Heat Shock Induces Apoptosis Independently of Any Known Initiator Caspase-activating Complex**

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Adaptive responses to mild heat shock are among the most widely conserved and studied in nature. More intense heat shock, however, induces apoptosis through mechanisms that remain largely unknown. Herein, we present evidence that heat shock activates an apical protease that stimulates mitochondrial outer membrane permeabilization and processing of the effector caspase-3 in a benzyloxycarbonyl-VAD-fluoromethyl ketone (polycaspase inhibitor)- and Bcl-2-inhibitable manner. Surprisingly, however, neither FADD-caspase-8 nor RAIDD-caspase-2 PIDDosome (p53-induced protein with a death domain) complexes were detected in dying cells, and neither of these initiator caspases nor the endoplasmic reticulum stress-activated caspases-4/12 were required for mitochondrial outer membrane permeabilization. Similarly, although cytochrome c was released from mitochondria following heat shock, functional Apaf-1- caspase-9 apotosome complexes were not formed, and caspase-9 was not essential for the activation of caspase-3 or the induction of apoptosis. Thus, heat shock does not require any of the known initiator caspases or their activating complexes to promote apoptotic cell death but instead relies upon the activation of an apparently novel apical protease with caspase-like activity.

Two pathways, referred to as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways, are widely regarded as being responsible for most, if not all, caspase-dependent apoptosis (1, 2). In both cases, caspase cascades are initiated through formation of a protein complex that contains a specific adapter protein and its associated “initiator” caspase (i.e. caspase-2, -8, -9, -10, or -12) (3). Once activated, most initiator caspases proteolytically activate the downstream “effector” caspases-3, -6, and/or -7, which in turn cleave specific cellular substrates resulting in chromatin compaction, membrane blebbing, and cell shrinkage. Stimulation of death receptors (such as CD95 and tumor necrosis factor (TNF) receptor 1) with their cognate ligands or agonistic anti-
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the Apaf-1 apoptosome, which in turn activates the effector caspases -3 and -7 (13). However, the caspase-12 gene is not expressed in the vast majority of humans because of a premature stop codon. Thus, it has been proposed that caspase-4 may substitute for caspase-12 in man. Indeed, caspase-4 is cleaved upstream of mitochondria in Bcl-2- or Bcl-xL-overexpressing cells, but similar to caspase-2, it must engage the intrinsic pathway to induce apoptotic cell death (14).

Responses to heat shock are among the most highly conserved and important in nature. Consequently, numerous studies have focused on how uni- and multicellular organisms cope with this common stress (15). Previous work makes it clear that up-regulation of various heat shock proteins (Hsps), including Hsp-70 and to a lesser extent Hsp-27 and Hsp-90, represents an adaptive response that can protect cells from subsequent pro-apoptotic stimuli. However, few studies have rigorously examined the mechanism(s) by which heat shock induces cell death. In this study, we have demonstrated that intense heat shock induces caspase-dependent apoptosis but does so via mechanisms that do not involve any of the established initiator caspase-activating complexes and thus may represent an archaic and novel pathway to cell death.

EXPERIMENTAL PROCEDURES

Cell Lines—E6.1, neo/Bcl-2, A3, caspase-8-deficient, and FADD-deficient Jurkat T cell strains were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% penicillin/streptomycin, and for neo/Bcl-2 overexpressors, G418. Ras/Myc-transformed apaf-1−/− and caspase-9−/− mouse embryonic fibroblasts (MEFs), primary wild-type and caspase-2−/−/ caspase-12−/− MEFs, and transformed caspase-12−/− MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin. All cells were grown in a humidified atmosphere of 5% CO2 in air at 37 °C.

Cell Death Treatments—Cells were challenged with the DNA-damaging agents etoposide (60 μM) or cisplatin (100 μM); the kinase inhibitor staurosporine (250 nM); or the death receptor ligands anti-CD95 (CH-11, 100 ng/ml), or a combination of TNF (300 ng/ml) and anti-CD95 (clone 4C1, Medical Biological Laboratories), mouse anti-caspase-2 (clone 1C12, Cell Signaling Technology), mouse anti-caspase-8 (clone 1C12, Cell Signaling Technology), mouse anti-caspase-10 (Pharmingen), and mouse anti-cytochrome c (clone 7H8.2C12, Pharmingen).

