Caspase-8 Activation and Bid Cleavage Contribute to MCF7 Cellular Execution in a Caspase-3-dependent Manner during Staurosporine-mediated Apoptosis*

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There are at least two distinct classes of caspases, initiators (e.g. caspases-8, -9, and -10) and effectors (e.g. caspase-3). Furthermore, it is believed that there are two distinct primary apoptotic signaling pathways, one of which is mediated by death receptors controlled by caspases-8/-10, and the other by the release of cytochrome c and activation of a caspase-9/Apaf1/cytochrome c apoptosome. However, several recent reports have demonstrated that caspase-8, and its substrate Bid, are frequently activated in response to certain apoptotic stimuli in a death receptor-independent manner. These results suggest that significant cross-talk may exist between these two distinct signaling arms, allowing each to take advantage of elements unique to the other. Here we provide evidence that activation of caspase-8, and subsequent Bid cleavage, does indeed participate in cytochrome c-mediated apoptosis, at least in certain circumstances and cell types. Furthermore, the participation of activated caspase-3 is essential for activation of caspase-8 and Bid processing to occur. Although caspase-8 activation is not required for the execution of a cytochrome c-mediated death signal, we found that it greatly shortens the execution time. Thus, caspase-8 involvement in cytochrome c-mediated cell death may help to amplify weaker death signals and ensure that apoptosis occurs within a certain time frame.

Apoptotic signals are generally believed to be mediated through a hierarchy of caspase activation controlled by one of two distinct pathways that are associated with either caspase-8 (i.e. death receptors) or caspase-9 (i.e. mitochondria) (1). However, caspase-8 activation has been observed in situations that are apparently independent of death receptor involvement (2–6). In these studies caspase-8 activation is nevertheless FADD-dependent, and the activation of caspase-9 occurs normally, although in some situations the release of cytochrome c may occur in a caspase-independent manner (3, 7). It is therefore possible that caspase-8 activation in a death receptor-independent manner may represent a physiologically distinct, and possibly via caspase-6, in vitro and in cell-free extracts (9), whereas another suggested that formation of a caspase-8/Apaf1/cytochrome c complex or direct activation of caspase-8 by caspase-9 might be responsible for these activities (8). Neither study, however, was able to determine whether caspase-9-mediated activation of caspase-8 is physiologically relevant or merely a bystander effect. Here we show that caspase-3 is required for cytochrome c-mediated activation of caspase-8 in vivo, resulting in caspase-8-mediated activation of Bid as well as a substantial reduction in the cellular execution time. Furthermore, FADD-DN expression effectively blocks caspase-8 activation and Bid cleavage. Perhaps more importantly, FADD-DN expression in MCF7/Casp3 cells substantially diminishes the enhanced cellular execution time observed in MCF7/Casp3 cells undergoing staurosporine (STS)-induced apoptosis. Even though these results are based upon the enforced expression of certain gene products within cells, they are nonetheless consistent with results from others (2–6) indicating that caspase-8 can function in a receptor-independent manner, possibly in a cell- and/or signal-type specific manner. Thus, although not essential for apoptosis, caspase-3-mediated caspase-8 activation may be physiologically relevant for at least some caspase-9-mediated death signals, as suggested by studies of Bid–/– mice (10), by serving to amplify weaker death signals in certain cells.

MATERIALS AND METHODS

Cell Lines, Expression Constructs, Transfections, and Quantitation of Apoptotic Cells—Human MCF7 breast carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. STS-induced apoptosis was accomplished by incubating cells with 1.0 μM of STS for different lengths of time as indicated. Apoptotic cells were examined by a light microscope for morphologic changes, such as the characteristic appearance of plasma membrane blebbing and nuclear condensation. Apoptotic cells were quantified by either trypan blue uptake or fluorescence-activated cell sorter analysis of annexin V/propidium iodide staining, TUNEL (terminal dUTP nick end-labeling) staining, and by the standard tumor necrosis factor cytotoxicity assay (12, 13). STS-induced cytochrome c release from mitochondria into cytosol was analyzed according to the methods used by Yang et al. (26).

