Inhibition of Ced-3/ICE-related Proteases Does Not Prevent Cell Death Induced by Oncogenes, DNA Damage, or the Bcl-2 Homologue Bak

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Abstract. There is increasing evidence for a central role in mammalian apoptosis of the interleukin-1β–converting enzyme (ICE) family of cysteine proteases, homologues of the product of the nematode "death" gene, *ced-3.* Ced-3 is thought to act as an executor rather than a regulator of programmed cell death in the nematode. However, it is not known whether mammalian ICE-related proteases (IRPs) are involved in the execution or the regulation of mammalian apoptosis. Moreover, an absolute requirement for one or more IRPs for mammalian apoptosis has yet to be established. We have used two cell-permeable inhibitors of IRPs, Z-Val-Ala-Asp.fluoromethylketone (ZVAD.fmk) and *t*-butoxy carbonyl-Asp.fluoromethylketone (BD.fmk), to demonstrate a critical role for IRPs in mammalian apoptosis induced by several disparate mechanisms (deregulated oncogene expression, ectopic expression of the Bcl-2 relative Bak, and DNA damage–induced cell death). In all instances, ZVAD.fmk and BD.fmk treatment inhibits characteristic biochemical and morphological events associated with apoptosis, including cleavage of nuclear lamins and poly-(ADP-ribose) polymerase, chromatin condensation and nucleosome laddering, and external display of phosphatidylserine. However, neither ZVAD.fmk nor BD.fmk inhibits the onset of apoptosis, as characterized by the onset of surface blebbing; rather, both act to delay completion of the program once initiated. In complete contrast, IGF-I and Bcl-2 delay the onset of apoptosis but have no effect on the kinetics of the program once initiated. Our data indicate that IRPs constitute part of the execution machinery of mammalian apoptosis induced by deregulated oncogenes, DNA damage, or Bak but that they act after the point at which cells become committed to apoptosis or can be rescued by survival factors. Moreover, all such blocked cells have lost proliferative potential and all eventually die by a process involving cytoplasmic blebbing.

Apoptosis is an innate cell suicide mechanism invoked in disparate situations, both physiological and pathological, to ablate unwanted, damaged, or potentially neoplastic cells. Apoptosis is classically defined by a characteristic series of morphological changes in the doomed cell, including membrane blebbing, cell shrinkage, chromatin condensation, and DNA cleavage, and culminating in fragmentation of the cell into membrane-bound apoptotic bodies whose surfaces express potent triggers for phagocytosis. A cell suicide program appears to have arisen early in metazoan evolution and its basal machinery has been substantially conserved. The key genetic protagonists of programmed cell deaths in the nematode worm, *Caenorhabditis elegans*, are two "killer" genes, *ced-3* and *ced-4*, that are required for the execution of the death program and *ced*-9, the "protector" gene, which mitigates the actions of the killer genes. *ced-9* and *ced-3* each have numerous mammalian homologues: *ced-9* is related structurally and functionally to the family of death-regulating genes of which the oncogene *bcl-2* is the prototype (Hengartner and Horvitz, 1994) and the *ced-3* product is structurally and functionally homologous to a class of mammalian cysteine proteases, of which the prototype is the interleukin-1β–converting enzyme (ICE)[1] (Yuan et al., 1993). To date, no vertebrate homologue for *ced-4* has been identified.

ICE was originally described as the cysteine protease required for the cleavage of pro-interleukin-1β at Asp^{116}-

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1. *Abbreviations used in this paper*: 4-OHT, 4-hydroxytamoxifen; BD.fmk, *t*-butoxy carbonyl-Asp.fluoromethylketone; FLICE, FADD-like ICE; ICE, interleukin-1β-converting enzyme; IGF, insulin-like growth factor; IRP, ICE-related protease; MACH, MORT1-associated CED-3 homologue; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly-(ADP-ribose) polymerase; RT, room temperature; ZVAD.fmk, benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl)-fluoromethylketone.

Ala^{117} to generate the active cytokine (Cerretti et al., 1992; Thornberry et al., 1992). ICE is but one of an emerging family of ICE-related proteases (IRPs), whose known members include Ich-1/Nedd-2 (Kumar et al., 1994; Wang et al., 1994), Ich-2/TX/ICE_{rel}II (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), CPP32/Apopain/Yama (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), Mch2 (Fernandes-Alnemri et al., 1995*a*), Mch3/ICE-LAP-3 (Duan et al., 1996*a*; Fernandes-Alnemri et al., 1995*b*), ICE-LAP-6 (Duan et al., 1996*b*), ICE_{rel}III/TY (Faucheu et al., 1995; Munday et al., 1995) and FADD-like ICE/MORT1-associated CEP-3 homologue (FLICE/MACH) (Boldin et al., 1996; Muzio et al., 1996). Of these, ICE, CPP32β, Mch2, and FLICE/MACH have been directly implicated in apoptosis: FLICE/MACH (Boldin et al., 1996; Muzio et al., 1996) and ICE (Enari et al., 1995; Kuida et al., 1995) are required for induction of apoptosis through the TNF-R1 and CD95/Fas signaling pathways. FLICE/MACH is directly activated upon ligand binding to either TNF-R1 or CD95/Fas receptors (Boldin et al., 1996; Muzio et al., 1996), and ICE activity is required (Enari et al., 1995; Los et al., 1995) for downstream activation of a CPP32β-like protease necessary for apoptosis to occur (Enari et al., 1996). CPP32β has also been implicated as a general component of the apoptotic program (Nicholson et al., 1995; Tewari et al., 1995) and is a component of prICE, a proteolytic extract derived from apoptotic chick cells that induces condensation and fragmentation of interphase nuclei (Lazebnik et al., 1995*a*). Mch2 cleaves nuclear lamins (Orth et al., 1996; Takahashi et al., 1996), an activity also present in prICE (Lazebnik et al., 1995*b*).