RNA Interference—Jurkat T cells (107 cells) were transfected by electroporation with 16 μg of double-stranded RNA oligonucleotides (Dharmacon) targeted against a luciferase control sequence (siCtrl: 5′-AA-CTTACGCTGAGTA-ACT TCGA-dTdT-3′) or a previously verified caspase-2 sequence (siC2–510: 5′-AA-CTTCCAGCT-GCC ATATAGG-dTdT-3′) (18). After 24 h, viable cells were purified using a Ficoll-Paque density gradient and replated at 106 cells/ml in fresh medium for an additional 24 h prior to treatment (18).

Gel Filtration Studies—Cell lysates were prepared from control or apoptotic cells and fractionated by size exclusion chromatography using a fast protein liquid chromatography protein purification system with a Superose-6 HR 10/30 column (Amersham Biosciences), as previously described (17). Fractions were subsequently mixed with 5× loading buffer, resolved by SDS-PAGE, and immunoblotted with a rabbit polyclonal antibody to PIDD (Alexis Biochemicals) or mouse monoclonal antibodies to RAIDD (clone 4B12, MBL) or caspase-2 (clone 35, BD Transduction Laboratories).

RESULTS

Heat Shock Induces a Unique Caspase Processing Profile That Cumulates in Apoptosis—In our initial experiments, we noted that diverse pro-apoptotic stimuli, including DNA damaging agents, Ser/Thr kinase inhibitors, p38/JNK activators, death receptor ligands, lysomotropic agents and ER stressors, stimulated time-dependent cleavage of caspase-9 at both Asp-315 (p35 caspase-9) and Asp-330 (p37 caspase-9) (Fig. 1A), whereas heat shock induced processing almost entirely at Asp-330 (Fig. 1B). Because cleavage of caspase-9 at Asp-315 and Asp-330 is generally mediated by the Apaf-1 apoptosome and the effector caspase-3, respectively (6), the data suggested that caspase-9 cleavage following heat shock was not mediated by the apoptosome but was instead a relatively late caspase-3-dependent event. In subsequent dose-response experiments, heat shock induced a steady increase in the activation of caspase-3 between 42 and 45 °C, which correlated with the formation of p37 caspase-9 and the amount of apoptosis (Fig. 1C, lanes 2–5). However, as previously observed, there was little evidence for the formation of p35 caspase-9 at any temperature. Therefore, our results suggested that heat shock might utilize an apical caspase other than caspase-9 to initiate its caspase cascade and promote apoptosis.
In an effort to determine the importance of caspases for heat shock-induced apoptosis, as well as the sequential order of caspase activation, we next examined heat-shocked cells pretreated with either the polycaspase inhibitor Z-VAD-fmk or the caspase-3/7 selective inhibitor DEVD-CHO (20). Heat shock alone initiated the activation of all apoptosis-related caspases (i.e. caspases -2, -3, -6, -7, -8, and -9), except for caspase-10, and induced ~50% apoptosis by 6 h, all of which was prevented by Z-VAD-fmk (Fig. 2A, lanes 1 – 3; Fig. 2B, left panels). Next, by carefully titrating DEVD-CHO, we established a concentration of this inhibitor, which did not prevent the initial processing of procaspase-3 to its intermediate p20 form (i.e. did not inhibit the apical protease responsible for cleaving procaspase-3 at Asp-175) but totally inhibited the subsequent autocatalytic conversion of caspase-3 from its p20 to its p19/17 forms (Fig. 2A, lane 4). In this context, selective inhibition of caspase-3 also prevented cleavage of a number of direct caspase-3 substrates, including poly(ADP-ribose) polymerase, caspase-6, and as predicted above, caspase-9 (Fig. 2A, lane 4). To our surprise, however, DEVD-CHO also prevented the processing of the remaining initiator caspases -2 and -8 (Fig. 2A, lane 4). Thus, our initial studies with pharmacological caspase inhibitors suggested that heat shock induced the activation of caspase-3 via a Z-VAD-fmk-inhibitable protease. This apical protease, however, did not appear to be any of the known classical initiator caspases, as they were all processed downstream of caspase-3.

