Cycloheximide-induced T-cell Death Is Mediated by a Fas-associated Death Domain-dependent Mechanism*

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Cycloheximide (CHX) can contribute to apoptotic processes, either in conjuction with another agent (e.g. tumor necrosis factor-a) or on its own. However, the basis of this CHX-induced apoptosis has not been clearly established. In this study, the molecular mechanisms of CHX-induced cell death were examined in two different human T-cell lines. In T-cells undergoing CHX-induced apoptosis (Jurkat), but not in T-cells resistant to the effects of CHX (CEM C7), caspase-8 and caspase-3 were activated. However, the Fas ligand was not expressed in Jurkat cells either before or after treatment with CHX, suggesting that the activation of these caspasess does not involve the Fas receptor. To determine whether CHX-induced apoptosis was mediated by a Fas-associated death domain (FADD)-dependent mechanism, a FADD-DN protein was expressed in cells prior to CHX treatment. Its expression effectively inhibited CHX-induced cell death, suggesting that CHX-mediated apoptosis primarily involves a FADD-dependent mechanism. Since CHX treatment did not result in the induction of Fas or FasL, and neutralizing anti-Fas and anti-tumor necrosis factor receptor-1 antibodies did not block CHX-mediated apoptosis, these results may also indicate that FADD functions in a receptor-independent manner. Surprisingly, death effector filaments containing FADD and caspase-8 were observed during CHX treatment of Jurkat, Jurkat-FADD-DN, and CEM C7 cells, suggesting that their formation may be necessary, but not sufficient, for cell death.

The apoptotic process is now known to involve the well orchestrated interactions of cell death receptors, death receptor adaptors, caspasess, and Bcl-2 family members (1–6). Although a number of stimuli have been reported to result in the up-regulation of the Fas receptor and its ligand (e.g. UV, c-Myc, and certain chemotherapeutic drugs), there are many other stimuli for which the mechanism responsible for their action is still unknown (7–10). An example of the latter is the ability of cycloheximide (CHX)† to either promote or inhibit apoptosis in divergent cell types and in response to varying death stimuli (11–16). A large body of evidence has shown that CHX can potentiate, and in some cases (e.g. TNF-a stimulation and staurosporine) be necessary, for the apoptotic effects of certain death stimuli (12–14, 16). The studies of Martin et al. (13) and Tuschida et al. (14) further indicated that CHX, independently of other stimuli, is also capable of promoting apoptosis in a number of transformed cell lines and normal cells. Jacobson et al. (16) have shown that staurosporine- and staurosporine/cycloheximide-induced death is mediated by a caspase-3-like activity that is blocked by Z-VAD-FMK, a synthetic tripeptide inhibitor that demonstrates broad caspase specificity. More recently, Woo et al. (17) demonstrated that bone marrow neutrophils from caspase-3-/- mice no longer undergo CHX-induced apoptosis, indicating that caspase-3 expression is most likely required for this type of cell death.

The ability of CHX to induce cell death varies considerably from one cell line to another, suggesting that the continuous synthesis of a regulatory protein that blocks apoptosis is required for the normal growth of these CHX-sensitive cell lines (12, 13). Sensitivity to CHX is not necessarily determined by cell type alone since cell lines from the same tissue and stage of development (e.g. Jurkat and CEM C7 T-cells) can be affected in very different ways. Furthermore, although cell death triggered by cell-surface receptors (e.g. Fas, DR3, and TNF receptor-1) requires an adaptor protein such as FADD to promote an apoptotic signal, cell death triggered by other stimuli (e.g. E1A, c-Myc, and Adriamycin) may not (18–24). Therefore, it is of interest to determine the basis of the cellular differences that result in cellular sensitivity or insensitivity to agents like CHX as well as the mechanism of CHX-induced apoptosis.

