and mTRAF1-HA were incubated for 1 h at 4 °C. Immunoprecipitation was carried out with Myc beads or control beads as described previously, and Western blot analysis was performed using rabbit polyclonal antibodies against the HA tag. E, interactions of the caspase-8 D73A mutant with TRAF1 or TRAF2. The experiment was performed as described for Fig. 4A. F and G, TRAF1 and TRAF2 interact with caspase-8-prodomain (residues 1–180), protease domain (residues 217–479), and MRITβ1 isoform. The experiment was performed as for Fig. 4A. The lack of communoprecipitation of GFP-HA demonstrates the specificity of the interactions. The expression of caspase-8 deletion constructs and MRITβ1 is shown by Western analysis on the cell lysates. H, the interaction of caspase-8 and TRAF5. 293T cells were transfected with expression vectors encoding FLAG-tagged caspases along with HA-tagged TRAF3 and GFP-HA. The experiment was performed as described in Fig. 4. I, FADD cannot displace TRAF1 and TRAF2 from caspase-8-prodomain. 293T cells were transfected with the indicated plasmids, and the experiment was performed as described above for A. Western blot analysis on total cellular lysates indicates equivalent expression of TRAFs and FADD protein in various lanes (middle and lower panels). I.P., immunoprecipitate; L, lysate; C, control antibody beads; F, Flag beads; mlg, mouse immunoglobulin.
interaction (data not shown). Caspase-8 also failed to commu-
nonprecipitate GFP, which served as a negative control (Fig. 4, B, F, and G). The ability of caspase-8 to interact with TRAF1 protein was confirmed using bacterially expressed and purified proteins, which demonstrated that this interaction does not require the presence of any intermediate bridging protein(s) that could be present in 293T cells (Fig. 4D).

We also tested the interaction between caspase-8 D73A and the TRAFs. As shown in Fig. 4E, this mutant of caspase-8 successfully communoprecipitated TRAF1 or TRAF2. These results indicate that activation of the JNK pathway involves interaction of specific residue(s) of caspase-8 prodomain with the TRAFs and not the simple act of binding of the two proteins.

We next sought to determine which domains of caspase-8 interact with the TRAF proteins. Both the prodomain (amino acids 1–180) and the caspase homology domain (amino acids 217–479) of caspase-8 could communoprecipitate TRAF1 or TRAF2 (Fig. 4, F and G). The ability of the prodomain of caspase-8 to interact with TRAF proteins was unexpected, since a deletion construct encoding the homologous prodomain of MRIT/Casper was recently shown not to interact with the TRAF proteins (12). To resolve this discrepancy, we tested the ability of the naturally occurring MRIT/b1 isomorf to communoprecipitate TRAF1. Like the prodomain construct used in the previous study (12), the MRIT/b1 isomorf contains both DEDs and has additional 37 amino acids at the COOH terminus (11). MRIT/b1 successfully communoprecipitated TRAF1 (Fig. 4F), suggesting that the additional amino acids at the COOH terminus may contribute to the stability of the MRIT/b1 isomorf or alternatively to its interaction with TRAF1. Taken together, the above results suggest that the DED-containing proteins interact with TRAF family members through multiple domains.

The TRAF proteins are characterized by the presence of a TRAF domain at their COOH termini, which, based on its functions and sequence conservation among different family members, can be further subdivided into TRAF-N and TRAF-C subdomains (39). For example, the TRAF-N domain of various family members is required for self-association and association with the cellular inhibitors of apoptosis proteins, whereas the TRAF-C domain is required for interaction with receptors and signaling molecules RIP and TNFR1-associated death domain (TRADD) (39). We used deletion mutagenesis to map the domain(s) of TRAF1 that are necessary for its interaction with caspase-8. As shown in Fig. 4H, a C-terminal deletion mutant of TRAF1 (encoding residues 1–262), which is missing the TRAF-C domain, could weakly interact with caspase-8. However, an almost complete absence of interaction with caspase-8 was observed in experiments that utilized a construct missing the TRAF-N domain as well (encoding residues 1–190). These results are in agreement with the previously demonstrated interactions between MRIT/Casper and TRAF2 and suggest the importance of an intact TRAF domain for the interaction with caspase-8 and its homologs.

**Effect of FADD on Caspase-8-TRAF Interaction**—Caspase-8 is recruited to the DISC through the interaction of its DED-containing prodomain with the DED of the adaptor molecule FADD. Recruitment to FADD leads to autoproteolytic activation of caspase-8, resulting in activation of the caspase cascade and eventual cell death. We were interested in determining whether FADD can influence the interaction between caspase-8 and TRAF proteins. Coexpression of FADD had no significant effect on the interaction between full-length caspase-8 and either TRAF1 or TRAF2 protein (data not shown). We reasoned that these results could be due to the inability of FADD to influence the interaction between the protease domain of caspase-8 and the TRAF proteins. Therefore, the above experiment was repeated with the prodomain of caspase-8 and the TRAF proteins. As shown in Fig. 4I, FADD led to displacement of TRAF1 or TRAF2 proteins from the caspase-8 prodomain. These results suggest that the multidomain interactions of TRAFs with the DED-containing proteins probably contribute to the stability of the interaction, thereby allowing the formation of multiprotein signaling complex. Moreover, these results argue against the possibility that the caspase-8-TRAF interactions are mediated via a FADD-TRADD bridge.