Heat Shock Activates an Apical Protease Capable of Inducing MOMP and a Loss in $\Delta \Psi_m$—Inhibition of the apical protease with Z-VAD-fmk not only prevented the activation of caspase-3 but also inhibited MOMP (e.g. mitochondrial release of cytochrome c or Smac into the cytoplasm) as well as cell death up to 24 h following heat shock (Fig. 2A, lanes 2 and 3; Fig. 2B, left panels) (supplemental Fig. S1). By contrast, inhibition of caspase-3 with DEVD-CHO did not significantly inhibit cytochrome c release but did partially inhibit Smac release or more likely inhibited its degradation by the proteasome (21). DEVD-CHO also failed to inhibit the overall amount of heat shock-induced cell death but did promote a shift in the mode of cell death from apoptosis (PS<sup>+</sup>) to necrosis (PS<sup>-</sup>PI<sup>+</sup>) (Fig. 2A, lane 4; Fig. 2B, left panels). Therefore, collectively, these results indicated 1) that the heat-activated apical protease was responsible for inducing MOMP and 2) that downstream caspase-3 activity was required for execution of the apoptotic program, but in its absence, cells nevertheless died by necrosis because of severe mitochondrial injury brought on by the apical protease. DEVD-CHO also failed to prevent a loss in $\Delta \Psi_m$ following heat shock (Fig. 2B, right panels). This result was somewhat surprising given that caspase-3 is thought to be primarily responsible for triggering reductions in $\Delta \Psi_m$ by cleaving and inactivating NDUFS1, a complex 1 component of the mitochondrial respiratory chain (22). Thus, the heat shock-activated apical protease could induce a loss in $\Delta \Psi_m$ via caspase-3-independent mechanisms.

Heat Shock Induces Apoptosis Independently of the Apaf-1-Caspase-9 Apoptosome—Although processing of caspase-9 appeared to be a relatively downstream event, as already noted, cytochrome c was released during heat shock-induced apoptosis (Fig. 2A, lane 2). Therefore, in an effort to determine whether heat shock inhibited the formation and/or activity of the Apaf-1 apoptosome, we fractionated lysates from control and heat-shocked cells (by Superose-6 gel filtration chromatography) and compared them with naïve lysates activated with cytochrome c and dATP (cytochrome c/dATP). Heat shock induced partial oligomerization of Apaf-1 into large molecular weight complexes (~700 kDa), which were similar in size to those apoptosome complexes formed in cytochrome c/dATP-activated lysates (Fig. 3A, fractions 8 –12). How-
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**FIGURE 2.** Heat shock activates an apical protease that induces MOMP, a loss in Δψm, and caspase-3 activation. E6 Jurkat T cells were pretreated for 1 h with MeSO (DMSO, vehicle), the polycaspase inhibitor Z-VAD-fmk (40 μM), the caspase-3/7-selective inhibitor DEVD-CHO (150 μM), or the caspase-1/4 inhibitor YVAD-CHO (150 μM) and were subsequently heat-shocked for 2 h at 45 °C followed by a 4-h incubation at 37 °C. A, cells were then immunoblotted for all human apoptosis-related caspases, poly(ADP-ribose) polymerase, cytochrome c release into the cytosol, and Hsp70. −−− denotes a crop within the same gel. Caspase inhibitors had no effect on control cells, as shown in Fig. 7B, cell death (annexin V/PI) and Δψm (TMRE) measurements were determined by flow cytometry, as indicated under “Experimental Procedures.” * denotes nonspecific bands; aCasp-6 and pCasp-10 refer to the active and proforms of caspases-6 and -10, respectively.

However, caspase-9 was not recruited to the Apaf-1 complex nor was there any evidence for apoptosome-dependent processing of caspase-9 at Asp-315 (data not shown; Figs. 1 and 2A). We considered the possibility that recruitment of caspase-9 might be inhibited by Hsp-70 or Hsp-90, because both are up-regulated in response to sublethal exposures to heat shock, and both reportedly inhibit the apoptosome (23). However, following a lethal exposure to heat shock, there was no increase in the expression of either Hsp-70 (Fig. 2A, lanes 1–2) or Hsp-90 at 6 h (data not shown). Moreover, pretreatment of cells with the global translation inhibitor cycloheximide did not potentiate (nor did it inhibit) heat shock-induced MOMP, caspase-3 activation, or apoptosis (supplemental Fig. S2).