The transformed human T-cell lines Jurkat and CEM C7 are representative of a similar T-cell developmental stage, and they are equally sensitive to agonistic anti-Fas mAbs and TNF-a (25, 26). However, they exhibit disparate responses when exposed to CHX. We therefore set out to determine which apoptotic signaling pathway components are involved in CHX-induced Jurkat cell death as well as the basis for the CEM C7 cellular resistance to CHX. Here we demonstrate that disruption of normal FADD function inhibits apoptotic signaling in these cells. This may indicate that in addition to its role as an adaptor that links TNF-related receptors to caspase activation, FADD mediates certain apoptotic signals through a receptor-independent pathway(s). Finally, we demonstrate that FADD and caspase-8 (FLICE) coalesce into what appear to be perinuclear death effector filaments (DEFs) (27, 28) in wild-type Jurkat, Jurkat-FADD-DN, and CEM C7 cells treated with CHX, even though only the wild-type Jurkat cells apoptose. These results suggest that the redistribution of FADD and caspase-8 into these filament structures is necessary, but not sufficient, for cell death to occur.

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‡ The abbreviations used are: CHX, cycloheximide; TNF, tumor necrosis factor; Z- benzoyloxy carbonyl- FMK, fluoromethyl ketone; mAb, monoclonal antibody; DEF, death effector filament; STS, staurosporine; FACS, fluorescence-activated cell sorter; PARP, poly(ADP-ribose) po-

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EXPERIMENTAL PROCEDURES

Cell Lines, Expression Constructs, and Transfections—Human Jurkat cells and CEM C7 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. This particular Jurkat cell line contains a mutation in the bax gene that results in a functionally inactive, truncated protein (29). For Fas-induced cell death analysis, cells at a density of 1.5 × 10⁶ were treated with an anti-Fas monoclonal antibody, CH-11 (Kamiya Biomedical Co.), at 100 ng/ml for 0, 2, 4, 6, and 8 h. CHX-induced cell death was performed by incubating the cells in 20 μg/ml CHX for 6 h, and staurosporine (STS)-induced apoptosis was accomplished by incubating cells with various concentrations (0.1, 0.3, 0.5, 0.8, and 1.0 μM) of STS for 4 h. Apoptotic cells were examined with a light microscope for appearance of plasma membrane blebbing and by TUNEL assay (30), and the percentage of apoptotic cells was determined as described below.

The Jurkat-Neo and Jurkat-FADD-DN cell lines were generated by resuspending 10⁶ Jurkat cells in 0.3 ml of the cell growth medium containing 12 μg of either pcDNA3.0 or pcDNA3.0/FADD-DN (kindly provided by Dr. V. Dixit), which were then electroporated using a 4-mm gap cuvette at 0.25 kV and 960 microfarads. Cells were cultured for 48 h before addition of G418 (Genetics, Life Technologies, Inc.) at 1 mg/ml. After 4 weeks of selection in the G418 medium, a subpopulation of non-clonal, transfected cells expressing a moderate to high level of FADD-DN was selected by exposing cells to an agonistic anti-Fas mAb (CH-11) at 100 ng/ml for 72 h. The surviving cells (~20% of the original population) were then expanded. In contrast to FADD-DN-transfected Jurkat cells, virtually no pcDNA3.0-transfected Jurkat cells (~0.1%) survived this treatment. FACS analysis demonstrated that most of the Jurkat-FADD-DN cells (~95%) expressed levels of cell-surface receptor equivalent to the original Jurkat cell population and the Jurkat-Neo cells. By Western blot analysis, the Jurkat-FADD-DN cells were found to express levels of wild-type FADD and caspase-8 equivalent to the levels expressed in the Jurkat-Neo and Jurkat cells.

Cell Lysis and Immunoblotting—For Western blotting, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 mM benzamidine, and 1 mM 4-(2-aminoethyl)benzenesulfonl fluoride (Boehringer Mannheim). 50 μg of cell lysate was separated by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore Corp.). The resulting membrane was blocked with 10% skim milk and incubated with a designated primary antibody, and the signals were detected by use of an ECL Western blotting kit (Amersham Pharma Biotech). The anti-caspase-8/FLICE mAb (C15) (31) was kindly provided by Drs. M. E. Peter and P. H. Krammer, whereas the anti-caspase-3 (Transduction Laboratories, Inc.) antibodies were obtained commercially.