All members of the ICE-related protease family share a predilection for cleavage of their substrates after an aspartate residue at the P1, usually followed by a small residue at the P1′ position, and all are synthesised as proenzymes that are activated by cleavage at critical aspartate residues that themselves conform to the substrate consensus for ICE family proteases. Thus, the IRPs are presumed to exist within hierarchies of auto- and trans-cleavage. For example, proICE can be activated by auto-cleavage (Thornberry et al., 1992), and proCPP32β can be activated by ICE (Tewari et al., 1995) as well as by the cytotoxic T cell granule serine protease granzyme B (Darmon et al., 1995). ICE-related protease processing releases an NH_2-terminal prodomain of varying length in different IRPs and cleaves the remaining polypeptide to yield two subunits (p20 and p10 for human ICE) that form the active $(p20{:}p10)_2$ enzyme (Walker et al., 1994; Gu et al., 1995*b*). The larger subunit contains the catalytic cysteine, but both subunits are required for enzyme activity.

Some clues as to how activated IRPs trigger apoptosis come from identification of potential IRP targets. CPP32β cleaves poly-(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994; Gu et al., 1995*a*) and DNA-dependent protein kinase (Casciola Rosen et al., 1995), both involved in aspects of DNA damage sensing and repair, whereas Mch2 cleaves nuclear lamins (Orth et al., 1996; Takahashi et al., 1996). Other targets of various IRPs are the sterol regulatory element–binding proteins SREBP-1 and SREBP-2 (Wang et al., 1995), the 70-kD protein of the U1-snRNP (Casciola Rosen et al., 1994), PKCδ (Emoto et al., 1995), and various components of the cytoskeleton such as actin (Mashima et al., 1995) and Gas2, a component of the microfilament system (Brancolini et al., 1995). However, it is unclear which, if any, of these targets is responsible for the cell blebbing, condensation, and fragmentation that characterizes apoptosis.

In the nematode, genetic evidence favors the notion that the IRP Ced-3 is part of the "execution machinery" of nematode cell death since, in *ced-3*–deficient mutants, no cell deaths occur (Ellis and Horvitz, 1986). However, the increased complexity and potential hierarchical relationships between mammalian IRPs make it unclear which proteases might act as executors of apoptosis and which might serve to regulate those executors. It is also unclear whether certain mammalian IRPs comprise a "final and obligate common pathway" activated by all triggers of mammalian apoptosis.

One way to approach these questions is to use inhibitors of IRPs based upon the preferred peptide substrate specificity of these enzymes. In this paper, we use two broad-spectrum cell permeable IRP inhibitors—benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (ZVAD.fmk) and *t*-butoxy carbonyl-Asp.fluoromethylketone (BD.fmk)—to demonstrate that IRPs are involved in mammalian apoptosis induced by disparate signals. Most importantly, however, we show that inhibition of IRPs blocks only part of the classical apoptotic program, generating doomed cells that are arrested before the program has completed.

Materials and Methods

Cell Culture and Cell Lines

Rat-1 fibroblasts expressing the β-oestradiol and the 4-hydroxytamoxifen (4-OHT)–dependent conditional alleles of c-Myc (Rat-1/c-MycER and Rat-1/c-MycER™, respectively) were isolated as described (Littlewood et al., 1995). Rat-1 fibroblasts expressing Bak from a Gal-4 element under the control of the 4-OHT–activatable chimeric GalER-VP16 transcription factor (Braselmann et al., 1993) have also been described (Rat-1/Bak cells) (Chittenden et al., 1995), as have Rat-1/c-MycER cells constitutively expressing Bcl-2 (Rat-1/c-MycER/Bcl-2) (Fanidi et al., 1992). A further Rat-1 cell line, constitutively expressing the adenovirus E1A (12S) protein (Rat-1/E1A), was isolated by infection of Rat-1 cells with the recombinant retrovirus pBabeNEO containing the entire open reading frame of 12S E1A under the control of the virus LTR and subsequent selection and cloning. Expression of 12S E1A was verified by immunoblotting. Rat-1 cells expressing E1A (12S) or empty vector controls were washed twice in serum-free medium and subsequently cultured in serum-free conditions. Cells were observed for 48 h using time lapse videomicroscopy. Rat-1/E1A cells exhibited marked apoptosis compared to controls demonstrating the ability of E1A to induce apoptosis in the absence of serum. Rat-1/c-MycER™, Rat-1/c-MycER/Bcl-2, and Rat-1/E1A cell lines were maintained in DME supplemented with 10% FCS and 1 mg/ml gentamicin. Rat-1/Bak cells were maintained in phenol-red–free DME supplemented with 10% charcoal-dextran–stripped FCS and 1 mg/ml gentamicin. Cells were selected using puromycin at 5 μg/ml for Rat-1/c-MycER™, neomycin at 1 mg/ml for Rat-1/E1A cells, and with puromycin at 5 μg/ml and neomycin at 1 mg/ml for Rat-1/Bak cells. Cells were passaged by standard trypsinization and seeded directly onto tissue culture plastic. Cells were serum-depleted by culture in 0.05% FCS for 48 h. Both Myc and Bak expression were induced by addition of 4-OHT to the cell cultures to a final concentration of 100 nM.

