Active Caspases and Cleaved Cytokeratins Are Sequestered into Cytoplasmic Inclusions in TRAIL-induced Apoptosis

Marion MacFarlane, Wendy Merrison, David Dinsdale, and Gerald M. Cohen

Medical Research Council Toxicology Unit, University of Leicester, Leicester LE1 9HN, United Kingdom

Abstract. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis, in transformed human breast epithelial MCF-7 cells, resulted in a time-dependent activation of the initiator caspases-8 and -9 and the effector caspase-7. Cleavage of caspase-8 and its preferred substrate, Bid, preceded processing of caspases-7 and -9, indicating that caspase-8 is the apical initiator caspase in TRAIL-induced apoptosis. Using transient transfection of COOH-terminal–tagged green fluorescent protein fusion constructs, caspases-3, -7, and -8 were localized throughout the cytoplasm of MCF-7 cells. TRAIL-induced apoptosis resulted in activation of caspases-3 and -7, and the redistribution of most of their detectable catalytically active small subunits into large spheroidal cytoplasmic inclusions, which lacked a limiting membrane. These inclusions, which were also induced in untransfected cells, contained cytokeratins 8, 18, and 19, together with both a phosphorylated form and a caspase-cleavage fragment of cytokeratin 18. Similarly, in untransfected breast HBL 100 and lung A 549 epithelial cells, TRAIL induced the formation of cytoplasmic inclusions that contained cleaved cytokeratin 18 and colocalized with active endogenous caspase-3. We propose that effector caspase-mediated cleavage of cytokeratins, resulting in disassembly of the cytoskeleton and formation of cytoplasmic inclusions, may be a characteristic feature of epithelial cell apoptosis.

Key words: Bid • caspase immunolocalization • epithelial cells • intermediate filaments • K18

Introduction

Apoptosis is a process of fundamental importance to multicellular organisms that enables control of cell populations and the removal of damaged or potentially harmful cells (Arends and Wyllie, 1991). A apoptosis occurs in two phases: an initial commitment phase followed by an execution phase involving cytoskeletal disruption, membrane blebbing, condensation and fragmentation of chromatin, and the formation of apoptotic bodies (Earnshaw, 1995). Caspases, a family of aspartate-specific cysteine proteases, play a critical role in the execution phase of apoptosis and are the key effectors responsible for many of the dramatic morphological and biochemical changes of apoptosis (for reviews see Cohen, 1997; Thornberry and Lazebnik, 1998). Caspases are proteolytically cleaved at specific aspartate residues, generating a large and small subunit that together form the active enzyme. Caspases can be divided into two classes: (1) initiator caspases, with long prodomains, such as caspases-8 and -9, which either directly or indirectly activate (2) effector caspases, such as caspases-3, -6, and -7 (Srinivasula et al., 1996; Cohen, 1997; Thornberry and Lazebnik, 1998). During the execution phase of apoptosis, active effector caspases cleave intracellular substrates including poly(ADP-ribose) polymerase (Lazebnik et al., 1994) and the cytoskeletal components actin (Kiyal et al., 1996) and lamins (Takahashi et al., 1996).

Caspase-mediated proteolytic cleavage of lamins facilitates nuclear collapse, which is so widely used to distinguish apoptotic cells (Rao et al., 1996). More recently, it has been reported that several type I and type II intermediate filament proteins, which comprise the acidic (K9–K20) and basic cytokeratins (K1–K8) are dramatically reorganized in cells during apoptosis (Caulin et al., 1997). Cytokeratin 8 (K8) and cytokeratin 18 (K18) are the major components of intermediate filaments in simple or sin-

Abbreviations used in this paper: GFP, green fluorescent protein; K8, K18, and K19, cytokeratins 8, 18, and 19, respectively; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; YVAD.CMK, acetyl-Tyr-Val-Ala-Asp chloromethyl ketone; z-DEVD.AFC, benzyloxycarbonyl-Asp-Glu-Val-Asp aminofluoromethyl coumarin; z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone.

The Journal of Cell Biology, Volume 148, Number 6, March 20, 2000 1239–1254
http://www.jcb.org
gle layer epithelial tissues (Moll et al., 1982). Both K8 and K18 are subject to posttranslational modification by phosphorylation (Ku and Omary, 1994). The reorganization of K8/K18 filaments during apoptosis is associated with the phosphorylation of K18 on serine 53 (previously referred to as serine 52 relative to posttranslational processed protein; Ku and Omary, 1994). Furthermore, K18 is cleaved in vitro by recombinant caspases, and such cleavage may initiate the orderly processing of filament proteins during apoptosis (Caulin et al., 1997).

Triggering of the CD95 (Fas/APO-1) receptor with its cognate ligand or agonistic antibody results in receptor trimerization and recruitment of FADD/MORT1, which in turn binds to the death effector domains of caspase-8, resulting in its activation (Boldin et al., 1996; Muzio et al., 1996). A caspase-8 activates all known caspases in vitro (Srinivasula et al., 1996), it is a prime candidate for an initiator caspase in many other forms of receptor-mediated apoptosis. Mitochondria also have been proposed to act as an amplifier in CD95-induced apoptosis with their involvement being more important in certain cell types (Kuwana et al., 1998; Scaffidi et al., 1998). Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, is a cytosolic substrate of caspase-8, which when cleaved, translocates to the mitochondria where it induces the release of cytochrome c (Wang et al., 1996; Li et al., 1998; Luo et al., 1998). In the presence of cytochrome c, dATP, and A paf-1, procaspase-9 can act as an initiator caspase, leading to the activation of the downstream effector caspases-3, -6, and -7 (P. Li et al., 1997). It remains to be determined whether this mitochondrial amplification loop, activated after CD95 receptor engagement, is also involved in other forms of receptor-mediated apoptosis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), like CD95, is a member of the tumor necrosis factor (TNF) ligand family and induces apoptosis by binding to the receptors TR-1 (also called DR-4) and TR-2 (also called DR5/TRICK-2/KILLER; for review see Ashkenazi and Dixit, 1998). Induction of apoptosis via this pathway is thought to be mediated through caspase activation, although the caspases involved or their order of activation in intact cells has not been demonstrated clearly. Regulation of TRAIL-induced apoptosis is, in part, controlled by the relative levels of the death receptors TR-1 and TR-2 and the decoy receptors TR-3 (also called TRID/DcR1/LIT) and TR-4 (also called DcR2/TRUNDD; Ashkenazi and Dixit, 1998). Specifically, expression of the decoy receptors in cell lines correlates, in some instances, with resistance to TRAIL-induced apoptosis (Pan et al., 1997; Sheridan et al., 1997); thus, their relative levels may contribute to the reported selective cytotoxic potential of TRAIL in some tumors (Walczak et al., 1999).