Induction of Caspase Cleavage and Activation, PARP Assays, Caspase Inhibitors, and Cell Death Assays—Anti-Fas mAb (CH-11), CHX-, and STS-induced caspase activation in Jurkat and CEM C7 cells was monitored by cleavage of the endogenous caspase substrate PARP using Western blot analysis. Cytochrome c release from mitochondria was determined according to a previously published protocol (33). Experiments involving the effects of caspase inhibitors on cytochrome c release in the presence of CHX were performed as follows. Jurkat cells were incubated in 50 μM Z-FA-FMK, Z-YVAD, Z-IEPD, Z-DEVD, or Z-VAD-FMK (Enzyme System Products) before addition of CHX to cells to initiate cell death. Apoptotic cells were detected by TUNEL assay (Boehringer Mannheim) according to the manufacturer’s protocol. The percentage of apoptotic cells during agonistic CH-11 antibody treatment was quantitated by FACS (32). The percentage of apoptotic cells during CHX treatment was quantitated by FACS for annexin V-positive and propidium iodide-negative cells (34, 35). Briefly, 10⁶ Jurkat cells were treated with CHX for 6 h, and the cells were then stained with FITC-conjugated annexin V and propidium iodide.

Indirect Immunofluorescence Analysis—Jurkat, Jurkat-FADD-DN, Jurkat-Neo, or CEM C7 cells were prepared for immunofluorescence by cytoospin. These cells were either left untreated or treated with an agonistic anti-Fas mAb for 4 h, with 20 μg/ml CHX for 6 h, or with 20 μg/ml CHX for 6 h in the presence of a 50 μM concentration of the caspase inhibitor Z-VAD-FMK before being prepared for analysis (32). Cells were then fixed and stained with an anti-FADD mAb and anti-caspase-8 polyclonal antiserum (goat anti-human IgG, SC-6134, Santa Cruz) as described previously (36). The anti-FADD mAb was detected using a rhodamine-conjugated anti-mouse secondary antibody (Southern Biotechnology, Birmingham, AL), and the anti-caspase-8 polyclonal antibody was detected using an FITC-conjugated anti-goat secondary antibody (Southern Biotechnology) with an Olympus BX-50 fluorescent microscope as described (36).

RESULTS

Ability of Cycloheximide to Induce Cell Death in Jurkat and CEM C7 T-cell Lines—It has been shown that CHX can augment many different death signals, presumably through its ability to inhibit the synthesis of a protective factor, as well as induce apoptosis on its own (12–14, 16). In fact, cell lines derived from the same source (e.g., lymphoid cells) respond quite differently when exposed to CHX; some (e.g. Jurkat T-cells) rapidly undergo apoptosis, whereas others (e.g. CEM C7 T-cells) do not (25, 26). To explore the possible molecular mechanisms involved in this response, we examined the ability of CHX to induce cell death in Jurkat and CEM C7 cells. As little as 5 μg/ml CHX was capable of inducing apoptosis in Jurkat cells (Fig. 1) within 4–6 h, whereas even 20 μg/ml did not induce CEM C7 cell death over a 48-h time period (data not shown). For these initial experiments, cleavage of the nuclear caspase substrate PARP in CHX-treated Jurkat cells was used as a biochemical marker of apoptosis (Fig. 1A). Annexin V staining of cell-surface phosphatidylserine was used as a measure to determine the percentage of apoptotic cells present (Fig. 1A). Furthermore, a much higher level of cytochrome c was released from the mitochondria into the cytosol of CHX-treated Jurkat cells compared with CHX-treated CEM C7 cells (Fig. 2). To demonstrate that cytochrome c release was not due to physical disruption of the mitochondria, proteins corresponding to the cytoplasmic and mitochondrial fractions of the CHX-treated Jurkat and CEM C7 cells were Western-blotted with an antibody to cytochrome oxidase (subunit II) (Fig. 2). Cytochrome oxidase is a protein that is not released from the mitochondria during apoptosis (37). If the cytoplasmic cytochrome c in the Jurkat cells were generated by physical disruption of the mitochondrial or mitochondrial contamination, one would expect to see cytochrome oxidase present in this cell fraction as well. No cytochrome oxidase was detected in the cytoplasmic fraction from the CHX-treated Jurkat cells, whereas a small amount was detected in the CEM C7 cells. The former result demonstrates that the mitochondria from the Jurkat cells were intact, whereas the latter result suggests that the CEM C7 cell mitochondria were slightly damaged, and/or the cytosol was contaminated with a low level of mitochondria. This explains the small amount of cytochrome c in the cytoplasmic fraction from the CHX-treated CEM C7 cells (Fig. 2).