Despite the evidence against the involvement of the Apaf-1 apoptosome in heat shock-induced apoptosis, it remained plausible that an undetectable amount of p35 caspase-9, activated within the apoptosome, might process small amounts of caspase-3 and initiate a robust caspase amplification loop. We therefore examined both apaf-1−/− and caspase-9−/− MEFs for their apoptotic responses to heat shock, as well as to tumor necrosis factor-α/cycloheximide (24). Heat shock triggered MOMP (loss of cytochrome c from mitochondria) and apoptosis in both the Apaf-1 and caspase-9-deficient MEFs and importantly, caused a time-dependent increase in caspase-3-like DEVDase activity (Fig. 3, B and C). Using an antibody that recognizes only the processed forms of caspase-3, we confirmed that caspase-3 was activated within 4 h of heat shock. TNF, which stimulates the formation of initiator FADD-caspase-8 complexes, likewise activated caspase-3 (Fig. 3, B and C). We subsequently confirmed the phenotype of the Apaf-1- and caspase-9-deficient cells by incubating them with cytochrome c/dATP. As expected, neither Apaf-1 nor caspase-9-deficient lysates could independently support cytochrome c/dATP-dependent activation of caspases -9 and -3 but readily did so when combined (50:50 mix) prior to activation (supplemental Fig. S3). Thus, functionally active apoptosome complexes were not formed in response to heat shock, but more importantly, were not essential for heat shock-induced MOMP, caspase-3 activation, or cell death.

**Heat Shock Does Not Utilize ER Stress-activated Caspases to Induce Apoptosis**—Given that heat can induce protein misfolding, we speculated that heat shock might induce apoptosis through an ER stress pathway. ER stress reportedly induces apoptosis independently of the apoptosome by activating the apical caspase-12 (mouse), or possibly caspase-4 (human), both of which are localized to the outer ER membrane (11–14). Nevertheless, neither caspase-4 nor caspase-1 (another pro-inflammatory caspase) were expressed in our Jurkat T cell model (Fig. 4A), and YVAD-CHO, which potently inhibits both caspases (20), failed to prevent heat shock-induced MOMP, caspase activation, or cell death (Fig. 2A, lane S). Consistent with these results, caspase-12-deficient MEFs also displayed no defects in their ability to undergo MOMP or activate caspase-3 following heat shock (Fig. 4B). Moreover, although calpains are thought to activate ER-associated caspases, neither PD150605 nor Z-VF-CHO, two distinct mechanism-based calpain

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inhibitors, inhibited the activation of caspases or prevented apoptosis (Fig. 4C). Therefore, although heat shock likely induces protein misfolding, it did not utilize caspases -1, -4, -12, or calpains to induce apoptosis.

**Bcl-2 Inhibits Heat Shock-induced MOMP and Cell Death, but Does Not Prevent the Upstream Processing of Procaspase-3**—Z-VAD-fmk clearly inhibited an apical protease responsible for heat shock-induced MOMP. However, it remained unclear whether MOMP was required for the activation of caspase-3 (e.g. by stimulating the release of a factor from mitochondria other than cytochrome c, which led to the activation of caspase-3) or whether the apical protease simultaneously activated caspase-3 upstream of mitochondria. To address these questions, we utilized cells stably transfected with Bcl-2 (a potent inhibitor of MOMP) and reasoned that if the apical protease could directly process procaspase-3 upstream of mitochondria, then Bcl-2 should not entirely prevent this cleavage from occurring. In fact, as previously demonstrated, Bcl-2 prevented MOMP and cell death induced by the agonistic CD95 antibody CH-11 but did not inhibit the initial caspase-8 activity in its proform (25). Therefore, Bcl-2 inhibited heat shock-induced MOMP, as well as cell death, but did not prevent (although it delayed) the initial processing of procaspase-3 to its p20 form (Fig. 5, lane 12).

In each instance, Bcl-2 likely prevented the autocatalytic conversion of caspase-3 from its p20 to its fully mature p17 form, because it inhibited the release of Smac from mitochondria (Fig. 5, lanes 12, 16 and 18) and thus preserved the inhibition of p20 caspase-3 by IAPs (16). Indeed, the addition of recombinant Smac to lysates prepared from heat-shocked Bcl-2-overexpressing cells resulted in increased caspase-3 DEVDase activity relative to non-treated cells (supplemental Fig. S4) (16). Similar to DEVD-CHO, IAP inhibition of caspase-3 activity also inhibited caspase amplification, resulting in a decrease in caspase-8 and -2 processing (Fig. 5, lanes 12, 16, and 18) (16). Thus, first and foremost, the data demonstrated that the heat-activated apical protease could induce the initial processing of procaspase-3 to its p20 form, upstream of mitochondria. Furthermore, they implicated a role for Smac in propagating the caspase amplification loop and promoting cell death by antagonizing the inhibition of p20 caspase-3 by IAPs (see Fig. 9).