We, and others, have recently shown that distinct caspase cascades are initiated during CD95-induced apoptosis (Scaffidi et al., 1998; Sun et al., 1999). We now demonstrate that during TRAIL-induced apoptosis in MCF-7 cells, the initiator caspase-8 is activated at the apex of a caspase cascade. Apoptosis induced in MCF-7, breast HBL100 and lung A549 epithelial cells results in the formation of spheroidal cytoplasmic inclusions. In HBL100 and A549 cells, these inclusions contain cleaved cytokeratin,
tage (Becton Dickinson), and populations of cells exhibiting low or high intensity green fluorescence were collected for analysis by Western blotting.

**Electron Microscopy and Immunogold Cytochemistry**

Nonadherent cells were dislodged by swirling the medium before removal, and spun down in a swing-out rotor at 3,000 g. The pellets, like the cells referred to acetylated tubulin, clone AC15, specific for M30 antibody specifically labels apoptotic epithelial cells and does not age of apoptotic nuclei with ~35% apoptosis detected by 6 h. To determine which caspases were activated and their order of activation, Western blot analysis was performed. In untreated MCF-7 cells, caspase-8 was present primarily as two isoforms of ~55 kD (Fig. 1 A, lane 1), corresponding to caspase-8a and -8b (Scaffidi et al., 1997). Induction of apoptosis by TRAIL resulted in a time-dependent processing of caspase-8 initially to two fragments of ~43 and 41 kD, corresponding to cleavage of the small subunit from the 43- and 41-kD fragments (Fig. 1 A, lanes 2–7). An increase in the processing of caspase-8 (to yield fragments of 41 and 43 kD) was first observed 30 min after TRAIL treatment. Untreated MCF-7 cells contained the 46-kD proform of caspase-8 (Fig. 1 A, lanes 2–7). A n increase in the processing of caspase-8 to yield fragments of 41 and 43 kD was first observed 30 min after TRAIL treatment. Untreated MCF-7 cells contained the 46-kD proform of caspase-8 (Fig. 1 A, lane 1), which on induction of apoptosis by TRAIL, was processed in a time-dependent manner to yield fragments of 37 and 35 kD (Fig. 1 A, lanes 2–7), as a result of cleavage at A sp 315 and A sp 330 (Srinivasula et al., 1998). Caspase-7, a major effector caspase, was present in MCF-7 cells primarily as its intact 35-kD proform (Fig. 1 A, lane 1). Treatment with TRAIL resulted in a time-dependent processing of caspase-7, which was accompanied by the formation of the 19-kD catalytically active large subunit (Fig. 1 A, lanes 2–7). Processing of caspases-7 and -9 was first observed 60–120 min after treatment with TRAIL.

Bid is a specific proximal substrate of caspase-8 in the CD 95 signaling pathway (Li et al., 1998; Luo et al., 1998). Cleavege of Bid by caspase-8 realizes its potent proapoptotic activity, which is important for the release of cyto-
Therefore, we wished to investigate whether cleavage of Bid also occurred during TRAIL-induced apoptosis. Bid was present in MCF-7 cells as a 26-kD protein, which, after exposure to TRAIL, was cleaved initially to yield a major fragment of 15 kD together with a minor 13-kD fragment. This cleavage was first observed at 45 min (Fig. 1 A), which is consistent with an early activation of caspase-8. Our data demonstrate the sequential activation of caspase-8 and Bid, followed by activation of caspases-7 and -9. These results support the hypothesis that caspase-8 is the apical caspase in TRAIL-induced apoptosis.

Redistribution of Caspases-3, -7, and -8 during TRAIL-induced Apoptosis in MCF-7 Cells

We have previously shown that a redistribution of effector caspases occurs during CD95-induced apoptosis in vivo (Chandler et al., 1998). Therefore, we wished to investigate the subcellular distribution of caspases in intact cells during TRAIL-induced apoptosis. As our polyclonal antibodies to caspases-7 and -8 were unsuitable for immunocytochemistry, we constructed initiator and effector caspases containing green fluorescent protein (GFP) at their COOH termini (Fig. 1 B). To facilitate the localization of these GFP-tagged caspases, they were transiently transfected into MCF-7 cells, an adherent cell line. To ensure that GFP fusion at the COOH terminus did not interfere with the recognition of caspase-3, -7, and -8, these proteins were expressed in MCF-7 cells (0.2 nmol/mg/min) and were incubated with recombinant TRAIL (1 µg/ml). Levels of caspase-3 activity were measured by immunoblotting using an anti-GFP antibody (top) or an antibody that recognizes the p17 subunit of caspase-3 (bottom). The caspase-3-GFP structure outlines the boundaries of the two caspase subunits and the NH2-terminal prodomain (Pro). Cleavage to generate fully processed enzyme occurs at A sp-175, A sp-9, and A sp-28.