Reagents and Antibodies

4-OHT was obtained from SEMAT (St. Albans, UK). A 1 mM stock solution in 100% ethanol was prepared, stored at −20°C, and diluted in DME as required. ZVAD.fmk and BD.fmk were obtained from Enzyme Sys-

tems Products (Dublin, CA). Stock solutions in DMSO were prepared at 50 mM, stored at −80°C, and used as required. Etoposide/VP16, phalloidin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Poole, UK). FITC-labeled annexin V was obtained from Bender & Co. GmbH (Vienna, Austria). Secondary antibodies and enhanced chemiluminescence were obtained from Amersham International (Amersham, UK).

Polyclonal antibody to adenovirus E1A protein was the gift of Dr. N. Jones, Imperial Cancer Research Fund, London, UK (ICRF). Polyclonal antibody to PARP (C-2-10) was the gift of Dr. W. Earnshaw (Edinburgh University, Scotland, UK). The anti–lamins A and C antibody (131C3) was the gift of Dr. Y. Raymond (Institut du Cancer de Montreal, Montreal, Canada), together with the control antibody MOPC 21C. The actin antibody was the gift of Dr. Fulvia Verde, ICRF.

Time-Lapse Videomicroscopy

Time-lapse videomicroscopic images were acquired on an inverted phase contrast microscope (Diaphot; Nikon, Inc., Mellville, NY) equipped with epifluorescence and collected on either BetaCam or sVHS video tape under the control of an external animation controller (EOS Electronics AV Ltd., Barry, Wales, UK) as described (Evan et al., 1992). Apoptotic cell deaths were scored at the point when they were fully detached and rounded. Cells with the "blebbing" phenotype were deemed to be alive while they remained adherent and actively blebbing and similarly scored as dead once fully detached and rounded.

Western Blotting

Cleavage of poly(ADPribose) polymerase, nuclear lamins A and C, and actin was analyzed by SDS-PAGE and subsequent transfer to nitrocellulose membrane (Immobilon P, Amersham International). Membranes were blocked in TNT buffer (1% TBS, 0.5% Tween 20, and 5% skimmed milk) for 30 min and then incubated in antibodies to PARP (Lamarre et al., 1986), actin, or lamins A and C antibody at 4°C for 12 h in TNT buffer. Membranes were washed twice in TNT and then incubated for 1 h at room temperature (RT) with anti–mouse or anti–rabbit HRP-conjugated secondary antibody as appropriate. Finally, membranes were washed twice in TNT buffer and once in TBS and 0.5% Tween 20, and bound antibodies were visualized by enhanced chemiluminescence.

Electron Microscopy and Immunocytochemistry

Standard electron microscopic techniques were used as follows. Cells were pelleted, fixed for 1 h at RT in 2.5% glutaraldehyde in Sorensens buffer, postfixed in osmium tetroxide in the same buffer, and embedded in Araldite resin. Sections were cut on a Reichert ultracut, stained with uranyl acetate and lead citrate, and viewed on an electron microscope (EM 10 CR; Carl Zeiss, Inc., Thornwood, NY). PARP expression and localization was determined immunocytochemically as previously described (Lamarre et al., 1986). Briefly, cells were grown on 8-well chamber slides and fixed with 100% methanol for 10 min at −20°C. Fixed cells were incubated with primary antibody for 60 min at RT in PBS followed by five washes in PBS buffer containing 0.1% Tween 20. After incubation in appropriate FITC-labeled secondary antibodies, cells were viewed using a confocal microscope (model MRC 1000; BioRad Labs, Hercules, CA). Lamins were identified immunocytochemically using anti–lamin A+C antibody (1:100), incubated with cells previously fixed as described above, and preblocked in 10% FCS and PBS. After three washes in PBS, cells were incubated with secondary antibody as described above. For actin staining, cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed in PBS at RT, and then permeabilized with 0.2% Triton in PBS for 5 min. Cells were then rinsed in PBS and incubated with 0.1 μg/ml FITC-phalloidin in PBS for 20 min at RT. Finally, cells were washed several times in PBS, mounted, and viewed as above.