**Heat Shock Induces Apoptosis Independently of FADD and Caspase-8**—Based on our studies with DEVD-CHO and Bcl-2 overexpression, caspase-8 appeared to be processed rather late in the heat shock-induced caspase cascade (Fig. 2A, lane 4; Fig. 5). Recent reports, however, suggest that, in some cases, caspase-8 may exhibit proteolytic activity in its proform (25). Therefore, to definitively rule in/out an essential role for caspase-8 (or the caspase-8/10 adapter protein FADD) in heat shock-induced apoptosis, we utilized Jurkat T cells that lacked FADD and Smac, and the induction of apoptosis, whereas these events were largely inhibited in the
caspase-8 and FADD-deficient cells (Fig. 6, lanes 10–12). In contrast, both etoposide and heat shock induced the activation of all caspases and triggered cell death in the parental, caspase-8, and FADD-deficient cells (Fig. 6, lanes 4–9). Importantly, the absence of caspase-8 had no effect on the release of cytochrome c or Smac from mitochondria in heat-shocked cells (Fig. 6, lanes 7 and 8), ruling out its essential role as the apical protease responsible for MOMP. The absence of caspase-8 did slightly inhibit the processing of procaspase-3 (Fig. 6, lanes 7 and 8), but this was likely due to a disruption in the normal caspase amplification loop, wherein once activated downstream of caspase-6, caspase-8 can participate in the cleavage of additional procaspase-3 (27). Thus, neither caspase-8 nor FADD played an essential role in mediating heat shock-induced MOMP, the loss in Δψm, caspase-3 activation or apoptosis.

Heat Shock Does Not Require Caspase-2 to Induce MOMP or the Activation of Caspase-3, nor Does It Stimulate the Formation of a RAIDD/Caspase-2 PIDDosome Complex—Caspase-2 also appeared to be activated downstream of caspase-3 following heat shock (Figs. 2A and 5). Nevertheless, to rule out an essential role for caspase-2 in heat shock-induced apoptosis, we examined primary wild-type and caspase-2-deficient MEFs and found that both were equally sensitive to heat shock-induced MOMP, caspase-3 activation, and apoptosis (Fig. 7A). Previous reports, however, have suggested that caspase-2 may be uniquely important for apoptotic signaling in tumor cells (10, 28). Therefore, we also heat shocked E6 Jurkat T cells in the presence of Z-VDVAD-fmk, a potent inhibitor of both caspases -2 and -3 (29). Because Z-VDVAD-fmk, similar to DEVD-CHO, can inhibit the activity of processed caspase-3 (29), it prevented the autocatalytic cleavage of caspase-3 from its p20 to its p19/17 forms and inhibited the downstream cleavage of all caspases, including caspase-2 (Fig. 7B, lanes 7 and 8) (data not shown). Nevertheless, Z-VDVAD-fmk failed to

![FIGURE 4. Inflammatory and ER stress-related caspases or calpains are not required for heat shock-induced apoptosis. A, naive lysates from E6 Jurkat T cells and THP.1 monocytic cells were immunoblotted for the inflammatory caspases -1 and -4, 8, caspase-12 (Casp-12) and calpain 8 (calpain-8) respectively. B, E6 Jurkat T cells were pretreated with the polycaspase inhibitor Z-VAD-fmk (40 μM) or one of two calpain inhibitors, Z-VC-CHO (10 – 80 μM) or PD150606 (10 – 80 μM). The cells were then heat-shocked, Western blotted for caspases -9 and -3, and analyzed for cell death by flow cytometry. - - - denotes a crop within the same gel.](image-url)

![FIGURE 5. Heat shock-induced MOMP and caspase-3 processing are regulated by Bcl-2 and an apical protease located upstream of mitochondria. E6 Jurkat T cells, stably transfected with Bcl-2 or vector alone (Neo), were treated with either heat shock (HS) (45 °C, 2 h) or CH-11 (100 ng/ml) and subsequently analyzed at 6–24 h for cell death, losses in Δψm, and release of cytochrome c (Cyt. c) and Smac/DIABLO. - - - denotes a crop within the same gel. S, supernatant; P, pellet; pro, proform of caspase.](image-url)
inhibit the initial processing of procaspase-3 to its p20 form and did not prevent MOMP or the loss in $\Delta \psi_{m}$, suggesting once again that caspase-2 was not the apical protease responsible for heat shock-induced apoptosis (Fig. 7B, lane 8).