Figure 1. Caspase-8 is the most apical caspase in TRAIL-induced apoptosis in MCF-7 cells. (A) MCF-7 cells were incubated for up to 2 h, either alone or in the presence of recombinant TRAIL (1 µg/ml) and the time course of cleavage of the initiator caspases-8 and -9, the effector caspase-7, and the regulatory molecule Bid were assessed by Western blot analysis as described in Materials and Methods. The asterisk marks nonspecific protein bands detected by the caspase-8 antibody in MCF-7 cells. (B) MCF-7 cells were transiently transfected with pEGFP-N1 or caspase-3-GFP and, 16 h after transfection, were incubated for up to 6 h with recombinant TRAIL (1 µg/ml). Cells were analyzed by immunoblotting using an anti-GFP antibody (top) or an antibody that recognizes the p17 subunit of caspase-3 (bottom). The caspase-3-GFP structure outlines the boundaries of the two caspase subunits and the NH2-terminal prodomain (Pro). Cleavage to generate fully processed enzyme occurs at A sp-175, A sp-9, and A sp-28.
redistribution of green fluorescence into large spheroidal inclusions was clearly visible in the cytoplasm of apoptotic cells (Fig. 2, D and F). Interestingly, in these cells, almost all of the active effector caspases were redistributed into the large inclusions, with little active caspase detectable elsewhere in the cell (Fig. 2, D and F). In contrast, treatment of cells with TRAIL resulted in a redistribution of active caspase-8 into small punctate inclusions in the cytoplasm of apoptotic cells (Fig. 2 H). Treatment of MCF-7 cells with anti-CD95 resulted in similar changes in caspase

Figure 2. TRAIL-induced apoptosis results in redistribution of caspases-3, -7, and -8. MCF-7 cells were transiently transfected with pEGFP-N1 (A and B), caspase-3-GFP (C and D), caspase-7-GFP (E and F), or caspase-8-GFP (G and H). At 24 h after transfection, cells were incubated either alone (A, C, E, and G) or in the presence of recombinant TRAIL (1 μg/ml; B, D, F, and H) for 6 h, stained with propidium iodide to label DNA, and analyzed by confocal microscopy. GFP exhibits an even cellular distribution: orange/yellow fluorescence being evident in the nucleus because of colocalization of GFP and propidium iodide. Note the redistribution of caspases-3 and -7-GFP into large spheroidal inclusions in apoptotic MCF-7 cells, whereas caspase-8 exhibits a discrete punctate distribution in cells exhibiting altered nuclear morphology. Bar, 10 μm.
distribution (data not shown). Thus, after TRAIL or CD95-induced apoptosis, a marked redistribution of caspases was observed, which differed between the effector caspases-3 and -7 and the initiator caspase-8.

Neither Mitotracker nor Calnexin Colocalize with GFP-tagged Caspases-3, -7, or -8

The TRAIL-induced redistribution of GFP-tagged caspases-3, -7, and -8 into intracytoplasmic spheroidal inclusions led us to investigate the nature of these structures. Using the selective dye MitoTracker red, mitochondria were clearly visible by confocal microscopy in control MCF-7 cells as discrete punctate structures throughout the cytoplasm, particularly in the perinuclear region (Fig. 3 A). No colocalization of caspase-3 with the mitochondria of MCF-7 cells was apparent when this distribution was compared with that of the GFP-tagged caspase-3. Treatment of caspase-3-transfected cells with TRAIL, followed by costaining with MitoTracker red, confirmed the subcellular redistribution of GFP-tagged caspase-3 into large spheroidal inclusions, and revealed that these structures were distinct from the mitochondria of apoptotic cells (Fig. 3 B). Similarly, no colocalization of calnexin, an ER transmembrane protein, was observed with either GFP-tagged caspase-3 in control MCF-7 cells (Fig. 3 D) or with the caspase-associated spheroidal inclusions induced by TRAIL (Fig. 3 E). Induction of apoptosis by TRAIL in caspase-7-transfected MCF-7 cells again resulted in the formation of large spheroidal inclusions that did not colocalize with mitochondria or the ER (Fig. 3, C and F). Similarly, actin, a major component of the cytoskeleton, did not colocalize with caspase-3 or -7 in control MCF-7 cells or after TRAIL treatment (data not shown). Furthermore, the small punctate inclusions detected in TRAIL-induced apoptotic MCF-7 cells transfected with GFP-tagged caspase-8 did not colocalize with mitochondria, ER, or the actin cytoskeleton (data not shown).

TRAIL Induces Spheroidal Inclusions in the Cytoplasm of Untransfected and Transfected MCF-7 Cells

The identity of the GFP-tagged caspase-3 and caspase-7–associated spheroidal inclusions in TRAIL-treated MCF-7 cells was further examined at the ultrastructural level. Initially, we confirmed that these inclusions were also formed in untransfected cells. Sections through untreated cultures showed a predominance of large cells, up
Caspase-3 and -7 express GFP in nonadherent cells (Table I). When cells transfected with the presence of keratin-containing inclusions in 12% of these cells to be indistinguishable from control cells either Lipofectamine or GFP alone revealed the majority of filamentous material were also observed (Table I). These inclusions (Fig. 4, B and C) were sharply demarcated from the nuclear envelope and dispersal of the nucleolus (Fig. 4 D). Similar nuclear changes and large cytoplasmic spheroidal inclusions were observed after anti-CD95 treatment of cells transfected with GFP-tagged caspase-3 or -7 (data not shown). In contrast, cells treated with TRAIL or anti-CD95 after transfection with caspase-8 did not undergo either of these changes. However, these cells contained the small cytoplasmic inclusions observed in nontransfected cells that had been treated with these agents (data not shown). Thus, transfection with GFP-tagged caspases-3 or -7, but not caspase-8, followed by treatment with TRAIL or anti-CD95 resulted in an exaggerated apoptotic phenotype compared with cells treated with TRAIL or anti-CD95 alone. This exaggerated phenotype was characterized by pronounced nuclear disruption and the formation of the large spheroidal inclusions (Fig. 4, compare B and D), which corresponded in size and distribution to those observed by confocal microscopy (Figs. 2 and 3).