The effects of treatment of Jurkat cells with 20 μg/ml CHX on PARP cleavage, release of cytochrome c, and annexin V staining in Jurkat cells during 8 h of exposure were assessed (Fig. 1B). All three were induced with similar kinetics. Since these results suggested that caspase-dependent apoptosis was being triggered, we examined the effect of CHX treatment on the processing of several caspases (Fig. 1C). Caspase-3 (CPP32/YAMA) activation, as judged by the appearance of the 17-kDa subunit, started to occur within 2 h. The processing of caspase-2 and caspase-8, as judged by the appearance of their 18-19-kDa subunits, started to occur within 2–4 h of CHX treatment. This suggests either that our ability to detect activation of caspase-8 was limited by the sensitivity of the reagents we used or that caspase-3 is responsible for the processing and activation of caspase-8 during CHX-induced Jurkat cell death.

FADD-DN Expression Inhibits Cycloheximide- and Staurosporine-Induced T-cell Death—Since caspase-8 is activated in CHX-treated Jurkat cells, and it appears that this activity...
might be linked to a cellular commitment to death, we decided to examine the effects of a dominant-negative form of FADD on this process. Others have shown that expression of a FADD-DN protein, containing the death domain but not the death effector domain, is capable of effectively inhibiting a number of different receptor-mediated apoptotic signals (18, 21, 38). The same FADD-DN expression construct was stably introduced into Jurkat cells, and the truncated protein was constitutively expressed along with endogenous FADD (Fig. 3A). Jurkat cells transfected with the empty expression vector (Neo) were used as a control. Since it has been shown that expression of the FADD-DN protein effectively inhibits Fas-mediated cell death, we examined the ability of these cells to survive Fas receptor oligomerization. As measured by cell viability (FITC-conjugated annexin V staining) and two different biochemical markers (i.e. PARP cleavage and caspase-8 processing), these Jurkat-FADD-DN cells were resistant to Fas-mediated apoptosis induced by an agonistic anti-Fas mAb (Fig. 3A).

We next examined the ability of CHX and STS, a broad spectrum protein kinase inhibitor that induces caspase-dependent apoptosis independently of cell-surface death receptors (16, 39), to trigger cell death in the Jurkat-FADD-DN cells. PARP cleavage and cell death, as measured by the percentage of annexin V-positive cells, were significantly inhibited in the FADD-DN cells as compared with the Neo controls as the cells were exposed to increasing amounts of CHX (Fig. 3B). However, the ability of STS to induce PARP cleavage and apoptosis was not significantly diminished. The effect of FADD-DN expression on the ability of CHX and STS to induce the release of cytochrome c from mitochondria, which is involved in the propagation of several apoptotic signals (40), was examined next. FADD-DN expression did not significantly inhibit the release of cytochrome c in the CHX- or STS-treated cells (Fig. 3B).
FIG. 3. Demonstration that the expression of the FADD-DN protein in Jurkat cells substantially inhibits the ability of CHX to induce apoptosis. In A, the left panel shows the expression of the FADD-DN protein in stably selected Jurkat cells compared with the Neo vector control as detected by Western blot analysis with an anti-FADD mAb. The middle panel demonstrates that PARP cleavage resulting from Fas receptor oligomerization induced by treatment with an agonistic mAb is inhibited in Jurkat cells expressing the FADD-DN protein as compared with Jurkat cells containing the Neo vector only. Below this panel, the percentage of apoptotic cells quantitated by FITC-conjugated annexin V staining is indicated. The right panel demonstrates that caspase-8/FLICE processing induced by Fas receptor oligomerization is also inhibited in Jurkat cells expressing the FADD-DN protein as compared with the Neo vector control cells. FLICE was detected with the C15 mAb previously described (31). B shows the comparison of PARP cleavage, and cytochrome c release induced by treatment of Jurkat cells expressing the FADD-DN protein or containing the Neo control vector with increasing amounts of either CHX (0, 5, 10, and 20 \( \mu \)g/ml) or STS (0, 0.1, 0.3, and 0.5 \( \mu \)M). The percentage of apoptotic cells was quantitated by FITC-conjugated annexin V staining and is indicated below the panels demonstrating PARP cleavage. C shows the cleavage of caspase-8, resulting in the production of its characteristic partial products and the p18 subunit, by increasing amounts of either CHX (0, 5, 10, and 20 \( \mu \)g/ml) or STS (0, 0.1, 0.3, and 0.5 \( \mu \)M) in Jurkat-Neo control cells or Jurkat-FADD-DN cells. 40 \( \mu \)g of cellular protein was Western-blotted with a mAb to caspase-8/FLICE (C15) (31). D shows the cleavage of caspase-3, resulting in the production of its p17 subunit, by increasing amounts of either CHX (0, 5, 10, and 20 \( \mu \)g/ml) or STS (0, 0.1, 0.3, and 0.5 \( \mu \)M) in Jurkat-Neo control cells or Jurkat-FADD-DN cells. 40 \( \mu \)g of cellular protein was Western-blotted with anti-caspase-3 polyclonal antiserum.
addition, although the FADD-DN protein inhibited CHX-induced cell death as measured by the use of an FITC-conjugated anti-annexin V antibody, it did not have a similar effect on STS-induced apoptosis (Fig. 3B).