The MCF7/neo, MCF7/Casp3, MCF7/FADD-DN, MCF7/Bcl-xL, MCF7/FADD-DN/Casp3, and MCF7/Bcl-xL/Casp3, MCF7/Fas, and MCF7/Fas/Casp3 cell lines were generated by resuspending 10⁷ MCF7/Fas/Casp3 cell lines in 0.8 ml of phosphate-buffered saline containing 20 μg of pCDNA3.0, pCDNA3.0/Casp3, pCDAN3.0/FADD-DN (both Casp3 and FADD-DN were kindly provided by Dr. V. Dixit), pCDNA3.0/Bcl-xL (the pCDNA3.1 contain hygromycin selection marker), and pSVFF/Vfas, which were then electroporated using a 4-mm gap cuvette at 0.330 kV, 960 μF. Cells were then cultured for 48 h before addition of G418 (Geneticin, Life Technologies, Inc.) at 1 mg/ml, hygromycin at 0.3 mg/ml, or both G418 and hygromycin at 0.5 and 0.15 mg/ml, respectively. After 4 weeks selection in the G418 medium and

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One study it was shown that caspase-3 can activate caspase-8, possibly via caspase-6, in vitro and in cell-free extracts (9), whereas another suggested that formation of a caspase-8/Apaf1/cytochrome c complex or direct activation of caspase-8 by caspase-9 might be responsible for these activities (8). Neither study, however, was able to determine whether caspase-9-mediated activation of caspase-8 is physiologically relevant or merely a bystander effect. Here we show that caspase-3 is required for cytochrome c-mediated activation of caspase-8 in vivo, resulting in caspase-8-mediated activation of Bid as well as a substantial reduction in the cellular execution time. Furthermore, FADD-DN expression effectively blocks caspase-8 activation and Bid cleavage. Perhaps more importantly, FADD-DN expression in MCF7/Casp3 cells substantially diminishes the enhanced cellular execution time observed in MCF7/Casp3 cells undergoing staurosporine (STS)-induced apoptosis. Even though these results are based upon the enforced expression of certain gene products within cells, they are nonetheless consistent with results from others (2–6) indicating that caspase-8 can function in a receptor-independent manner, possibly in a cell- and/or signal-type specific manner. Thus, although not essential for apoptosis, caspase-3-mediated caspase-8 activation may be physiologically relevant for at least some caspase-9-mediated death signals, as suggested by studies of Bid–/– mice (10), by serving to amplify weaker death signals in certain cells.

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hygromycin, single cell clones were selected by limiting dilution. Bulk populations of transfected cells at early state of tissue culture, as well as clonal cell lines, were used in experiments.

Cell Lysis and Immunoblotting—For immunoblotting, 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)benzenesulfonyl fluoride (Roche Molecular Biochemicals). 50 μg of cell lysate was separated by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore). The resulting membrane was blocked with 10% skim milk, incubated with a designated primary antibody, and the signals were detected by use of an ECL Western blotting kit (Amersham Life Science, Inc). The following antibodies were used for immunobLOTS: caspase-2 (Pharmingen, catalog no. 13951A), caspase-3 (Transduction Laboratories, catalog no. C31720), Bcl-xL (Transduction Laboratories, catalog no. 22630), caspase-8 (mAb C15, provided by M. Peter and P. Kramer and Pharmingen, catalog no. 66231A), FADD (Transduction Laboratories, catalog no. F36620), caspase-7 (Transduction Laboratories, catalog no. M64620), caspase-6 and -10 (Upstate Biotechnology, catalog nos. 06691 and 06836), caspase-9 (4748), Bid (Santa Cruz Biotechnology, catalog no. SC-65-38), and cytochrome c (Pharmingen, catalog no. 65981A).

RESULTS AND DISCUSSION

Certain caspases (e.g., caspase-8) and their substrates are not proteolytically processed in the human MCF7 (a mammary adenocarcinoma) cell line, which does not express functional caspase-3 due to deletion within exon 3 of CASP3 gene (11). MCF7 cells were stably transfected with a caspase-3 expression vector or a vector control, and caspase-3 expression confirmed in a clonal MCF7/Casp3 cell line (Fig. 1A, left panel) to examine the role of caspase-3 in STS-mediated apoptosis. These studies were initiated because we observed that caspase-8 processing, as compared with caspase-3 processing, was slightly delayed temporally in human Jurkat T-cells treated with staurosporine (data not shown). Caspase-3 is readily activated in MCF7/Casp3 cells treated with STS for 2 h, with the maximal level of activation
achieved after 6 h (Fig. 1A, middle panel). In agreement with previous reports (12, 13), we found that caspase-3 is not required for STS-induced apoptosis in MCF7 cells (Fig. 1B, compare MCF7/Neo and MCF7/Casp3 during 17 and 24 h of STS treatment). However, the expression of functional caspase-3 greatly enhanced the sensitivity of these cells to STS, substantially reducing the cellular execution time (Fig. 1B). Identical results were obtained using several other single cell MCF7/Casp3 clones and a mixed MCF7/Casp3 cell population (data not shown).