### Cytokeratins 8, 18, and 19 Are Redistributed into the Large Spheroidal Inclusions

TRAIL-induced formation of large spheroidal inclusions in cells transfected with effector caspases was accompanied by the loss of detectable cytofilaments (Fig. 4 D); therefore, the subcellular distribution of cytofilament proteins in apoptotic cells was investigated. Immunogold labeling of ultrathin sections indicated that the spheroidal inclusions contained cytokeratins but not β-actin, tubulin, or vimentin. A specific mAb to K18 produced intense, specific immunogold labeling of the spheroidal inclusions of cells transfected with GFP-tagged caspase-3 or -7 and treated with TRAIL (Fig. 5 A and data not shown). Intermediate filament disorganization and reorganization occurs during mitosis and is associated with phosphorylation, primarily on serine 53 of K18 (Ku and Omary, 1994). Immunogold labeling with antibody 3055, which specifically recognizes the K18 phosphorylated on serine 53 (P-K18; Liao et al., 1995), clearly demonstrated its accumulation in the spheroidal inclusions (Fig. 5 D). mAbs specific to the two other major cytokeratins present in MCF-7 cells, namely K8 and 19 (Moll et al., 1982), also labeled these spheroidal inclusions, whereas antibodies to K13, 15, and 16 did not (data not shown). There were no obvious inclusions in untreated MCF-7 cells, but the filament network was labeled by K8, 18, and 19 (Fig. 5 B, inset, and data not shown). In nontransfected TRAIL-treated cells, these antibodies labeled the distinctive sheaves of filaments (Fig. 5 B) and the small inclusions, which characterized these cells. In contrast, antibody M30, which specifically recognizes a caspase cleavage site within K18, did not label either the filament network or the sheaves of filaments (data not shown), but it did label the spheroidal inclusions (Fig. 5 C). M30 recognizes a neo-epitope, DALD, in the COOH terminus of K18 exposed early during apoptosis (Leers et al., 1999). Furthermore, colabeling of the spheroidal inclusions with antibody 3055 (5 nm immunogold) and the M30 antibody (10 nm immunogold) was also observed (Fig. 5 D). Thus, the distinctive large spheroidal inclusions in apoptotic MCF-7 cells contained K8, 18, and 19 as well as P-K18, and resulted from redistribution and cleavage of the intermediate filaments.

---

**Table I. TRAIL Induces Cytokeratin-containing Inclusions in Epithelial Cells**

| Cell line | Control Adherent cells | Control Nonadherent cells | TRAIL Adherent cells | TRAIL Nonadherent cells |
|-----------|------------------------|---------------------------|----------------------|------------------------|
| MCF-7     | 0                      | 4                         | 0.7                  | 12                     |
| A549      | 0                      | 6                         | 1.5                  | 64                     |
| HBL100    | 0                      | <1                        | 0.5                  | 64                     |

Cells were treated with TRAIL (1 μg/ml) for 7 h and nonadherent cells were centrifuged. Insufficient nonadherent cells were obtained in control cultures to yield a cell pellet. Both adherent and nonadherent cells were fixed and labelled with immunogold using a mixture of mAbs to cytokeratins 8, 18, and 19. The percentage of cells containing cytokeratin inclusions was determined. Intermediate filaments were positively labelled in all untreated cells.

*The number in parentheses indicates the maximum diameter (expressed in μm) of the cytokeratin-containing inclusions.*
TRAIL-induced Colocalization of Active Effector Caspases and Cleaved Cytokeratin 18

We wished to determine whether the GFP-containing spheroidal inclusions observed by confocal microscopy in TRAIL-induced MCF-7 cells transfected with GFP-tagged caspases-3 and -7 (Fig. 2, D and F) were associated with cleaved cytokeratins. Using the M30 antibody, we performed immunocytochemistry on caspase-3-GFP-transfected MCF-7 cells that had been exposed to TRAIL for 6 h. Immunolabeling for cleaved K18 was only present in apoptotic cells and was predominantly localized in discrete cytoplasmic structures and on the periphery of large spheroidal inclusions (Fig. 3 H), which resembled those inclusions previously shown to contain caspase-3 or -7 in transfected cells (Figs. 2, D and F, and 3 G). A mouse monoclonal antibody M30 only recognizes cleavage of K18 at DALD397S; we cannot exclude the possibility that cleavage at VEVD238A has also occurred either before or in parallel with sequestration of cleaved K18 into spheroidal inclusions after TRAIL-induced apoptosis. Preferential immunolabeling
of the periphery of inclusions by antibody M30 was almost certainly the result of their impermeability to antibodies as immunogold studies, using ultrathin sections, showed uniform labeling throughout the inclusions (Fig. 5 D). Despite these difficulties with antibody accessibility, it was evident that GFP-tagged caspase-3 and cleaved K18 were colocalized in large spheroidal inclusions present in MCF-7 cells after exposure to TRAIL (Fig. 3 I). Similar results were obtained when HeLa cells, transfected with caspase-3- or -7-GFP and exposed to TRAIL, were colabeled with antibody M30 (data not shown). Thus, activated caspases-3 and -7 are associated with cleaved K18 in apoptotic MCF-7 cells, further supporting the hypothesis that effector caspases are primarily responsible for the ordered dismantling of the filament structure in epithelial cells during apoptosis.

Figure 5. Ultrastructural localization of cytokeratins in control, caspase-3, and caspase-7-transfected MCF-7 cells after treatment with TRAIL. (A) The cytoplasm of a cell transfected with caspase-3, and exposed to TRAIL for 6 h, is largely devoid of labeling for K18, but the label is concentrated over large (asterisks) and small spheroidal inclusions. Remnants of the dense, fibrillar component (black arrowheads) are evident alongside the enlarged granular component (curved black arrow) of the nucleolus. The inset is a threefold magnification of the area outlined. (B) In a nontransfected cell, exposed to TRAIL for 6 h, K18 is localized in sheaves (double white arrows) present throughout the cytoplasm. The inset shows the diffuse distribution of cytokeratin 18-labeled intermediate filaments in control cells. (C) The cytoplasm of a cell transfected with caspase-7, and exposed to TRAIL for 6 h, contains a large spheroidal cytoplasmic inclusion (asterisk), together with two smaller inclusions (curved white arrows) which are labeled with 10 nm immunogold after incubation with the M30 antibody, which is specific to the cleaved form of K18. The rest of the cell, including a remnant of the nucleolar dense fibrillar component (black arrowhead) is devoid of label. The inset is a threefold magnification of the area outlined. (D) The cytoplasm of a cell transfected with caspase-7 and exposed to TRAIL for 6 h. Labeling by both the M30 antibody (10 nm immunogold) and the 3055 antibody, which is specific to the phosphorylated form of this cytokeratin (5 nm immunogold), is evident over a large spheroidal inclusion (asterisk). The inset is a threefold magnification of the area outlined. Bars, 1 μm.