Assays of Cell Viability

Mitochondrial dehydrogenase activity, used as a marker of cell survival, was assayed by the ability of cells to convert soluble MTT into an insoluble formazan reaction product (Mosmant, 1983). 10 μl of MTT solution (5 mg/ml in PBS) was added to each well of a 96-well plate containing 3,000 cells in 100 μl of medium. The reaction was stopped after 2 h at 37°C by removal of the supernatant and addition of 200 μl of acid propan-2-ol. The plates were left at RT for 15 min in the dark, and absorbance difference at 570–630 nm was measured using a microplate reader and % survival calculated as (Experiment − Blank)/(Control − Blank) × 100%, where the blank is medium + MTT without cells and the Control was the signal from wells with maximum cell survival, i.e., with serum and no 4-OHT.

Cell survival was also assayed by a direct microscopic assessment of the proportion of cells able to exclude the vital dye, trypan blue. Rat-1/c-MycER™ cells were grown in 3-cm tissue culture dishes, changed to serum-free medium for 48 h, and then 4-OHT ± ZVAD.fmk/BD.fmk was added (time 0). At the time points indicated, cells were trypsinized and washed in DME, and the number of trypan blue–positive cells was assessed by counting a minimum of 200 cells on a hemocytometer.

Flow Cytometric Analysis of Cells

Adherent cells were trypsinised, fixed in 70% ethanol, and stained with propidium iodide. Flow cytometric analysis was carried out on a Becton-Dickinson (Mountain View, CA) FACSstar plus.

Annexin V Binding to External Phosphatidyl Serine

Cells undergoing apoptosis in the presence or absence of ZVAD.fmk or BD.fmk were observed by time-lapse videomicroscopy. At the time that a significant proportion of the cells were either apoptoptic (no ZVAD.fmk/BD.fmk) or blebbing (plus ZVAD.fmk/BD.fmk), the culture was flooded with FITC–annexin V. The same microscopic field as had been observed thus far was then examined by fluorescence and phase microscopy to detect bound annexin V and examine cell morphology, respectively.

Results

ZVAD.fmk and BD.fmk, Inhibitors of ICE-related Proteases, Delay but Do Not Prevent Cell Death Induced by Multiple Signals

Apoptosis can be triggered by a wide range of influences, including oncogene deregulation, DNA damage, and expression of killer genes. To determine the general relevance of IRPs to apoptosis, we examined the effect of the cell-permeable IRP inhibitors, ZVAD.fmk and BD.fmk, upon apoptosis induced in Rat-1 fibroblasts by a number of agents. Apoptosis was induced by activation of a deregulated conditional allele of c-Myc (Littlewood et al., 1995), expression of the adenovirus protein E1A (Rao et al., 1992), DNA damage in combination with c-Myc expression using the topoisomerase II inhibitor etoposide (Fanidi et al., 1992), or expression of the killer member of the Bcl-2 family, Bak (Chittenden et al., 1995). Both E1A (Debbas and White, 1993) and etoposide (Lowe et al., 1993) induce apoptosis via a p53-dependent mechanism; c-Myc may act partly via p53 (Littlewood et al., manuscript in preparation) and Bak killing is p53 independent (T. Chittenden, unpublished data).

The Rat-1 fibroblast cell lines were induced, activated, or treated with drug, as appropriate, in the presence or absence of ZVAD.fmk or BD.fmk. Cells were monitored by time-lapse videomicroscopy and scored as dead when they became detached from the substratum. Both ZVAD.fmk and BD.fmk afforded excellent protection from apoptosis in all cases, regardless of the trigger (data for ZVAD.fmk shown in Fig. 1). Thus, all these triggers of apoptosis act via a ZVAD.fmk- or BD.fmk–sensitive agent, presumably one or more of the IRPs.

As shown in Fig. 1, the degree of protection afforded by ZVAD.fmk or BD.fmk is profound. Nonetheless, under the sustained pro-apoptotic influence of c-Myc, E1A, Bak, or continuous DNA damage, all cells were eventually observed to detach from the substratum, undergo partial