Studies by Tinel and Tschopp (7) suggest that procaspase-2 can be recruited to a RAIDD PIDDosome complex following DNA damage, where it resides in its active but unprocessed form. Therefore, because it was unclear whether Z-VDVAD-fmk could inhibit full-length procaspase-2, we fractionated lysates from control, heat-shocked, and etoposide-treated cells and analyzed them for the formation of caspase-2 PIDDosome complexes (Fig. 7C, lane 12). The RAIDD-caspase-2 PIDDosome complex was originally identified using an in vitro model of caspase-2 activation, wherein naïve lysates prepared from PIDD-overexpressing cells were incubated at 37 °C for 1 h (7). Therefore, to validate our assay, we examined Jurkat T cell lysates under the same conditions and found that indeed both procaspase-2 and PIDD-DD co-eluted in higher molecular weight fractions 5–14 ($M_{r} \sim 400,000–1,500,000$) (Fig. 7C, Lysate). However, RAIDD did not co-elute with this large caspase-2 complex, consistent with a previous report (30). Thus, we could find no evidence for the formation of a “native” RAIDD-caspase-2 PIDDosome complex (at least in apoptotic Jurkat T cells) following exposure to heat shock or the DNA-damaging agent etoposide.

**Caspases -2 and -8 Do Not Play Redundant Roles in Heat Shock-induced Apoptosis**—Although there was no a priori reason to suspect that caspases -2 and -8 might compensate for one another to promote heat shock-induced apoptosis, we heat-shocked caspase-8-deficient Jurkat T cells that were first depleted of caspase-2 by RNA interference or pretreated with the caspase-2 inhibitor Z-VDVAD-fmk. Transient transfection with caspase-2-specific small interfering RNAs reduced the expression levels of caspase-2 by >90% (Fig. 8A, lanes 1 and 3) in accordance with observed transfection efficiencies. However, loss of caspase-2 expression in caspase-8-deficient cells failed to prevent the activation of caspase-3, a loss in $\Delta \psi_{m}$, or the induction of apoptosis (Fig. 8A, lanes 2 and 4). Moreover, pretreatment of caspase-8-deficient Jurkat T cells with Z-VDVAD-fmk did not inhibit the initial processing of caspase-3 to its p20 form but did inhibit autocatalytic processing to its p17 form and the downstream processing of caspase-2, as already described (Fig. 8B, lanes 2 and 4; Fig. 7B, lane 8). More importantly, however, it once again failed to significantly inhibit either the loss in $\Delta \psi_{m}$ or cell death (Fig. 8B, lanes 2 and 4). Thus, in summary, neither active pro- nor processed caspase-2 played an essential role in triggering heat shock-induced apoptosis, and caspases -2 and -8 did not play redundant roles in promoting heat shock-induced cell death.

**DISCUSSION**

Elegant studies by Strasser, Vaux, and colleagues (31–33), using *apaf-1* $^{-/}$ and *caspase-9* $^{-/}$ hematopoietic cells and MEFs, indicate that neither Apaf-1 nor caspase-9 is essential for apoptosis induced by certain pro-apoptotic stimuli, including cytokine withdrawal, dexamethasone, and γ-irradiation. They propose that, similar to CED-9 in the worm *Caenorhabditis elegans*, Bcl-2 may regulate the activation of a heretofore unidentified apical caspase in mammals, which directly activates caspase-7 and induces apoptosis. They further argue that this apical caspase may have evolved so as to stimulate MOMP and induce the formation of the Apaf-1-caspase-9 apotosome, which primarily serves to accelerate cell death by activating caspase-3 and initiating a caspase amplification loop. Herein, we have reported that heat shock activates a pathway, which appears to be remarkably similar to that described above, yet contains some significant differences. Indeed, we have found that heat shock activates an apical protease, which is located upstream of mitochondria and is responsible for stimulating MOMP (Figs. 5 and 9). However, this heat-activated protease differs from that previously described (31, 32), in that it is effectively inhibited by Z-VDVAD-fmk, and it can activate caspase-3 independently of the Apaf-1-caspase-9 apotosome (Figs. 2, 3, B and C; and 9). Indeed, even in cells that overexpressed Bcl-2, the heat-activated apical protease still managed to stimulate processing of procaspase-3 to its intermediate p20 form, although it required significantly more time to do so and the overall amount of processing was dramatically reduced (Fig. 5, lane 12). These results are consistent with the notion that Bcl-2 may directly inhibit the activation of the apical protease. However, Bcl-2 also inhibited the release of Smac from mitochondria, which likely pre-
vented a caspase amplification loop by preserving the inhibition of caspase-3 by IAPs (Fig. 9) (34).