MacFarlane et al. Caspases and Cytokeratins Colocalize in Apoptosis 1247
**Effector Caspases Induce Cleavage of Cytokeratins 8, 18, and 19 during TRAIL-induced Apoptosis**

To determine the integrity and phosphorylation state of the cytokeratin proteins during apoptosis, MCF-7 cells were transfected with caspase-7-GFP, treated with TRAIL for 5 h, and sorted into two populations containing either untransfected cells or cells transfected with caspase-7-GFP (Fig. 6 A). Control MCF-7 cells contained K8, 18, and 19 as intact proteins of 52, 48, and 40 kD, respectively. Treatment of untransfected or caspase-7-GFP–transfected MCF-7 cells with TRAIL resulted in cleavage of K19 to yield a fragment of ~38 kD and cleavage of K8 to yield a fragment of ~46 kD and possibly one of ~48 kD (Fig. 6 A). Cleavage of both K8 and K19 has been reported during radiation-induced apoptosis of breast tumor cells (Prasad et al., 1998). Although K8 cleavage also occurs in the nonsmall-cell lung cancer cell line, MRC65 (van Engelund et al., 1997), its cleavage may be a cell type–specific phenomenon as it is relatively spared during apoptosis of several other epithelial cell types (Caulin et al., 1997; K u et al., 1997). In the MCF-7 cells treated with TRAIL for 5 h, K18 was cleaved to yield a fragment of ~26 kD but, in the cells transfected with caspase-7-GFP, an additional cleavage product of ~19 kD was also detected (Fig. 6, A lane 3). This result implicated effector caspases in the degradation of the cytoskeleton in agreement with previous work that identified a specific caspase cleavage site in K18 during drug and UV-induced apoptosis (Caulin et al., 1997). Using recombinant caspases as well as various mutants, these authors demonstrated that caspase-3, -6, or -7 could cleave K18 at VEVD238, located within the non-α-helical L1-2 linker region, yielding a 26-kD NH2-terminal and a 22-kD COOH-terminal fragment. A caspase-3 or -7 COOH-terminal cleavage site, which results in cleavage of intact K18 to a 45-kD fragment or further processing of the 22-kD fragment to an ~19-kD fragment, was also noted but not identified (Caulin et al., 1997).

To further investigate the role of caspases in TRAIL-induced cleavage of cytokeratins, we exposed either control or transfected MCF-7 cells to TRAIL in the presence of two caspase inhibitors (Fig. 6 B). z-VAD.FMK is a broad spectrum caspase inhibitor, whereas YVAD.CMK is reported to be more specific for caspases-1 and -6 (Laezribnik et al., 1995; MacFarlane et al., 1997b). Treatment of control MCF-7 cells, or cells transfected with GFP alone, with TRAIL for 7 h resulted in cleavage of K18 to yield four immunoreactive products of ~45, 26, 22, and 19 kD (Fig. 6 B, lanes 2 and 4). In cells transfected with caspase-3-GFP either alone or treated with TRAIL, cleavage of K18 predominantly yielded the 19-kD product with small amounts of the other fragments (Fig. 6 B, lanes 5 and 6). The 26-kD but not the 22- or 19-kD fragments was also detected by the P-K18–specific antibody, 3055 (Fig. 6 B), supporting the suggestion that the 26- and 22-kD products are the NH2 and COOH terminally derived fragments, respectively, resulting from cleavage at VEVD238. (A, Fig. 6 C). The M30 antibody, which recognizes the neo-epitope DALD, labeled the 19-kD but not the 26- or 22-kD fragments (data not shown), suggesting that the 19-kD product was derived from further cleavage of the ~22-kD product at DALD237 (Fig. 6 C). Thus, in the absence of P-K18 are cleaved by effector caspases during TRAIL-induced apoptosis. (A) MCF-7 cells were transiently transfected with caspase-7-GFP and, 24 h later, were incubated with recombinant TRAIL (1 μg/ml) for a further 5 h. Cells were sorted by flow cytometry into two populations of cells exposed to TRAIL: untransfected cells and those expressing caspase-7-GFP. Control cells contained primarily the intact forms of all three cytokeratins (lane 1). In cells treated with TRAIL, processing of K8, 18, and 19 was detected, with K18 being more extensively processed in cells expressing caspase-7-GFP (lanes 2 and 3, respectively). The asterisks mark non-specific bands of ~49 and 47 kD detected by the K8 and K18 antibodies, respectively. (B) z-VAD.FMK blocks the cleavage of cytokeratins. MCF-7 cells were transiently transfected with pEGFP-N1 or caspase-3-GFP either in the absence or presence of YVAD.CMK (10–50 μM) or z-VAD.FMK (10–50 μM). Control cells, or cells transfected with GFP alone or caspase-3-GFP, were treated with recombinant TRAIL (1 μg/ml) for 7 h and analyzed by Western blot analysis. Extensive cleavage of K18 and P-K18, induced by transfection of caspase-3-GFP and a 7-h exposure to TRAIL, was blocked by YVAD.CMK. (C) A schematic representation of the potential fragments resulting from cleavage of K18 by effector caspases. The K18 antibody, CY90, detects intact K18 together with fragments of 45, 26, 22, and 19 kD. In contrast, antibody 3055, which is specific for K18 phosphorylated on serine 53, detects only K18 and a 26-kD fragment generated by cleavage at VEVD238.
of caspase-3, K18 was preferentially cleaved to a 26-kD fragment (Fig. 6 B, lanes 2 and 4), but in the presence of either caspase-3 (Fig. 6 B, lane 6) or caspase-7 (Fig. 6 A, lane 3), the additional 19-kD product was also observed. Pretreatment of cells with z-VAD.FMK (10–50 μM) inhibited apoptosis and cleavage of both K18 and P-K18 in a concentration-dependent manner (Fig. 6 B, lanes 7–9). A most complete inhibition was observed at the highest concentration (Fig. 6 B, lanes 7–9), which is consistent with the ability of z-VAD.FMK to inhibit the proteolytic activity of all known caspases (Garcia-Calvo et al., 1998). YVAD.CMK (10–50 μM), which caused only a slight inhibition of apoptosis (Fig. 6 B), did not markedly inhibit the processing of K18 or P-K18 (Fig. 6 B, lanes 10–12). In contrast, in untransfected MCF-7 cells exposed to TRAIL, YVAD.CMK (25 μM) completely inhibited formation of the 26- and 19-kD cleavage products of K18 (data not shown), which is consistent with previous reports that cleavage of K18 at VEVD↓A, is primarily mediated by caspase-6. Thus, the inability of YVAD.CMK to significantly inhibit K18 cleavage in the presence of exogenously transfected caspase-3 or -7 (Fig. 6 B) is consistent with cleavage of K18 occurring, in this instance, at both the caspase-3 and/or -7 preferred site, DALD↓S and the VEVD↓A site normally preferred by caspase-6.