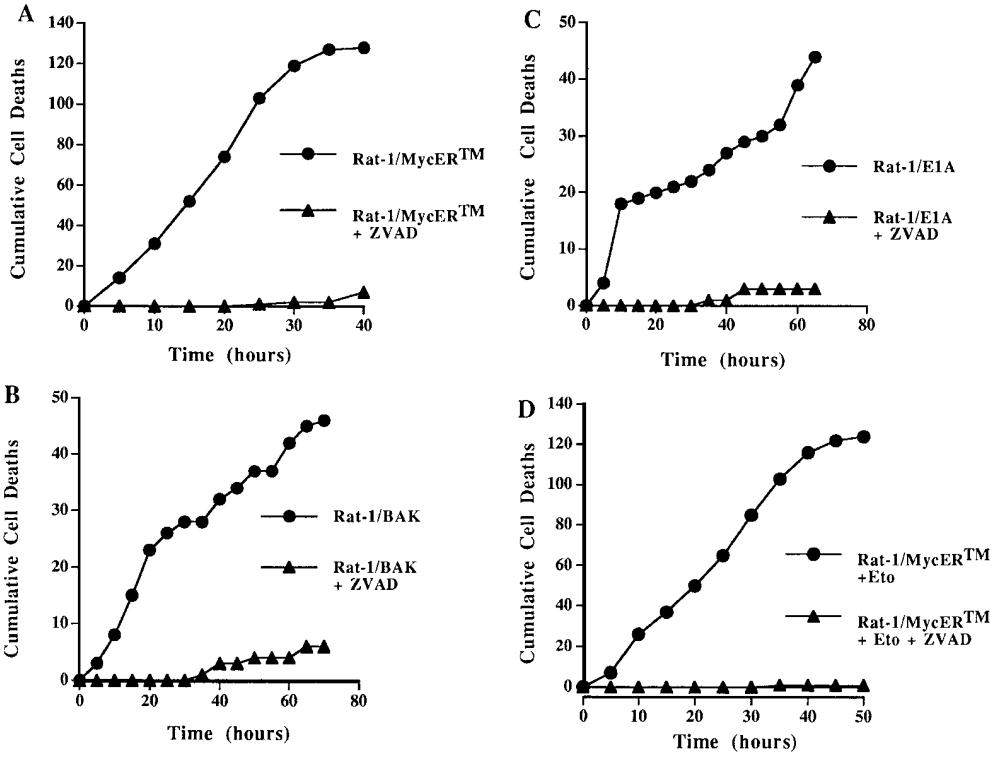


Figure 1. The cell-permeable inhibitors of ICE-related proteases, ZVAD.fmk and BD.fmk, suppress apoptosis induced by oncogenes, DNA damage, and Bak. Rat-1 cell cultures were observed by time-lapse videomicroscopy and images were collected at the rate of 12 frames/h. At the end of each 5-h period, the total number of apoptotic events (as determined by cell detachment) thus far was summed and plotted against time. Rates of apoptotic events in each culture are shown in the presence or absence of ZVAD.fmk (100 μM). Both ZVAD.fmk and tamoxifen (where applicable) were added at time 0. (*A*) Rat-1 cells expressing c-Myc-ER™ in the presence (▲) or absence (●) of ZVAD.fmk. c-Myc was activated by addition of 4-OHT (100 nM). (*B*) Rat-1 cells expressing Bak under the control of a GalER-VP16 promotor in the presence (▲) or absence (●) of ZVAD.fmk. The GalER-VP16 chimeric transcription factor was activated by addition of 4-OHT (to 100 nM), so inducing expression of Bak. (*C*) Rat-1 cells constitutively expressing the adenoviral E1A protein in the presence (▲) or absence (●) of ZVAD.fmk. (*D*) Rat-1 cells expressing c-MycER™ and treated with etoposide/VP16 (100 nM) in the presence (▲) or absence (●) of ZVAD.fmk. c-Myc was again activated by addition of 4-OHT (to 100 nM).

chromatin condensation, and eventually become permeable, even in the sustained presence of ZVAD.fmk or BD.fmk (see below). Neither higher levels of ZVAD.fmk or BD.fmk nor regular 24 hourly readdition of ZVAD.fmk or BD.fmk to the culture medium was able to delay the eventual demise of cells (data not shown), suggesting ZVAD.fmk and BD.fmk inhibition of cell death is eventually overwhelmed or bypassed. In all cases tested, the effects of ZVAD.fmk and BD.fmk were identical. Accordingly, primarily data for ZVAD.fmk is shown henceforth.

ZVAD.fmk Does Not Delay the Onset of Membrane Blebbing but Blocks Completion of the Apoptotic Program

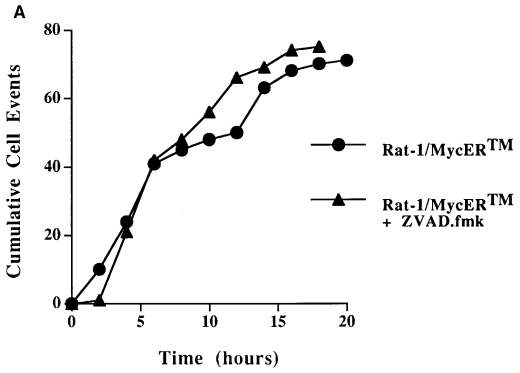
Time-lapse videomicroscopy permits observation both of the onset and the execution of individual apoptotic events within a cell culture. The onset of apoptosis is characterized by the sudden initiation of surface blebbing, together with cytoplasmic fragmentation and exfoliation, from which the name apoptosis derives. In fibroblastic cells, onset of blebbing is rapidly followed by nuclear condensation and collapse and total cell fragmentation, a process that typically takes between 30–60 min (Evan et al., 1992) (see Note Added in Proof for Web information). However, whereas an individual apoptotic event is rapid, individual cells within a culture, undergoing apoptosis induced by activation of c-Myc in low serum, demonstrate asynchronous initiation of apoptosis over a prolonged period. Since such apoptosis is cell cycle independent, the basis of this asynchronicity is unclear, although its incidence is greatly affected by levels of c-Myc protein and the presence of survival factors in the culture medium (Harrington et al., 1994).