**Delineation of a FADD-dependent Apoptotic Pathway in CHX-treated Cells**—There are at least two possibilities to explain how FADD-DN expression can interfere with CHX-induced Jurkat cell death. One is to block CHX-induced cytochrome c release; the other is to block caspase activation, downstream of cytochrome c release. To distinguish between these two possibilities, we first examined CHX-induced cytochrome c release in both Jurkat-Neo and Jurkat-FADD-DN cells. Fig. 3B shows that expression of the FADD-DN protein did not prevent cytochrome c release induced by CHX or STS. Conversely, FADD-DN expression inhibited caspase-8 activation in CHX- and STS-treated cells (Fig. 3C). Surprisingly, FADD-DN expression also efficiently inhibited caspase-3 activation induced by CHX treatment, but not by higher doses of STS (Fig. 3D). Since FADD-DN expression had no apparent effect on cytochrome c release in both CHX- and STS-treated cells, the inhibition of caspase-3 processing in CHX-treated Jurkat-FADD-DN cells may be the result of caspase-8 inhibition. This possibility is further supported by the fact that at higher doses of STS (>0.3 μM), caspase-8 processing continued to be inhibited by FADD-DN expression (Fig. 3C), whereas caspase-3 processing was not (Fig. 3D). Taken together, these results suggest that expression of the FADD-DN protein directly inhibits caspase-8 processing, whereas it may indirectly inhibit caspase-3 processing through its effects on caspase-8. The ability of FADD-DN expression to inhibit caspase processing is reflected by its ability to inhibit cell death, as judged by PARP cleavage and the percentage of FITC-conjugated annexin V-positive cells (Fig. 3B).

Based on these results, we decided to examine the effects of various caspase inhibitors on cytochrome c release in Jurkat cells treated with CHX. Fig. 4 shows that none of these caspase inhibitors prevented the release of cytochrome c from mitochondria during CHX-induced Jurkat cell death, whereas cell death was inhibited (data not shown). Others have shown that, significantly, caspase inhibitors do not prevent cytochrome c release induced by a number of non-receptor apoptotic stimuli, including STS (6). These data, coupled with that from the Jurkat cells expressing the FADD-DN protein, suggest that cytochrome c release is upstream of caspase activation in Jurkat cells undergoing apoptosis in response to CHX. We discuss the possible mechanisms and implications of these observations below.