**Role of JNK Activation in Caspase-induced Cell Death**—We next sought to determine the role of JNK activation in caspase-mediated cell death. We chose caspase-10 for these experiments, since it was found to be a more efficient inducer of cell death than caspase-8 or MRIT. The JBD of JIP-1 could partially block caspase-10-induced cell death in 293T and 293 cells (Fig. 5, A and B, and data not shown). The inhibitory role of the JBD of JIP-1 was more pronounced when the amount of caspase-10 plasmid was kept low and became less significant at higher levels of caspase-10 expression, at which point massive activation of the caspase cascade probably obviated any requirement of JNK activation for apoptosis (data not shown). Consistent with the role of JNK activation in caspase-mediated cell death, JBD of JIP-1 could effectively block apoptosis mediated by Fas/CD95 (Fig. 5C). However, we did not observe any significant inhibition of caspase-10-induced cell death by the caspase-8 D73A mutant, which could reflect the simultaneous activation of the caspase cascade. Taken together, the above results suggest that while JNK activation may facilitate caspase-10-induced apoptosis in the cells tested, it may not be essential for this process.

**Role of JNK Activation in MRIT-induced Cell Death**—Although MRIT is proteolytically inactive, its overexpression has been shown to lead to apoptosis (11, 12, 15, 16). Therefore, we were interested in testing the role of the JNK activation in MRIT-induced apoptosis. We began by performing a dose-response experiment to look for any correlation between the JNK- and apoptosis-inducing abilities of MRIT. As shown in Fig. 5D, transfection of MRIT-a1 led to a dose-dependent increase in both JNK activation and apoptosis, and there was a close correlation between the two activities, suggesting a causal link. To further test the involvement of the JNK pathway in MRIT-induced cell death, we tested the effect of blocking the JNK activation on MRIT-induced apoptosis. As shown in Fig. 5E, coexpression of JBD-JIP1 led to effective inhibition of MRIT-induced cell death in 293 EBNA cells. Taken together, the above results suggest a role for the JNK pathway in MRIT-induced cell death.

**DISCUSSION**

Death effector domain-containing caspases and caspase homologs have been extensively studied in the activation of programmed cell death. The proapoptotic ability of these caspases and caspase homologs has been so far attributed to their ability to proteolytically cleave and activate the downstream executioner caspases. In the present study, we provide evidence that transient overexpression of the DED-containing caspases and caspase homologs may lead to cell death by an indirect route involving activation of the JNK pathway.

Based on the available data, it is likely that DED-containing proteins activate the JNK pathway by virtue of their interaction with the TRAF proteins and subsequent activation of MEKK1. This conclusion is supported by the ability of dominant negative mutants of TRAF2 and MEKK1 to block caspase-8 prodomain-induced JNK activation. In addition to
MEKK1, we have also tested the involvement of ASK1 in caspase-8 prodomain-induced JNK activation. However, a kinase-inactive mutant of ASK1 (22) has been only partially effective in blocking caspase-8 prodomain-induced JNK activation in 293 EBNA cells, arguing against a major role of ASK1 in caspase-8-induced JNK activation.

The activation of the JNK pathway by the DED-containing proteins is unlikely to be secondary to the activation of caspase cascade and the resultant activation of stress-response genes based on the following evidence. First, this activity was localized to the prodomain of these proteins rather than the caspase homology domain. Second, active site mutants of caspase-8 and -10, which are proteolytically inactive, could efficiently activate the JNK pathway. Third, several non-DED-containing caspases failed to activate this pathway. Finally, CrmA and p35, two inhibitors of caspases and the apoptosis induced by them, could not block JNK activation by the caspase-8 prodomain.

It is unlikely that the caspase-8 and its homologs activate the JNK pathway due to the overexpression-induced reverse signaling via a caspase-8/10/MRIT-FADD-TRADD-death receptors-TRADD-TRAF2 pathway due to the following reasons. First, Shu et al. (12) have previously shown that Casper/MRIT could interact with both TRAF1 and TRAF2 in the yeast two-hybrid assay, which rules out the involvement of any bridging interaction.
protein(s) in this interaction. In fact, these researchers successfully isolated a total of nine independent clones of the TRAFs that interacted with Casper using the above assay, which points to the strength of the interaction (12). Second, we have similarly observed an interaction between bacterially expressed caspase-8 and TRAF1 in the present study. Third, a reverse signaling complex containing caspase-8/10/MRIT-TRADD-TRAF2 has never been demonstrated. In fact, Shu et al. (12) specifically looked for such a complex in the case of MRIT/Casper but failed to recruit MRIT/Casper to TNFR1 complex by the addition of TRADD, FADD, and TRAF2 either individually or in various combinations. Similarly, they failed to generate a complex consisting of MRIT, TRADD, and TRAF2 (12). We have similarly failed to detect the presence of endogenous FADD in the co-immunoprecipitated complex of caspase-8 with TRAFs (data not shown) and have found that cotransfection of FADD, in fact, decreases the binding between caspase-8 prodomain and TRAF1 or -2. Fourth, virally encoded DED-containing proteins, which have been previously shown to bind FADD (34–36), fail to activate the JNK pathway. Similarly, we have observed that the caspase-8 D73A mutant, which cannot activate the JNK pathway, continues to bind to FADD.