Others (9) have shown that caspase-3 and -6 are required for cytochrome c-mediated activation of caspase-8 in human cell-free extracts. However, a separate study in vivo revealed that caspase-6 is normally activated downstream of caspase-3 during Fas-mediated cell death (14). Significant activation of caspase-8 was observed in the STS-treated MCF7/Casp3, but not MCF7/Neo, cells (Fig. 1A, right panel). To establish that caspase-8 was being activated, the cleavage of the caspase-8 substrate Bid was examined by immunoblot (15–17). Bid cleavage is involved in the release of cytochrome c into the cytosol, and the subsequent formation of active caspase-9/Apaf1/cytochrome c complexes during receptor-mediated apoptosis (15–17). Although Bid was not cleaved in STS-treated MCF7/Neo cells, it was readily processed in STS-treated MCF7/Casp3 cells (Fig. 1B). However, caspase-3 can also cleave Bid, albeit far less efficiently, in vitro using human cell-free extracts (18). To determine whether caspase-3 contributes to the Bid cleavage observed here, a FADD-DN protein was expressed in these same cells (see Fig. 3A). Bid cleavage was effectively blocked (Fig. 1C), demonstrating that caspase-3, which is not sensitive to the effects of FADD-DN expression (19), does not contribute to Bid processing in vivo. In addition, Bcl-xL expression also blocks Bid processing (Fig. 1C), consistent with earlier reports (15–17).

Because a hierarchy of caspase activation was recently established using a human cell-free system (9), the kinetics of caspase-2, -6, -7, -9, and -10 proteolysis were also examined in both MCF7/Neo and MCF7/Casp3 cells treated with 1 μM STS for 8 h. As might be expected based on the results from the cell-free studies, we found that caspase-6, -8, and -10 were not processed in the absence of caspase-3, whereas caspase-2, -7, and -9 were (Fig. 2). Caspase-3 expression restored processing of caspase-6 and -8 as well as significantly increasing the percentage of apoptotic cells (Fig. 2, caspase-6, and Fig. 1A, caspase-8). However, processing of caspase-10 does not occur in STS-treated MCF7/Casp3, Jurkat, or HeLa cells (Fig. 2) (11), suggesting that it is not activated in vivo in response to release of cytochrome c in these cell lines. In contrast, caspase-10 processing is induced by cyto-

**Fig. 2.** Kinetics of caspase-2, -6, -7, -9, and -10 activation in STS-treated MCF7 and MCF7/Casp3 cells. The percentage of apoptotic cells was determined by trypan blue uptake and 4,6-diamidino-2-phenylindole staining of nuclei.

**Fig. 3.** Caspase-3 activation of caspase-8 contributes to staurosporine-induced apoptosis in MCF7 cells. A, demonstration of the proteins expressed in Neo, FADD-DN, FADD-DN + Casp3, Bcl-xL, or Bcl-xL + Casp3 MCF7 cells by immunoblot analysis. B and C, these cells were then treated with STS for the times indicated. The extent of apoptosis after 4, 8, and 24 h, derived from three independent experiments, was determined by trypan blue uptake (panel B). Activation of caspase-3 and -8 in each of the cell lines during this treatment was analyzed by immunoblot as indicated (panel C). D, STS-induced cytochrome c redistribution in the cytosol of the same cells shown in panels B and C was determined by immunoblot.
Caspase-8 Activation in Response to Cytochrome c