**Changes in the Subcellular Localization of FADD Occur during Fas-mediated and Cycloheximide-induced T-cell Death—How does FADD, a death receptor adaptor protein, mediate receptor-independent apoptotic signals?** Recently, it has been demonstrated that overexpression of either FADD or caspase-8 induces apoptosis through the formation of unique cellular filament structures that contain the death effector domains of these proteins (27, 28). Accordingly, these structures have been termed “death effector filaments,” and their formation is believed to be important for the execution phase of apoptosis. We reasoned that CHX might be inducing cell death through the formation of DEFS. To examine this possibility, we prepared untreated and CHX-treated Jurkat and CEM C7 cells for indirect immunofluorescence analysis using an anti-FADD mAb and anti-caspase-8 polyclonal antiserum. In the untreated Jurkat, Jurkat-FADD-DN, and CEM C7 cells, FADD and caspase-8 were dispersed throughout the cytoplasm, as expected (Fig. 5A). Conversely, in Jurkat cells undergoing CHX-induced apoptosis, FADD formed perinuclear filaments (Fig. 5B) that were very similar in appearance to what has been reported by others when FADD, its death effector domain, or caspase-8 is overexpressed (27). Caspase-8 was colocalized to these same filaments, consistent with the results of these investigators (27, 28). In addition, these aggregate structures did not colocalize with a known cytoskeletal element, tubulin, in these cells (Fig. 5D). In fact, tubulin remained equally distributed throughout the cytoplasm of CHX-treated Jurkat cells. This demonstrates that these perinuclear aggregate structures containing FADD and caspase-8 are not the result of the cytoplasm collapsing due to cell shrinkage during apoptosis. Somewhat surprisingly, CHX-treated CEM C7 T-cells and the Jurkat-FADD-DN cells also formed the FADD/caspase-8 perinuclear DEFS (Fig. 5, C and D). Finally, when we examined the distribution of FADD and caspase-8 in Jurkat cells treated with CHX in the presence of Z-VD-FMK, we found that the perinuclear DEFS disappeared, and the cells resumed their normal growth within a few hours (data not shown). This did not happen with the Jurkat cells. Thus, whereas CHX treatment resulted in the formation of DEFS in all three of these cell lines, only the wild-type Jurkat cells underwent apoptosis. This suggests that FADD/caspase-8 DEF formation may be necessary, but not sufficient, for cell death signals and that as yet unidentified “factors” may need to be recruited to this filament complex to initiate other apoptotic events (e.g. the caspase cascade).

**DISCUSSION**

Apoptosis can be initiated by a number of different stimuli. Although the mechanism of action for many of the cell-surface death receptors has been established (2, 42), the signaling mechanism(s) responsible for the action of many other apoptotic agents is less clear. Examples of the latter include cycloheximide, which can induce cell death either on its own or in concert with another signaling molecule (e.g. TNFα) (11–15), and STS, a broad spectrum protein kinase inhibitor that is a potent inducer of apoptosis (39). In addition, Jacobson et al. (16) have shown that both CHX and STS induce apoptosis in a number of different cell lines/types by a caspase-3-dependent pathway. Furthermore, not all cells, both in vitro and in vivo, are equally sensitive to the death-inducing effects of CHX (12–15, 17). Understanding the molecular mechanisms responsible for these differential effects may provide clues to how certain cells are “sensitized” to selected death agents while others are not as well as provide valuable information regarding novel receptor- and/or non-receptor-mediated apoptotic signaling pathways.

In this study, we have demonstrated that CHX signals cell death through a FADD-dependent mechanism that does not involve the action of the cell-surface Fas death receptor. This is
a rather surprising result since it has been assumed that FADD functions primarily as a death receptor adaptor molecule essential for the recruitment and activation of specific procaspases, such as caspase-8 and caspase-10 (18, 19, 21, 43–46). Using both FADD gene knockout and FADD-DN-overexpressing transgenic mice, it has recently been shown that in addition to its well known role in apoptotic signaling, FADD also contributes to certain aspects of cellular proliferation (24, 38). Although these studies suggest that FADD does not play a role in apoptosis induced by viral oncoproteins (e.g. E1A), c-Myc, or certain chemotherapeutic agents (e.g. Adriamycin), data from several other groups suggest that FADD might be important (9, 10, 46). Thus, FADD may function in a number of different contexts relevant to cellular proliferation as well as apoptotic signaling. Here we have shown that agents such as CHX mediate cell death in certain cells through a FADD-dependent mechanism. It should be noted, however, that the effect of the FADD-DN protein on this process in these cells does not need to be due to FADD itself. This phenotype could be the result of FADD-DN disruption of the binding of another protein to FADD. In addition, it appears unlikely that Fas or TNF receptor-1 and, most likely, DR3 are involved in the CHX-mediated Jurkat cell death we have studied here. Since the TNF-related receptors DR4 and DR5 induce apoptosis independently of FADD (47), it is improbable that they are involved in mediating CHX-induced cell death in these cells.