While both CrmA and p35 failed to block caspase-8-induced JNK activation, they successfully blocked the activation of this pathway by MRIT. This result is somewhat surprising, since CrmA does not directly bind to MRIT (12). However, since CrmA is known to bind to caspase-8 (12), it is possible that it blocks MRIT-induced JNK activation by blocking its interaction with caspase-8. Alternatively, MRIT may require activation by caspases for activation of the JNK pathway, and this step may be blocked by CrmA and p35.

An important discovery of the present study was the role of the JNK pathway in MRIT-induced cell death. Overexpression of MRIT has been shown to lead to cell death by several independent groups (11, 12, 15, 16). Based on the ability of CrmA to block MRIT-mediated cell death, we and others had previously concluded that MRIT induces apoptosis by activating the caspase cascade (11, 12, 15). However, overexpression of MRIT/CLARP in 293T cells, under the conditions where it induces significant apoptosis, failed to generate caspase activity (15). In the present study, we have discovered that CrmA is also capable of blocking MRIT-induced JNK activation, which provides an alternative explanation for the ability of CrmA to block MRIT-induced cell death.

A major limitation of the current study is that the activation of the JNK pathway is observed under conditions of overexpression of caspase-8 (and its homologs), and it is not clear whether the same effect would be observed under an endogenous level of expression of this protein. It can be further argued that the observed effect is an artifact of overexpression-induced aggregation of caspase-8. However, under endogenous conditions, caspase-8 is present in the cells as a minimally active zymogen and is even incapable of inducing cell death. Recruitment to the DISC allows aggregation of caspase-8 zymogens and generation of active caspase molecules via cross-proteolysis (8). Therefore, overexpression-induced aggregation of caspase-8 may mimic a physiological step in the activation of this protein and may not be entirely artificial. While not the perfect approach, it currently represents the only practical way for studying the signaling by cytotoxic proteins in their native form due to our inability to cross-link them using antibodies. We have also attempted to generate stable cell lines expressing the prodomain of caspase-8 with the hope of subsequently testing them for JNK activation. However, we have so far failed to generate such cell lines, which might reflect the cytotoxic effect of constitutive JNK activation.

The physiological role of caspase-8 in the JNK activation by death domain-containing receptors is also supported by two recent studies (40, 41). The first study compared the activation of the JNK pathway in embryonic fibroblast cell lines derived from caspase-8 +/- and caspase-8 -/- embryos in response to signaling via TNFR1, Fas/Apo1, and DR3 and concluded that the JNK pathway could be activated by these receptors in both cell lines (40). However, a careful analysis of this experiment has revealed a clear difference in the kinetics of JNK activation between the two groups of cells. For example, stimulation with TNF-α resulted in almost equivalent peak JNK activation between the two cell lines, which was observed after 10 min of treatment (40). However, while this JNK activation was sustained in the caspase-8 +/- cells for at least 30 min, there was a quick decline in the caspase-8 -/- cells and a return to the baseline level by the end of this time period (40). Essentially similar results were obtained when the JNK pathway was activated via Fas or DR3 signaling (40). caspase-8 -/- cells also appeared to lag behind the wild-type cells in the initiation of JNK activation response (40). Taken together, these results indicate that there is a kinetic difference in JNK activation between the caspase-8 +/- and -/- embryonic fibroblast cells and suggest that caspase-8 may play a contributory role in sustaining (and probably initiating) this response in these cells. Future studies should confirm this difference in additional tissues, since the contribution of caspase-8 to JNK activation may be tissue-specific, as demonstrated by its inability to activate the JNK pathway in the baby hamster kidney cells. The latter conclusion is further supported by another recent study that reported the isolation of a Jurkat T lymphocyte cell line deficient in caspase-8 that was resistant to Fas-induced cell death (41). Detailed analysis revealed that this cell line was not only incapable of activating caspases in response to Fas stimulation but, unlike in the study by Varfolomeev et al. (40), also completely failed to activate the JNK and p38 kinase pathways. This study suggests that caspase-8 may be essential for Fas-induced JNK activation in T cells (41) and underscores the importance of analyzing multiple tissues when testing the functional role of a gene/protein in knock-out animals. Finally, caspase-8 and its homologs may play a mutually redundant role in JNK activation by the death domain receptors, and it may require the knock-out of all three cellular DED-containing proteins to fully appreciate their contribution to this pathway.

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