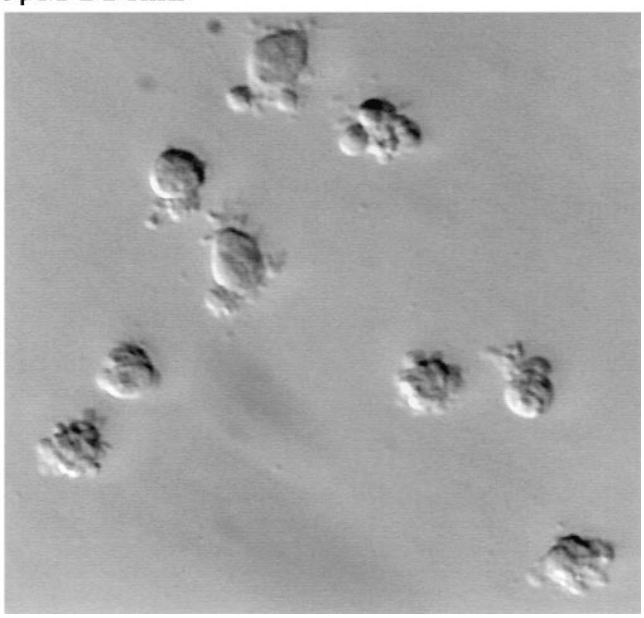
Using time-lapse videomicroscopy to observe cells undergoing c-Myc–induced apoptosis in the presence of either ZVAD.fmk or BD.fmk, we were surprised to find that neither inhibitor appears to delay the initiation of apoptosis within the cultures, as judged by the onset of blebbing. Indeed, the kinetics of onset of apoptotic events within ZVAD.fmk-treated cultures were superimposable on those of untreated cell cultures (Fig. 2 *A*). However, unlike untreated cells, ZVAD.fmk- or BD.fmk-treated cells did not rapidly condense and fragment but continued to bleb and boil for extended periods (Fig. 2 *B*). During this extended blebbing, often lasting days, cells remain adherent, exclude trypan blue, and exhibit physical activity, all suggestive of sustained metabolism (see Note Added in Proof for Web Information). This latter notion was supported by assaying mitochondrial activity of ZVAD.fmk-treated cells using the MTT assay, which showed continued mitochondrial dehydrogenase activity in such cells (Fig. 3 *A*).

Thus, ZVAD.fmk-treated cells appear to initiate apoptosis normally but then become arrested within the apoptotic program. Identical results were obtained when apoptosis was induced by E1A, etoposide, or Bak expression (data not shown).

Characterization of Apoptotic Cells Blocked with ZVAD.fmk

To investigate further the ZVAD.fmk-mediated block in the





death program, we examined a number of biochemical and morphological parameters of apoptosis in the ZVAD.fmk-treated blebbing cells. First, we examined the efficacy with which ZVAD.fmk inhibits intracellular IRPs by examining cleavage of known IRP substrates by a combination of Western blotting and confocal immunofluorescence microscopy. Cells were harvested from ZVAD.fmk-treated cultures in which 50–60% of cells exhibited the continuously blebbing phenotype as judged by time-lapse videomicroscopy (typically 24–36 h after induction of apoptosis), and cell lysates fractionated by SDS-PAGE and electroblotted. Blots were probed with antibodies to actin, lamins A+C, and PARP, all known IRP substrates (Lazebnik et al., 1994, 1995*b*; Gu et al., 1995*a*; Mashima et al., 1995; Oberhammer et al., 1994), and compared with equivalent lysates from control cultures without ZVAD.fmk. ZVAD.fmk inhibits cleavage of all proteins for up to 48 h (Fig. 4 *A*). We also examined the immunocytochemical distribution of actin, PARP, and lamin staining in cells with and without ZVAD.fmk treatment by immunofluorescence confocal microscopy (Fig. 4 *B*). The effect of ZVAD.fmk on actin distribution within cells was examined by staining permeabilized cells with phalloidin-FITC. This showed ZVAD.fmk-treated cells to be morphologically distinct from either log-phase viable or apoptotic cells, exhibiting very obvious cytoplasmic blebbing (Fig. 4 *B*). ZVAD.fmk treatment prevented the dispersal and reduction of PARP and lamin staining that is typically observed in apoptotic cells. Thus, as reported elsewhere (Fearnhead et al., 1995; Fletcher et al., 1995; Slee et al., 1996), ZVAD.fmk effectively inhibits intracellular cleavage of known IRP substrates.

