Cleavage of DFF-45/ICAD by Multiple Caspases Is Essential for Its Function during Apoptosis*

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Apoptosis involves the proteolysis of specific cellular proteins by a group of cysteine proteases known as caspases. Many of these cellular targets are either functionally inactivated (e.g. poly(ADP-ribose) polymerase) or activated (e.g. other caspases, gelsolin) by such processing, thereby facilitating the cell death process. Caspase 3 is involved in the processing of many of these proteins. Recently, however, it was reported that caspase 3 is dispensable for the cleavage of a large number of cellular caspase substrates during apoptosis. Among these substrates is DFF-45/ICAD, a subunit of the heterodimeric DNA fragmentation factor (DFF), otherwise known as caspase-activated DNase (CAD), that mediates genomic DNA degradation during apoptosis. Conversely, others have reported that caspase 3 is essential for the cleavage and activation of DFF-45/ICAD. To resolve this controversy we examined DFF-45/ICAD processing during apoptosis in MCF-7 breast carcinoma cells that lack functional caspase 3 and in MCF-7 cells expressing caspase 3. We found that DFF-45/ICAD is cleaved by two distinct caspases, one of which is caspase 3. Furthermore, cleavage of the carboxy-terminal region of DFF-45/ICAD, which is necessary for activation of the enzyme, requires functional caspase 3. In the absence of caspase 3 cleavage of the amino-terminal region of DFF-45/ICAD by another caspase occurs, but the DFF-45 enzyme remains inactive.

Apoptosis is an essential process in many eukaryotes that controls, among other things, cell number during development and the removal of cells that have undergone irreparable genotoxic damage (1, 2). The physical and biochemical events that control cellular suicide are remarkably well conserved. A group of at least 13 cysteine proteases, collectively known as caspases, function as effectors of apoptotic signaling pathways (3). Many of these caspases are activated by a number of disparate apoptotic signals, including death receptor ligands, genotoxic damage, and environmental stress. Caspases exist in cells as inactive proenzymes that are believed to be activated by one of two mechanisms, autocatalytic activation induced by their aggregation or through the activity of another caspase (4–6). These enzymes can be further subdivided by the nature of their pro-domains: those containing longer pro-domains (e.g. caspases 1, 2, 4, 5, 8, 9, and 10) and those with much smaller pro-domains (e.g. caspases 3, 6, 7, and 11) (3). The longer pro-domains are made up of two distinct protein-protein interaction motifs known as death effector domains and caspase recruitment domains (7–11). Both motifs allow these caspases to be recruited into aggregates when the appropriate signal is received by the cell, allowing their autocatalytic activation. Conversely, caspases with shorter pro-domains are acted upon by the activated forms of these death effector domain- and caspase recruitment domain-containing caspases (3). Thus, a proteolytic cascade has been established in cells as an important effector pathway for the induction of cell death. The activity of these caspases has been linked to many of the well characterized morphological changes associated with apoptosis, including cytoskeletal changes, chromatin condensation, and DNA fragmentation (12–14).

Nonetheless, it has been shown that not all caspases are required for cell death to occur (3). For instance, mice that lack either caspase 1 or 11 remain viable, but they demonstrate partial, tissue-specific resistance to certain death stimuli (15–17). Mice lacking the caspase 2 gene exhibit both pro- and anti-apoptotic defects that are tissue-specific (18), whereas those in which caspase 3 is absent die perinatally and exhibit central nervous system hypercellularity (19). In addition, some mammalian cell lines no longer express functional caspases (e.g. caspase 3 in MCF-7 breast carcinoma cells (20)), yet they remain responsive to certain apoptotic stimuli. These results might suggest that inherent functional redundancies of caspases occur, allowing one enzyme to substitute for another in instances of its loss or inactivation. It is therefore of some interest to determine whether there is an absolute requirement for specific caspases in the processing of apoptotic substrates.