To date, caspases -2, -8, and -10 (and more controversially caspases -4 and -12) are the only apical caspases activated upstream of mitochondria, which may in turn induce MOMP. During the completion of our studies, Tu et al. (35) reported that caspase-2 is the apical protease responsible for initiating heat shock-induced cell death (35). They identified caspase-2 largely based on the use of a biotinylated analog of VAD-fmk (bVAD-fmk), which they used to "trap" procaspase-2 in situ, early following heat shock (35). Nevertheless, we have demonstrated that neither primary cells from caspase-2-deficient mice nor tumor cells depleted of caspase-2 by RNAi display any defects in MOMP, caspase-3 activation, or apoptosis. Moreover, pharmacological inhibition of caspase-2 failed to inhibit apoptosis, and we could find no evidence for the formation of a trimolecular RAIDD-caspase-2 PIDDosome complex in heat-shocked cells (Fig. 7). One possible explanation could be that caspases -2 and -8 might play redundant roles in promoting heat shock-induced apoptosis. However, this could also be ruled out, as cells deficient in either caspase-8 or FADD remained sensitive to heat shock, and neither depletion of caspase-2 by RNA interference nor pharmacologi-
Cells were then assayed for caspase processing, losses in caspase-2 by RNA interference (a Z-VAD-fmk-inhibitable apical protease that stimulates MOMP, a loss in caspase-3 activation or apoptosis. Therefore, the identification of the heat-activated apical protease and the mechanism(s) by which it is activated and induces MOMP remain the primary focus of future studies.

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REFERENCES

1. Jiang, X., and Wang, X. (2004) Annu. Rev. Biochem. 73, 87–106
2. Danial, N. N., and Korsmeyer, S. J. (2004) Cell 116, 205–219
3. Boatright, K. M., and Salvesen, G. S. (2003) J. Biol. Chem. 278, 27709–27716
4. Walczak, H., and Krammer, P. H. (2000) Nat. Rev. Immunol. 1, 47–56
5. Barnhart, B. C., Alappat, E. C., and Peter, M. E. (2003) J. Cell Biol. 162, 677–686
6. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Mol. Cell 3, 251–259
7. Tinel, A., and Tschopp, J. (2004) Science 304, 843–846
8. Guo, Y., Srinivasula, S. M., Druhlé, A., Fernandes-Alnemri, T., and Alnemri, E. S. (2002) J. Biol. Chem. 277, 13430–13437

The identity of the heat-activated apical protease is currently unknown, but it does not appear to be any of the known mammalian initiator caspases previously associated with apoptosis. Subsequent attempts to implicate other cysteine proteases, such as calpains and cathepsins, in heat shock-induced apoptosis have thus far been unsuccessful (Fig. 4C) (data not shown). Intriguingly, however, heat shock has recently been shown to induce apoptotic-like cell death in plants, and two serine proteases (coined saspases) have been isolated that are reversibly inhibited by Z-VAD-fmk and prefer substrates with an aspartate residue in the P1 position (37). Thus, it is possible that heat shock may activate an upstream serine protease in mammals that exhibits caspase-like activity.

As already noted, the heat-activated apical protease stimulated apoptosis in both apaf-1−/− and caspase-9−/− MEFs, and processing of caspase-9 was found to be a late caspase-3-dependent event in Jurkat T cells (Figs. 1–3). However, cytochrome c was released following MOMP, and because it is currently unclear how heat shock inhibits the formation of active apoptosome complexes, we cannot rule out that the Apaf-1-caspase-9 apoptosome may amplify the caspase cascade downstream of mitochondria in some cell types, only that it is not essential for caspase-3 activation or apoptosis. Therefore, the identification of the heat-activated apical protease and the mechanism(s) by which it is activated and induces MOMP remain the primary focus of future studies.

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![FIGURE 8. Caspases -2 and -8 do not play redundant roles in promoting heat shock-induced apoptosis. Caspase-8-deficient Jurkat T cells were depleted of endogenous caspase-2 by RNA interference (a Z-VAD-fmk-inhibitable apical protease that stimulates MOMP, a loss in caspase-3 activation or apoptosis. Therefore, the identification of the heat-activated apical protease and the mechanism(s) by which it is activated and induces MOMP remain the primary focus of future studies.