Next, electron microscopy was performed on Rat-1/c-MycER™ cells induced to undergo apoptosis in the presence or absence of ZVAD.fmk (Fig. 5). 24 h after c-Myc activation in low serum, many cells in the non–ZVAD.fmk-treated population exhibited features of classical apoptosis, i.e., chromatin condensation, cell shrinkage, cytoplasmic blebbing, and cell fragmentation. In contrast, although cells from ZVAD.fmk-treated populations exhibited gross cytoplasmic blebbing and some dilatation of the endoplasmic reticulum, their organelle structure was otherwise well preserved. More remarkably, nuclei of ZVAD.fmk-treated cells resembled those of nonapoptotic cells, with reticular chromatin pattern bounded by intact nuclear lamina and membrane.

Figure 2. ZVAD.fmk and BD.fmk do not delay the onset of cell death, but block completion of apoptosis after the onset of membrane blebbing. (*A*) ZVAD.fmk does not delay onset of cell death but extends the kinetics of individual apoptotic events. Time-lapse videomicroscopic quantitation of onset of apoptosis induced in Rat-1/c-MycER™ cells by 4-OHT (100 nM) in the presence (▲) or absence (●) of ZVAD.fmk (to 100 μM). Time-lapse videomicroscopy was performed as before, but cells were scored for initiation of membrane blebbing. (*B*) Both ZVAD.fmk and BD.fmk show profound cytoplasmic blebbing 24 h after c-Myc induction in the absence of serum. Rat-1/c-MycER™ cells were serum starved for 48 h before addition of 4-OHT (100 nM) with and without 100 μM ZVAD.fmk or 5 μM BD.fmk. After 24 h, cells were examined by light microscopy using Hoffman optics. Both photographs show a representative field of blebbing cells treated with either ZVAD.fmk or BD.fmk.