Figure 7. TRAIL induces spheroidal inclusions in HBL100 cells. (A) Untreated adherent HBL100 cells display a regular nucleus (N) and are devoid of cytoplasmic inclusions. (B) After exposure to TRAIL for 7 h, detached cells show characteristic apoptotic nuclear morphology and small spheroidal cytoplasmic inclusions (black arrowheads). (C and D) These inclusions (black arrowheads) contain cleaved K18 as shown by immunogold labeling with antibody M30. The inset in C is shown at a higher magnification in D. Bars, 1 μm.
Redistribution of Cleaved Cytokeratins into Spheroidal Inclusions Is a Characteristic Feature of Epithelial Cells Undergoing Apoptosis

We wished to determine whether the spheroidal inclusions observed in transfected or untransfected MCF-7 cells exposed to TRAIL (Figs. 4 and 5) were also evident in other epithelial cells undergoing apoptosis. HBL 100, breast epithelial cells, and A 549, type II lung cells, were exposed to TRAIL and examined by ultrastructural immunocytochemistry. Sections through untreated cultures of HBL 100 (Fig. 7 A) and A 549 (Fig. 8 A) cells showed a predominance of large adherent cells with regular nuclear profiles. Treatment of these cells with TRAIL resulted in an increased incidence of apoptotic cells, characterized by detachment, rounding-up and chromatin margination (Figs. 7 B and 8 B). The hypercondensed mitochondria observed in apoptotic MCF-7 cells (Fig. 4) and apoptotic THP.1 cells (Dinsdale et al., 1999) were not observed in either HBL 100 or A 549 cells, suggesting that these changes are only associated with the apoptotic phenotype of certain cells.

Apoptotic HBL 100 cells were also characterized by the presence of small cytoplasmic inclusions (<1 µm diam) with an indistinct outline. These inclusions were labeled by antibodies to both intact (Table I) and cleaved K18 (Fig. 7, Figure 8 TRAIL induces spheroidal inclusions in A 549 cells. (A) Untreated adherent A 549 cells display a regular nucleus (N) and are devoid of cytoplasmic inclusions. (B) A fter exposure to TRAIL for 3 h, many detached cells display a characteristic apoptotic nuclear morphology and contain a range of spheroidal cytoplasmic inclusions (black arrowheads). (C) These inclusions contain K18 as shown by immunogold labeling. For clarity, only one inclusion/cell is marked (black arrowheads). (D) These inclusions (black arrowheads) also contain cleaved K18 as shown by immunogold labeling with antibody M30. Bars: (A, B, and D) 1 µm; (C) 10 µm.
In A549 cells exposed to TRAIL, many more and larger cytoplasmic inclusions were observed, and these were also labeled by both antibodies (Table I and Fig. 8, C and D). Thus, redistribution and cleavage of the cytokeratin network into cytoplasmic inclusions is a characteristic feature of a number of epithelial cell types undergoing apoptosis.

**Trail-induced Colocalization of Active Endogenous Caspase-3 with Cleaved Cytokeratin 18 in Apoptotic Epithelial Cells**

The recently characterized M30 antibody recognizes cleaved K18 in apoptotic cells, but does not recognize uncleaved K18 in viable or necrotic cells (Leers et al., 1999). In both untransfected MCF-7 and HBL100 cells exposed to TRAIL, M30 immunoreactivity was present only in apoptotic cells and localized in discrete cytoplasmic inclusions (Fig. 9, A and B). Similarly, the CM1 antibody recognizes only the p18 subunit but not the zymogen precursor of caspase-3 (Srinivasan et al., 1998). CM1 immunoreactivity was observed only in TRAIL-induced apoptotic HBL100 cells but not in nonapoptotic cells (Fig. 9 C). These studies confirmed the use of both these antibodies for specifically labeling apoptotic cells. Interestingly, both M30 (Fig. 9, A, B, D, and G) and CM1 (Fig. 9, C, E, and H) immunolabeling exhibited a discrete punctate cytoplasmic distribution in apoptotic cells. Furthermore, when apoptotic cells were colabeled with CM1 and M30 antibodies, the majority of active caspase-3 colocalized with the cytokeratin-containing inclusions (Fig. 9, D–I). These results demonstrated the redistribution and colocalization of both cleaved K18 and active endogenous caspase-3 into small cytoplasmic inclusions in both HBL100 and A549 cells undergoing apoptosis.

**Discussion**

**TRAIL Initiates a Caspase Cascade with Caspase-8 at the Apex**

Our demonstration of an early cleavage of caspase-8 and Bid, before activation of caspases-3 and -7, in MCF-7 cells (Fig. 1), strongly supports the hypothesis that caspase-8 is the most apical caspase in TRAIL-induced apoptosis. Activation of caspases-8 and -3 also has been observed in melanoma cells exposed to TRAIL (Griffith et al., 1998). Activated caspase-8 may either directly activate effector caspases or, via cleavage of Bid, lead to the involvement of mitochondria, indirect activation of caspase-9, and downstream effector caspases. Cleavage of Bid, a caspase-8 substrate, only previously has been reported in CD95 and TNF-α-induced apoptosis (Li et al., 1998). However, our data...
now demonstrate the participation of Bid in TRAIL-induced apoptosis. Recent studies using Bid-deficient mice have confirmed that Bid is a critical substrate for in vivo signaling by death receptor agonists, thereby mediating a mitochondrial amplification loop that is essential for apoptosis in selected cells (Yin et al., 1999).