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REFERENCES

1. Jiang, X., and Wang, X. (2004) Annu. Rev. Biochem. 73, 87–106
2. Danial, N. N., and Korsmeyer, S. J. (2004) Cell 116, 205–219
3. Boatright, K. M., and Salvesen, G. S. (2003) Curr. Opin. Cell Biol. 15, 725–731
4. Walczak, H., and Krammer, P. H. (2000) Exp. Cell Res. 256, 58–66
5. Barnhart, B. C., Alappat, E. C., and Peter, M. E. (2003) Semin. Immunol. 15, 185–193
6. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) Mol. Cell 3, 251–259
7. Tinel, A., and Tschopp, J. (2004) Science 304, 843–846
8. Guo, Y., Srinivasula, S. M., Druhlé, A., Fernandes-Alnemri, T., and Alnemri, E. S. (2002) J. Biol. Chem. 277, 13430–13437

The identity of the heat-activated apical protease is currently unknown, but it does not appear to be any of the known mammalian initiator caspases previously associated with apoptosis. Subsequent attempts to implicate other cysteine proteases, such as calpains and cathepsins, in heat shock-induced apoptosis have thus far been unsuccessful (Fig. 4C) (data not shown). Intriguingly, however, heat shock has recently been shown to induce apoptotic-like cell death in plants, and two serine proteases (coined saspases) have been isolated that are reversibly inhibited by Z-VAD-fmk and prefer substrates with an aspartate residue in the P1 position (37). Thus, it is possible that heat shock may activate an upstream serine protease in mammals that exhibits caspase-like activity.

As already noted, the heat-activated apical protease stimulated apoptosis in both apaf-1−/− and caspase-9−/− MEFs, and processing of caspase-9 was found to be a late caspase-3-dependent event in Jurkat T cells (Figs. 1–3). However, cytochrome c was released following MOMP, and because it is currently unclear how heat shock inhibits the formation of active apoptosome complexes, we cannot rule out that the Apaf-1-caspase-9 apoptosome may amplify the caspase cascade downstream of mitochondria in some cell types, only that it is not essential for caspase-3 activation or apoptosis. Therefore, the identification of the heat-activated apical protease and the mechanism(s) by which it is activated and induces MOMP remain the primary focus of future studies.

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9. Robertson, J. D., Gogvadze, V., Kropotov, A., Vakifahmetoglu, H., Zhivotovsky, B., and Orrenius, S. (2004) *EMBO Rep.* **5**, 643–648
10. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002) *Science* **297**, 1352–1354
11. Yoneda, T., Imaizumi, K., Oono, K., Uey, D., Gomi, F., Katayama, T., and Tohyama, M. (2001) *J. Biol. Chem.* **276**, 13935–13940
12. Nakagawa, T., and Yuan, J. (2002) *Science* **297**, 1352–1354
13. Yoneda, T., Imaizumi, K., Oono, K., Uey, D., Gomi, F., Katayama, T., and Tohyama, M. (2001) *J. Biol. Chem.* **276**, 13935–13940
14. Nakagawa, T., and Yuan, J. (2000) *J. Cell Biol.* **150**, 887–894
15. Soengas, M. S., Alarcon, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999) *Science* **284**, 156–159
16. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. (2002) *Science* **297**, 1352–1354
17. Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) *J. Cell Biol.* **144**, 281–292
18. Robertson, J. D., Enoksson, M., Suomela, M., Zhivotovsky, B., and Orrenius, S. (2002) *J. Biol. Chem.* **277**, 29803–29809
19. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) *J. Biol. Chem.* **272**, 9677–9682
20. Marsden, V. S., O’Connor, L., O’Reilly, L. A., Silke, J., Metcalf, D., Eker, P. G., Huang, D. C., Cecconi, F., Kuida, K., Toma, M. L., Roy, S., Nicholson, D. W., Vaux, D. L., Boullet, P., Adams, J. M., and Strasser, A. (2002) *Nature* **419**, 634–637
21. Marsden, V. S., Eker, P. G., Van Delft, M., Vaux, D. L., Adams, J. M., and Strasser, A. (2004) *J. Cell Biol.* **165**, 775–780
22. Tu, S., McStay, G. P., Boucher, L. M., Mak, T., Beere, H. M., and Green, D. R. (2006) *Nat. Cell Biol.* **8**, 72–77
23. Bratton, S. B., and Cohen, G. M. (2003) *Cell Death Differ.* **10**, 4–6
24. Berube, C., Boucher, L. M., Ma, W., Wakeham, A., Salmena, L., Hakem, R., Yeh, W. C., Mak, T. W., and Benchimol, S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 14314–14319
25. Coffeen, W. C., and Wolpert, T. J. (2004) *Plant Cell* **16**, 857–873