Fig. 4. A caspase-3/caspase-8 amplification loop sensitizes cells to anti-Fas mAb and STS. A, 10^6/ml Jurkat cells were treated with either anti-Fas monoclonal antibody (CH-11, 1 or 10 ng/ml), STS (0.1 μM), or both CH-11 and STS at the doses indicated for 4 h. Apoptosis was quantitated by TUNEL using fluorescence-activated cell sorter. Activation of caspase-3 (top panel) and caspase-8 (middle panel), DNA fragmentation (bottom panel), and percentage of cells with positive annexin-V surface staining (numbers below bottom panel) were determined. B, MCF7 cells expressing identical levels of Fas receptor in the absence (MCF7/Fas) and presence (MCF7/Fas/Casp3) of caspase-3 expression were treated with the indicated amounts of agonistic Fas mAb (CH-11). Activation of caspase-8 and caspase-3 was determined by immunoblot assay (12). C, analysis of the effect of 4 h of treatment with the indicated concentrations of STS, agonistic Fas mAb, or a combination of both on the activation of caspase-3 and -8 and the percentage of cell death in MCF7/Fas and MCF7/Fas/Casp3 cells. The percentage of apoptotic cells was determined by cellular uptake of trypan blue.

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The possible physiological relevance of cytochrome c-mediated caspase-8 activation is not clear. Because caspase-8 activation is also mediated by caspase-3 in response to STS, as well as other agents that chemically induce cell death (e.g. dexamethasone, etoposide), it suggests that activated caspase-8 may contribute to cytochrome c-mediated cell death. To examine this possibility, we determined the effects of FADD-DN (i.e. a dominant negative mutant of FADD) expression and Bcl-xL overexpression on STS-mediated activation of caspase-3 and -8, as well as the ability of these cells to undergo apoptosis. MCF7 cells were stably transfected with either a FADD-DN or Bcl-xL expression constructs alone, or co-transfected with a caspase-3 expression construct, and protein expression confirmed by Western blotting (Fig. 3A). As might be expected, Bcl-xL expression, with or without caspase-3, greatly reduced STS-induced cytochrome c redistribution from mitochondria into the cytosol (Fig. 3D, compare lanes 1, 2, and 3 to lanes 10, 11, and 12). Bcl-xL overexpression also prevented the activation of caspase-3 and -8 (Fig. 3C, compare lanes 1–3 and 4–6 to lanes 10–12). In addition, the ability of STS to induce cell death is significantly reduced by bcl-xL (Fig. 3B, compare percentage of cell death between STS-treated MCF7/Neo, MCF7/Bcl-xL, and MCF7/Casp3/bcl-xL cells). However, expression of the FADD-DN protein, with or without caspase-3, does not affect STS-induced cytochrome c redistribution (Fig. 3D, compare lanes 1–3 to lanes 7–9; data not shown). Although FADD-DN expression has no effect on STS-induced cell death in the absence of caspase-3 (Fig. 3B, compare percentage of cell death of Neo and FADD-DN cells), its co-expression with caspase-3 partially inhibits STS-induced cell death (Fig. 3B, compare percentage of cell death between MCF7/Casp3 and MCF7/Casp3/FADD-DN cells at 4 and 8 h). This difference becomes less significant after 16–18 h of exposure to STS, possibly reflecting the contribution of necrotic death. Once again, identical results were obtained using other single cell isolates as well as a mixed cell population (data not shown). Finally, the effect of FADD-DN expression on the cellular execution time of STS-treated MCF7/Casp3 cells was examined (Fig. 1B). A significant increase in execution time (7 versus 4.5 h for 50% cell death) was observed in the MCF7/Casp3/FADD-DN cells, which furthermore indicates that cytochrome c-mediated activation of caspase-8 significantly contributes to the overall death process in these cells.