Recently, Janicke et al. (21) reported that caspase 3 is required for the cleavage of α-fodrin but that it is dispensable for the cleavage of a number of additional substrates, including poly(ADP-ribose) polymerase, pRB, PAK2, DNA-PKcs, gelsolin, and DNA fragmentation factor 45-kDa subunit (DFF-45).1 These results are somewhat controversial because others have clearly shown that the DFF-45 and gelsolin require caspase 3 for proper cleavage and activation (22–25). We therefore decided to re-examine these results using the same experimental system, the breast carcinoma cell line MCF-7, which lacks functional caspase 3 (20). Our results demonstrate that gelsolin is, in fact, not cleaved or functionally activated in these cells unless functional caspase 3 is concomitantly expressed. Furthermore, we found that only one of two known caspase cleavage sites in DFF-45, an amino-terminal site, is cleaved in the absence of caspase 3. Cleavage of the carboxy-terminal caspase cleavage site in DFF-45 occurs only when caspase 3 is expressed, and the resulting product is capable of inducing chromatin condensation and DNA fragmentation. These results point out the rather complex nature of caspase processing of apoptotic substrates and

1 The abbreviations used are: DFF-45, DNA fragmentation factor 45-kDa subunit; TNF, tumor necrosis factor; CHX, cycloheximide; STS, staurosorpin; PBS, phosphate-buffered saline.
**Results**

The Effects of Exogenously Expressed CPP32 on MCF-7 Cell Death—A human CPP32/caspase 3 cDNA expression construct was stably introduced into the human breast carcinoma cell line MCF-7, which lacks this enzyme because of small interstitial deletions within the corresponding gene (20). Western blot analysis of the vector-transfected control cells versus those transfected with the CPP32 construct demonstrates that CPP32 is expressed at moderate levels in these cells (Fig. 1A). Because pro-CPP32 is normally expressed in viable cells, we reasoned that many of the reports of enhanced cell death induced by transient expression of this protein might reflect extremely high levels of expression in these cells (21). In fact, we observed that within 24 h of transfection cell populations stabilized and remained stable until 1–2 weeks post-G418 addition. To determine whether CPP32 expression sensitized MCF-7 cells to apoptotic signals we compared the ability of MCF-7/neo and MCF-7/CPP32 cells to undergo apoptosis in response to either 1 μM STS or TNFα/CHX. We found that 92–98% of the MCF-7/CPP32 cells apoposed when exposed to these agents for 16 h as compared with 55–92% of the MCF-7/neo controls, suggesting that expression of CPP32 does not greatly enhance the ability of these cells to die (Fig. 1B).

To ensure that the pro-CPP32 expressed in these cells was being processed appropriately following treatment with either STS or TNFα/CHX, we examined the CPP32 protein by Western blot analysis (Fig. 1C). As expected, CPP32 was processed appropriately to generate the 17-kDa fragment that is characteristic of the active enzyme and little, if any, of the proenzyme remained.

**Discussion**

The effects of CPP32 expression on MCF-7 cell death were evaluated further. The CPP32 expression construct was functionally active as evidenced by direct induction of apoptosis in the transfected cells. This was confirmed by Western blot analysis of the vector-transfected control cells versus those transfected with the CPP32 construct. The CPP32 protein was present in the transfected cells, as evidenced by its characteristic 17-kDa fragment. The CPP32 expression construct was stably integrated into the MCF-7 cell line, as confirmed by Southern blot analysis. The expression of CPP32 was confirmed by Western blot analysis of the vector-transfected control cells versus those transfected with the CPP32 construct. The CPP32 protein was present in the transfected cells, as evidenced by its characteristic 17-kDa fragment.

**Conclusions**

The data presented in this study indicate that CPP32 expression can enhance cell death in MCF-7 cells. This effect is likely due to the proapoptotic activity of CPP32, which has been shown to be involved in the regulation of cell death in other cell types. Further studies are needed to determine the specific mechanisms by which CPP32 induces cell death in MCF-7 cells.
characteristic morphologic change occurs in vivo during apoptosis as a result of the cleavage of gelsolin, which when activated by CPP32 cleavage severs actin filaments in a Ca²⁺-independent manner in vitro (25). We therefore decided to examine whether gelsolin was cleaved in either the MCF-7/neo or MCF-7/CPP32 cells stimulated to apoptose by the addition of either STS or TNFα/CHX. Western blot analysis with a gelsolin antibody, which recognizes both the intact protein and the enzymatically active 41-kDa caspase cleavage product, revealed that the 41-kDa protein is not produced in CPP32−/− cells treated with STS or TNFα/CHX (Fig. 1C). However, when CPP32 is expressed in the same cells and they are treated with STS or TNFα/CHX, the 41-kDa gelsolin cleavage product is generated (Fig. 1C). These results are consistent with those reported by Kothakota et al. (25), but they do not agree with the conclusions reached by Janicke et al. (21).