After TRAIL-induced apoptosis, the initiator caspase-8 redistributed within the cytosol into discrete punctate structures (Fig. 2), whereas the effector caspases-3 and -7 redistributed into large spheroidal inclusions (Figs. 2 and 3). These differences between initiator and effector caspases further emphasize their fundamentally different roles in receptor-mediated apoptosis in epithelial cells. In mouse liver, caspases-3 and -7 are initially cytosolic but, after CD95-induced apoptosis, show a differential subcellular redistribution (Chandler et al., 1998). The proform of caspase-3 also has been found primarily in the cytosol together with small amounts in the mitochondria of several cell types from rat and human tissues (Mancini et al., 1998; Samali et al., 1998; Zhivotovsky et al., 1999). However, in the present study, no colocalization of caspase-3-GFP with the mitochondria of MCF-7 cells was observed (Fig. 3), which is in agreement with the finding that the ratio of cytosolic to mitochondrial procaspase-3 varies between different tissues (Samali et al., 1998).

**Effector Caspases Promote an Exaggerated Apoptotic Phenotype**

MCF-7 cells are caspase-3 null (F. Li et al., 1997; Jänıcke et al., 1998) and, therefore, provide a good model to dissect the apoptotic pathways for which caspase-3 activation is necessary. A apoptosis in most cell types is not solely dependent on caspase-3, as mice with a homozygous deletion in the CASP-3 gene only exhibit hyperplasia and significant abnormalities in the brain (Kuida et al., 1996; Woo et al., 1998). Based on studies using caspase-3-null cells, it has been proposed that caspase-3 activity is required for DNA fragmentation and membrane blebbing (Jänıcke et al., 1998; Zheng et al., 1998), but is not essential for induction of apoptosis by either CD95 or TNF (F. Li et al., 1997). In the current study, the intermediate stage of chromatin condensation observed in MCF-7 cells exposed to TRAIL (Fig. 4) can be attributed to a partial fragmentation of DNA into kilobase pair fragments in the absence of DNA ladders (Brown et al., 1993). Overexpression of caspase-3 or -7, but not caspase-8, in cells exposed to TRAIL resulted in a more exaggerated apoptotic phenotype with larger cytokeratin-containing cytoplasmic inclusions and more pronounced nuclear changes (Figs. 4 and 5). These results are consistent with cleavage by effector, but not initiator, caspases of intracellular substrates, such as DFF (Liu et al., 1997) and cytokeratins, with appropriate motifs (e.g., DxxD). This further emphasizes the marked differences between initiator and effector caspases.

**TRAIL Induces the Redistribution of Effector Caspases into Cytokeratin-associated Spheroidal Inclusions**

Cytokeratins comprise the major intermediate filaments in the cytoplasm of epithelial cells and help to maintain cellular integrity. Disintegration of these filaments, resulting in the concentration of cytokeratins within cytoplasmic inclusions, has been reported in vivo in the Mallory bodies of injured hepatocytes and also in vitro in mitotic epithelial cells (Franke et al., 1982). Similar inclusions are observed in adenovirus-infected HeLa cells after cleavage of K18 by the viral L3 proteinase (Chen et al., 1993). The significance of cytokeratin reorganization during apoptosis only recently has been examined (van Engeland et al., 1997). The redistribution of cytokeratins into granular structures enriched for phosphorylated K18 has been reported in apoptotic cells (Caulin et al., 1997; Ku et al., 1997). Primarily based on studies with recombinant proteins, this redistribution has been proposed to be dependent on cleavage of type I keratins by effector caspases. Interestingly, phosphorylation of K18 at serine 53 does not render K18 a better substrate for effector caspases (Caulin et al., 1997; Ku et al., 1997).

Our biochemical and ultrastructural data provide new insight on the fate of cytokeratins during apoptosis. On exposure to TRAIL, the cytokeratin bundles characteristic of control cells (Fig. 5 B, inset) reorganized into filament sheaves found either free in the cytoplasm or associated with the periphery of small inclusions containing K8, -18, and -19 (Figs. 4, B and C, and 5 B). Cytokeratin disassembly was primarily mediated by effector caspases, as these inclusions were larger and more pronounced in MCF-7 cells in the presence of exogenously expressed caspases-3 or -7. Both the inclusions and the filament sheaves contained the phosphorylated form of K18, whereas commumunogold labeling with the M30 antibody demonstrated the presence of cleaved K18 only in the spheroidal inclusions (Fig. 5). These data are compatible with initial phosphorylation of K18 and the formation of filament sheaves, followed by cleavage of K18 at DALD397S, which results in the transformation of these sheaves into inclusions. However, we cannot exclude the possibility that the filament sheaves may contain K18 cleaved at other sites, such as VEVD238LA, which would not be recognized by the M30 antibody.

**Cytokeratin-associated Spheroidal Inclusions Are a Characteristic Feature of Epithelial Cell Apoptosis**

Particularly striking was the finding of spheroidal inclusions in TRAIL-treated epithelial cells. While initially observed in MCF-7 cells transfected with caspases-3 or -7, they were subsequently observed in a variety of untransfected TRAIL-treated epithelial cells from the breast (MCF-7 and HBL100), lung (A549), and cervix (HeLa; Figs. 4, 7, and 8, and data not shown). These inclusions contained cleaved K18, which, in both A549 and HBL100 cells, colocalized with active endogenous caspase-3 (Fig. 9). Interestingly, the inclusions were more prevalent in untransfected HBL100 and A549 than in MCF-7 cells most probably because the A549 and HBL100 cells possess sufficient caspase-3 to cleave cytokeratins and form cytoplasmic inclusions. The formation of such inclusions appears to be a general feature of TRAIL-induced apoptosis of epithelial cells. These observations raise the question as to why such inclusions are formed and why active effector caspases are sequestered within them. A ctive caspases may be required to ensure the ordered dismantling of the intermediate fila-
ment network in epithelial cells undergoing apoptosis. Ef-
fect caspases cleave several other cytoskeletal proteins
including nuclear lamins, fodrin, and actin together with
the cytoskeletal regulators Gas2 and gelsolin (Thornberry
and Lazebnik, 1998); thus, reflecting the requirement for
assembly of the cytoskeleton during the execution
phase of apoptosis. Alternatively, the sequestration and
subsequent degradation of active effector caspases may
prevent their escape from the cell and, therefore, their
ability to damage healthy neighboring cells. This latter
suggestion is compatible with the observation that apop-
tosis in tissues is normally limited to individual scattered
(Arends and Wyllie, 1991). Sequestration of active ef-
cessor caspases may be an important component of the
cell death program required to prevent the spread of cell
damage before removal of the apoptotic cell by phagocytosis.
We demonstrate that cytochrome disassembly and the
formation of caspase-associated spheroidal inclusions occurs
after TRAIL-induced apoptosis of lung, cervical, and non-
transformed breast epithelial cells. Therefore, we propose
that this phenomenon may be a characteristic feature of
epithelial cells undergoing apoptosis.