Although caspase-8 activation is rapid once the death-inducing signaling complex forms (20), caspase-3 directed activation of caspase-8 might help to amplify weak receptor-mediated death signals. A recent study of Bid-/- mice supports this hypothesis, because it was shown that Fas-induced apoptosis was impaired in hepatocytes but not thymocytes, indicating that rapid caspase-8 activation by recruitment to the DISC may be cell-type specific (10). Therefore, we decided that a comparison of the kinetics of caspase-8 activation and execution time in casepase-3 null, and positive, MCF7 cells stimulated to die by either receptor activation, chemical treatment, or a combination of the two, might be informative. To test this, caspase-8 activation was induced in Jurkat T-cells, MCF7/Fas cells, and MCF7/Fas/Casp3 cells derived from the MCF7/Fas...
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cells, using a low dose of an agonistic Fas antibody (CH-11), whereas caspase-9 activation was induced with STS. MCF7/Fas cell lines derived from single cell clones were used in these experiments to prevent variability in the Fas response that might arise due to differences in receptor levels. In addition, the minimal concentration of Fas mAb (10 ng/ml) and STS (0.1 μM) required to induce apoptosis in these experiments was determined (Fig. 4, panels A–C). Conversely, when Jurkat cells were treated with a combination of 10 ng/ml of CH-11 and 0.1 μM STS for 6 h (Fig. 4A). Conversely, when Jurkat cells were treated with a combination of 10 ng/ml of CH-11 and 0.1 μM STS for the same time, significant activation of caspase-3 and -8, DNA ladder, and cell death (~70%) was observed (Fig. 4A). Therefore, although the activation of either pathway with these stimuli is not sufficient to effectively promote apoptosis, activation of both pathways through the administration of the two stimuli simultaneously enhances cell death. However, it is also possible that some of this effect in the MCF7 cells may be due to high levels of caspase-3 expression, although several different, independent stable transfectants expressing variable levels of caspase-3 yielded the same results (data not shown). The apparent synergy between receptor-mediated and chemically induced apoptotic signals may not be surprising. As mentioned earlier, caspase-8 can activate caspase-9 through the processing of the cytosolic factor Bid, resulting in the release of cytochrome c into the cytosol and enhanced caspase-9 activation (10, 15–17). In addition, caspase-8 can be activated by caspase-9 in cells treated with agents that induce the cytochrome c/Apaf1/caspase-9 pathway (3, 21–23).

The MCF7/Fas and MCF7/Fas/Casp3 cell lines allowed us to examine the role of caspase-3 in caspase-9-mediated activation of caspase-8, at least in the context of this particular cellular environment. When increasing amounts of the agonistic CH-11 mAb were used to treat these cells, significantly enhanced activation of caspase-8 and cell death were observed in the cell line expressing caspase-3 (Fig. 4B). Similarly, when these cell lines were treated with 0.1 μM STS, 10 ng/ml CH-11, or a combination of the two, caspase-8 activation and cell death were significantly higher in the MCF7/Fas/Casp3 cells (Fig. 4C). Similar results were obtained in several other clonal MCF7/Casp3 cell lines, as well as the clonal MCF7/Fas/Casp3 cell lines derived from them (data not shown). Therefore, in this setting, caspase-3 appears to be capable of linking these two pathways through its ability to activate, directly or indirectly, caspase-8, resulting in increased levels of caspase-8 activation and enhanced cell death.

We conclude from the current studies that caspase-8 does not participate in STS-mediated apoptosis unless functional caspase-3 is expressed. We cannot rule out the possible involvement of caspase-6 downstream of caspase-3, because the processing of caspase-6 is greatly enhanced in MCF7 cells containing caspase-3. This result is consistent with the earlier cell-free and biochemical studies (9, 24). However, in contrast to results from the same cell-free system, we have demonstrated that caspase-3 does not cleave Bid directly in vivo (18). It is important to note that even in the cell-free system, caspase-3-mediated Bid cleavage much less effectively than caspase-8, suggesting that a distinct cytosolic factor was the caspase-8 substrate. Our results are consistent with this possible interpretation. Similarly, whereas cytochrome c addition to cell-free extracts can induce the processing of caspase-10 (9), STS-induced release of cytochrome c in vivo does not lead to the activation of caspase-10, although other caspases are activated and apoptosis ensues. Finally, the ability of FADD-DN expression to inhibit STS-induced apoptosis, as well as the time required for cellular execution, in the cells studied herein suggest that the activation of caspase-8 that occurs downstream of caspase-9 is physiologically relevant under these circumstances. Although caspase-8 gene knockout experiments clearly show that caspase-8 activation is not necessary for caspase-9-mediated apoptosis (25), they do not exclude the possibility that its activation may modulate weak death signals, perhaps in a cell/signal type-specific manner.

Such signal modulation might alter some parameters of the apoptotic process (e.g. the time required for a cell to die), as seen here (Fig. 1B). Thus, even though a hierarchy of caspase activation has been established in many circumstances, these results suggest that this hierarchy may not be absolute and that significant cross-talk between the death receptor and mitochondrial-signaling pathways can occur.

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