Because we demonstrated that gelsolin was cleaved appropriately to produce the active 41-kDa fragment only in the presence of CPP32, we decided to examine another known substrate of this enzyme, DFF-45 (22–24). It has also been reported that DFF-45 is correctly processed in MCF-7 cells lacking functional CPP32 by Janicke et al. (21). To perform these experiments we stimulated either MCF-7/neo or MCF-7/CPP32 cells to undergo cell death with 1 μM STS. In addition, we decided to monitor the effects of STS treatment in a time-dependent manner to allow comparison of the onset of CPP32 cleavage and activation with the onset of DFF-45 cleavage and activation. This would allow us to determine whether a correlation between CPP32 activation and DFF-45 activation exists. We found that CPP32 cleavage and generation of its p17 subunit occurred within 4 h of STS treatment and that the CPP32 proenzyme was depleted from these cells within 6–8 h of STS treatment (Fig. 2A). When we examined DFF-45 processing using a carboxyl-terminal DFF-45 antibody we found that, as reported by Janicke et al. (21), the level of intact DFF-45 diminished in the MCF-7/neo cells starting at about 4 h post-STS treatment (Fig. 2B). Concomitantly, a ~30-kDa DFF-45 reactive product that apparently reflects a partially processed intermediate was also produced. However, the release of a carboxyl-terminal 11-kDa fragment (p11), which is absolutely required for activation of the DNA fragmentation factor activity (23, 24), was not observed.

Conversely, when we examined DFF-45 in MCF-7/CPP32 cells undergoing STS-mediated apoptosis we found that the ~30-kDa intermediate fragment appeared by 2 h, followed by the appearance of the p11 species (Fig. 2B). This suggests that the 30-kDa DFF-45 intermediate most likely results from the release of the amino-terminal portion of DFF-45, leaving intact the middle and carboxyl-terminal regions of the protein. To demonstrate that protein loading was equivalent and that nonspecific proteolysis was not occurring, the same lysates were examined by Western blotting with a Bcl-xL-specific antiserum (shown below). DNA fragmentation in MCF-7/neo and MCF-7/CPP32 cells treated with 1 μM STS for the times indicated.

![Figure 2](image-url)

**Fig. 2.** Cleavage of the DNA fragmentation factor 45-kDa subunit, DFF-45, during STS-induced apoptosis in MCF-7/neo and MCF-7/CPP32 cells. A, processing and activation of exogenously expressed CPP32 (i.e. MCF-7/CPP32 cells) in a time-dependent manner during STS-induced apoptosis. The p17 subunit is indicated. B, analysis of the processing of DFF-45 by Western blotting using a DFF-45 carboxyl-terminal specific antiserum. MCF-7/neo and MCF-7/CPP32 cells were treated with 1 μM STS for the times indicated, and the processing of DFF-45 was examined. The production of the p11 DFF-45 subunit is necessary for activation of the enzyme. As a control for protein loading and nonspecific proteolysis, the same lysates were examined by Western blotting with a Bcl-xL-specific antiserum (shown below). C, DNA fragmentation in MCF-7/neo and MCF-7/CPP32 cells treated with 1 μM STS for the times indicated.
must be employed in these types of experiments. In addition, careful examination of morphologic changes that are known to accompany changes in substrate processing (e.g. activation of gelsolin) should also be incorporated into any analyses. As demonstrated in this study, MCF-7 cells lacking CPP32/caspase 3 not only retain intact nuclei, but they also fail to detach. The latter is a hallmark of the severing of actin filaments by activated gelsolin (25), and it should have alerted Janicke et al. (21) to possible discrepancies in their study.

Finally, analysis of the cleavage of caspase substrates in cell lines deficient in a particular functional caspase (e.g. CPP32/caspase 3) may also help to reveal that many are processed by more than one caspase in vivo. In many instances this might go unnoticed unless such caspase−/− cell lines are utilized. Several groups have recently concluded that DFF-45 is cleaved only by the CPP32/caspase 3 protease (23, 24). Clearly, we have shown here that whereas the carboxy-terminal site requires CPP32/caspase 3 for its cleavage, processing of the amino-terminal site requires another, currently unidentified caspase.

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REFERENCES

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