We thank the following for supplying us with valuable reagents: E. Al-
 nemri for caspase-3, -7 and -8 cDNA templates; D. Green for caspase-9
antibody; M. Jattella for MCF-7-Fas cells; D. Nicholson for caspase-3 anti-
obody; B. Omary for antibody 3055; A. Srinivasan for CM1 antibody; and
Wang for Bid antibody. We are grateful to Judy McWilliam and Tim
Smith for preparation of samples for electron microscopy.

Submitted: 1 June 1999
Revised: 4 February 2000
Acepted: 4 February 2000

References
Arends, M. J., and A. H. Wyllie. 1991. Apoptosis: mechanisms and roles in
pathology. Int. Rev. Exp. Path. 32:223–254.
Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signalling and modu-
lation. Science. 281:1305–1308.
Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involve-
mation of caspase-associated spheroidal inclusions occurs after TRAIL-induced apoptosis of lung, cervical, and non-
transformed breast epithelial cells. Therefore, we propose
that this phenomenon may be a characteristic feature of
epithelial cells undergoing apoptosis.

We thank the following for supplying us with valuable reagents: E. Al-
 nemri for caspase-3, -7 and -8 cDNA templates; D. Green for caspase-9
antibody; M. Jattella for MCF-7-Fas cells; D. Nicholson for caspase-3 anti-
obody; B. Omary for antibody 3055; A. Srinivasan for CM1 antibody; and
Wang for Bid antibody. We are grateful to Judy McWilliam and Tim
Smith for preparation of samples for electron microscopy.

Submitted: 1 June 1999
Revised: 4 February 2000
Acepted: 4 February 2000

References
Arends, M. J., and A. H. Wyllie. 1991. Apoptosis: mechanisms and roles in
pathology. Int. Rev. Exp. Path. 32:223–254.
Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signalling and modu-
lation. Science. 281:1305–1308.
Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involve-
mation of caspase-associated spheroidal inclusions occurs after TRAIL-induced apoptosis of lung, cervical, and non-
transformed breast epithelial cells. Therefore, we propose
that this phenomenon may be a characteristic feature of
epithelial cells undergoing apoptosis.

We thank the following for supplying us with valuable reagents: E. Al-
 nemri for caspase-3, -7 and -8 cDNA templates; D. Green for caspase-9
antibody; M. Jattella for MCF-7-Fas cells; D. Nicholson for caspase-3 anti-
obody; B. Omary for antibody 3055; A. Srinivasan for CM1 antibody; and
Wang for Bid antibody. We are grateful to Judy McWilliam and Tim
Smith for preparation of samples for electron microscopy.

Submitted: 1 June 1999
Revised: 4 February 2000
Acepted: 4 February 2000

References
Arends, M. J., and A. H. Wyllie. 1991. Apoptosis: mechanisms and roles in
pathology. Int. Rev. Exp. Path. 32:223–254.
Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signalling and modu-
lation. Science. 281:1305–1308.
Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involve-
mation of caspase-associated spheroidal inclusions occurs after TRAIL-induced apoptosis of lung, cervical, and non-
transformed breast epithelial cells. Therefore, we propose
that this phenomenon may be a characteristic feature of
epithelial cells undergoing apoptosis.
Sheridan, J. P., S. A. Marsters, R. M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C. L. Gray, K. Baker, W. I. Wood, et al. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 277:818–821.

Srinivasan, A., K. A. Roth, R. O. Sayers, K. S. Shindler, A. M. Wong, L. C. Fritz, and K. J. Tomaselli. 1998. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ. 5:1004–1016.

Srinivasula, S. M., M. Ahmad, T. Fernandes-Alemri, G. Litwack, and E. S. Alnemri. 1996. Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/CE-like cysteine proteases. Proc. Natl. Acad. Sci. USA. 93:14486–14491.

Sun, X.-M., M. MacFarlane, J. Zhuang, B. B. Wolf, D. R. Green, and G. M. Cohen. 1999. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem. 274:5053–5060.

Takahashi, A., E. S. Alemri, Y. A. Lazebnik, T. Fernandes-Alemri, G. Litwack, R. D. Moor, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. 1996. Cleavage of lamin A by Mch2 but not CPP32: multiple interleukin 1p-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc. Natl. Acad. Sci. USA. 93:8395–8400.

Thornberry, N. A., and Y. Lazebnik. 1998. Caspases: enemies within. Science. 281:1312–1316.

van Engeland, M., H. J. H. Kuijpers, F. C. S. Ramaekers, C. P. M. Rieteling-Spender, and B. Schutte. 1997. Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp. Cell Res. 235:421–430.

Walczak, H., R. E. Miller, K. A. Riall, B. Gliniak, T. S. Griffith, M. J. Lin, W. Chin, J. J. Jones, A. Woodward, T. L. E. et al. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. 5:157–163.

Wang, K., X. -M. Yin, D. T. Chao, C. L. Milliman, and S. J. Korsmeyer. 1996. BID: a novel BH3 domain-only death agonist. Genes Dev. 10:2859–2860.

Woo, M., R. Hakem, M. S. Soengas, G. S. Duncan, A. Shahinian, D. K. K. A. Hakem, M. McCurrach, W. Kho, S. A. Kaufman, et al. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12:806–819.

Yin, X.-M., K. Wang, A. Gross, Y. Zhao, S. Zinkel, B. Klocke, K. A. Roth, and S. J. Korsmeyer. 1999. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature. 400:886–891.

Zheng, T. S., S. F. Schlosser, T. D. A. Ho, R. Hingorani, I. N. Crispe, J. L. Boyer, and R. A. Flavell. 1998. Caspase-3 controls both cytoplasmic and nuclear events associated with Fas-mediated apoptosis in vivo. Proc. Natl. Acad. Sci. USA. 95:13618–13623.

Zhivotovsky, B., A. Samali, A. Gahm, and S. Orrenius. 1999. Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ. 6:644–651.