Why would FADD function in an apparently receptor-independent manner during apoptosis induced by agents such as CHX? Several recent reports suggest one possibility, namely, the formation of perinuclear filament structures that also recruit caspase-8 (27, 28), which may play a role in transducing cell death signals in the absence of an appropriate death-inducing signaling complex (DISC). Furthermore, Scaffidi et al. (48) have shown that so-called “type I” cells form the DISC and activate caspase-8 and caspase-3 very rapidly, whereas “type II” cells do not efficiently form the DISC and therefore activate these caspases over a much longer time. These investigators demonstrated that in type II (but not type I) cells, the overexpression of Bcl-2 or Bcl-xL blocked caspase-8 and caspase-3 activation as well as apoptosis. In separate studies, it was shown that Bcl-xL overexpression and, to a lesser extent, Bcl-2 substantially impaired the formation of FADD-containing DEFs in HeLa cells (28). Both Jurkat and CEM C7 cells have been classified as type II DISC-forming cells (HeLa cells have not been classified), and Bcl-2/Bcl-xL inhibit Fas-mediated apoptosis in these cell lines (48). The marked similarities between the ability of these cells to rapidly form a functional DISC and the ability of Bcl-2/Bcl-xL to inhibit cell death might suggest
that these processes are related to the formation of DEFs in the absence of an appropriate DISC. Such an interpretation would also be consistent with the data presented here concerning CHX-mediated cell death. Additional study of the similarities and differences between type I and II cells as well as their ability/ inability to form DEFs in response to different apoptotic stimuli may help to answer this question.

Perhaps even more surprising is the ability of the FADD-DN protein to inhibit apoptosis, but not the formation of the DEF-like structures or the release of cytochrome c, in response to CHX. Furthermore, caspase inhibitors do not block the release of cytochrome c from mitochondria or prevent the formation of DEF-like structures in cells treated with CHX. Although these results suggest that the release of cytochrome c is not sufficient to cause apoptosis under these circumstances, they do not rule out the possibility that its release is required for cell death. Salvesen and co-workers (49) have recently shown that procaspase-3 is the major physiological substrate of caspase-8 and that the processing of procaspase-9 in death receptor-mediated apoptosis requires the presence and activation of caspase-3. As these authors suggested, the mitochondrion-mediated death signal may function as an accelerator of the death signal mediated by these receptors. They also suggested that cells that are programmed to undergo cell death during their development, such as those associated with the immune response (e.g. Jurkat T-cells), have developed the death receptor-caspase-8-caspase-3 activator complex to allow rapid apoptotic signaling. Finally, it has recently been demonstrated that the processing of procaspase-9 is blocked in cells by Akt protein kinase phosphorylation of Ser-186 of procaspase-9 (41). Therefore, it is possible that the phosphorylated form of procaspase-9 prevents cell death in the presence of cytochrome c released after treatment with CHX. This possibility is consistent with the ability of high levels of STS to induce apoptosis, even in the presence of the FADD-DN protein (Fig. 3B). These issues definitely warrant further study.

Finally, we have shown that the formation of FADD- and caspase-8-containing DEFs occurs in response to CHX in cells that apoptose as a result of this treatment as well as in cells that do not. The recent studies of Siegel et al. (27) and Perez and White (28) concerning these DEFs dealt with their formation as a result of the overexpression of FADD and/or caspase-8 rather than their induction by exogenously administered apoptotic stimuli. Here we demonstrate that such DEFs are also formed in response to exogenous stimuli, possibly independently of cell-surface death receptors, consistent with the interpretation of Siegel et al. (27). However, we have also shown that these FADD- and caspase-8-containing DEFs are generated in cells that do not undergo apoptosis as a result of CHX treatment (i.e. CEM C7) as well as in Jurkat cells protected from this apoptotic signal by the expression of a FADD-DN protein or treatment with the caspase inhibitor Z-VAD-FMK. This suggests that although the formation of DEFs may be necessary for apoptosis induced by certain stimuli, it may not be sufficient for the propagation of a death signal. Since both FADD and caspase-8 were found to be in the DEFs of the non-apoptosing cells, we also suggest that the recruitment of an additional factor and/or a specific modification may be necessary for execution of cell death.

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