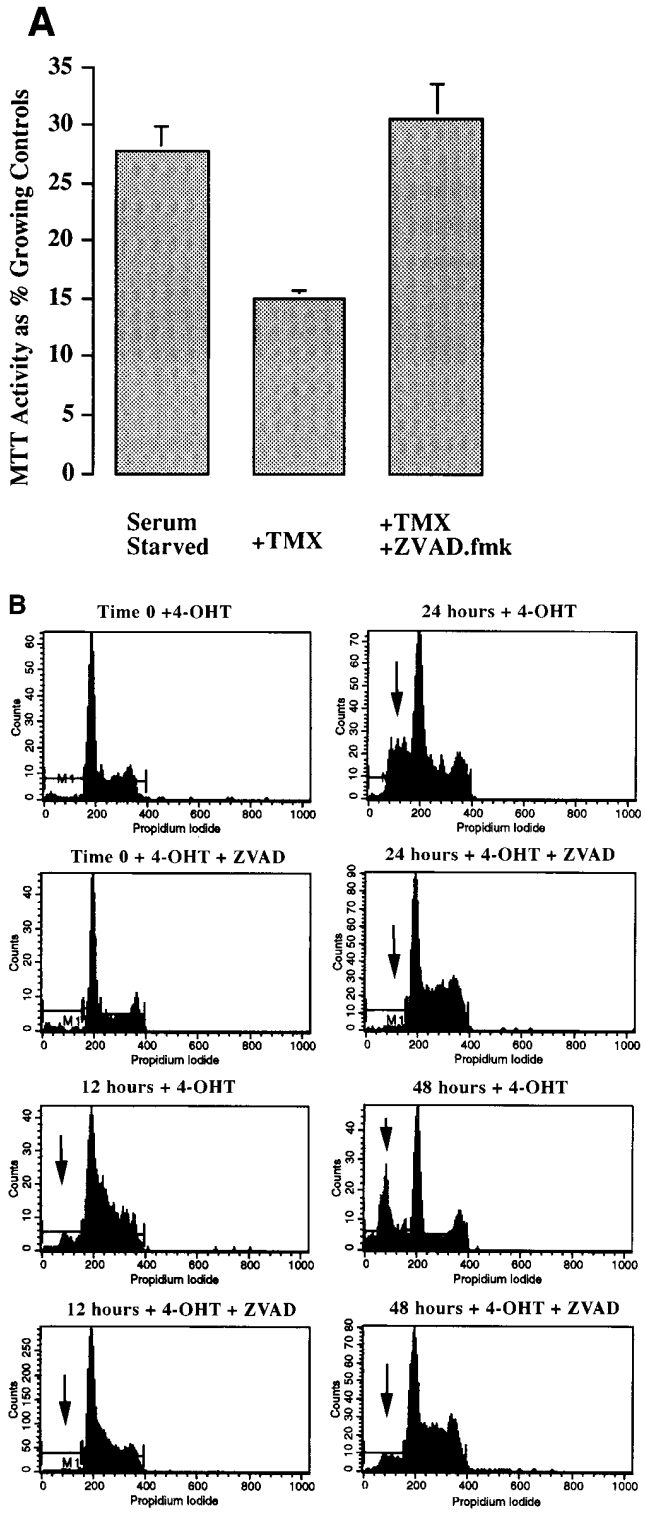


Figure 3. ZVAD.fmk delays the onset of characteristic markers of apoptosis. (*A*) ZVAD.fmk delays destruction of mitochondrial integrity. Rat-1/c-MycER™ cells were plated into 96-well plates in DME containing 10% FCS at a density of approximately 3,000 cells per well. After 48 h of culture, the medium was replaced with serum-free medium and the cells left a further 48 h. Cells were washed again, and the medium was replaced with either growth medium containg 100 nM 4-OHT or 4-OHT plus ZVAD.fmk, as indicated. Mitochondrial integrity was then assayed at various times by a photometric MTT assay. Bars represent means of quadruplicate culture wells ± SDs. Data shown are from a single representative experiment. (*B*) Flow cytometric analysis of Rat-1/c-MycER™ cells undergoing c-Myc–induced apoptosis in cells in the presence or absence of ZVAD.fmk. At time 0, 4-OHT ± ZVAD.fmk was added to serum-deprived cells. Cells were then harvested, at time points indicated, by trypsinization. Cells were fixed in ethanol, stained with propidium iodide, and examined by flow cytometry. The sub-G1 peak characteristic of apoptotic cells is absent from the ZVAD.fmk-treated populations.

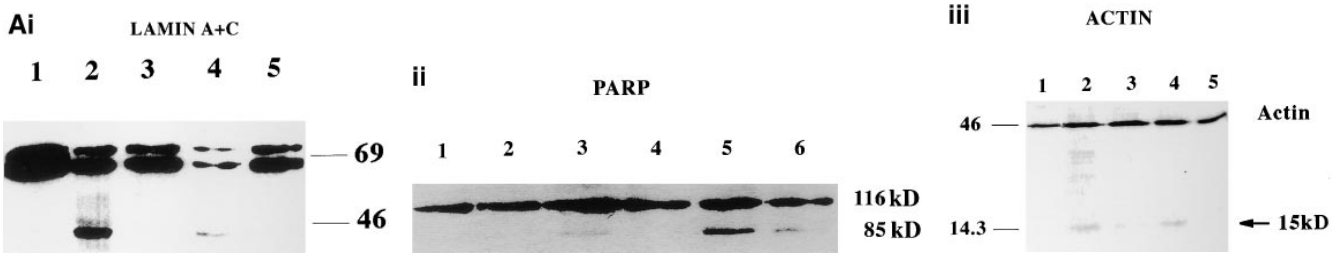
We next examined integrity of DNA in ZVAD.fmk-treated blebbing cells. Flow cytometric analysis demonstrated that ZVAD.fmk suppresses the appearance of cells with sub-G1 content of DNA characteristic of apoptotic cells (Fig. 3 *B*). Consistent with this, no degradation of DNA into typical oligonucleosomal fragments (Wyllie, 1980) was detected in DNA extracted from ZVAD.fmk-treated cells up to 48 h after c-Myc activation (data not shown).

Apoptosis is classically associated with loss of membrane phospholipid asymmetry: inactivation of a critical "flippase" results in expression of phosphatidylserine on the cell surface, a signal for recognition and ingestion of apoptotic cells by macrophages (Fadok et al., 1992). Surface phosphatidylserine expression was assessed by binding of FITC-labeled annexin V and visualized by fluorescence microscopy (Koopman et al., 1994). Rat-1/c-MycER™ cells undergoing normal apoptosis exhibited annexin V binding, whereas no binding was detected on blebbing cells blocked with ZVAD.fmk or live cells (Fig. 6). Thus, ZVAD.fmk blocks apoptosis before destruction of membranes, mitochondria, or loss of phosphatidylserine asymmetry.

As discussed above, ZVAD.fmk-treated cells induced to undergo apoptosis and exhibiting the blebbing phenotype for several days do eventually detach and become permeable to vital dyes. Analysis of these "late-stage" ZVAD.fmk-treated cells revealed a ragged surface membrane, vacuolated cytoplasm, partial chromatin condensation (although less than in "normal" apoptotic cells), and eventual membrane permeability (data not shown). We conclude that these cells appear to have undergone a partial apoptotic cell death, although this will require further characterization.

ZVAD.fmk Inhibits Apoptosis Downstream of the Antiapoptotic Cytokine IGF-1 or the Apoptosis Suppressor, Bcl-2

Both cytokines (Harrington et al., 1994) and Bcl-2 (Fanidi et al., 1992) are effective at suppressing fibroblast apoptosis, which is most evident as a delay in onset of apoptosis, with the extent of the delay depending upon levels expression of c-Myc and the abundance of IGF-1 or Bcl-2 (Evan et al., 1992; Fanidi et al., 1992; Harrington et al., 1994). Intriguingly, time-lapse videomicroscopic analysis reveals that, while IGF-1 and Bcl-2 delay the onset of apoptosis (as judged by the start of membrane blebbing), they do not inhibit completion of the program once initiated. Cells proceed from blebbing to final fragmentation within 20–60 min, essentially the same kinetics as cells cultured in the absence of IGF-1 or overexpression of *bcl-2* (Fig. 7, *A* and *B*).



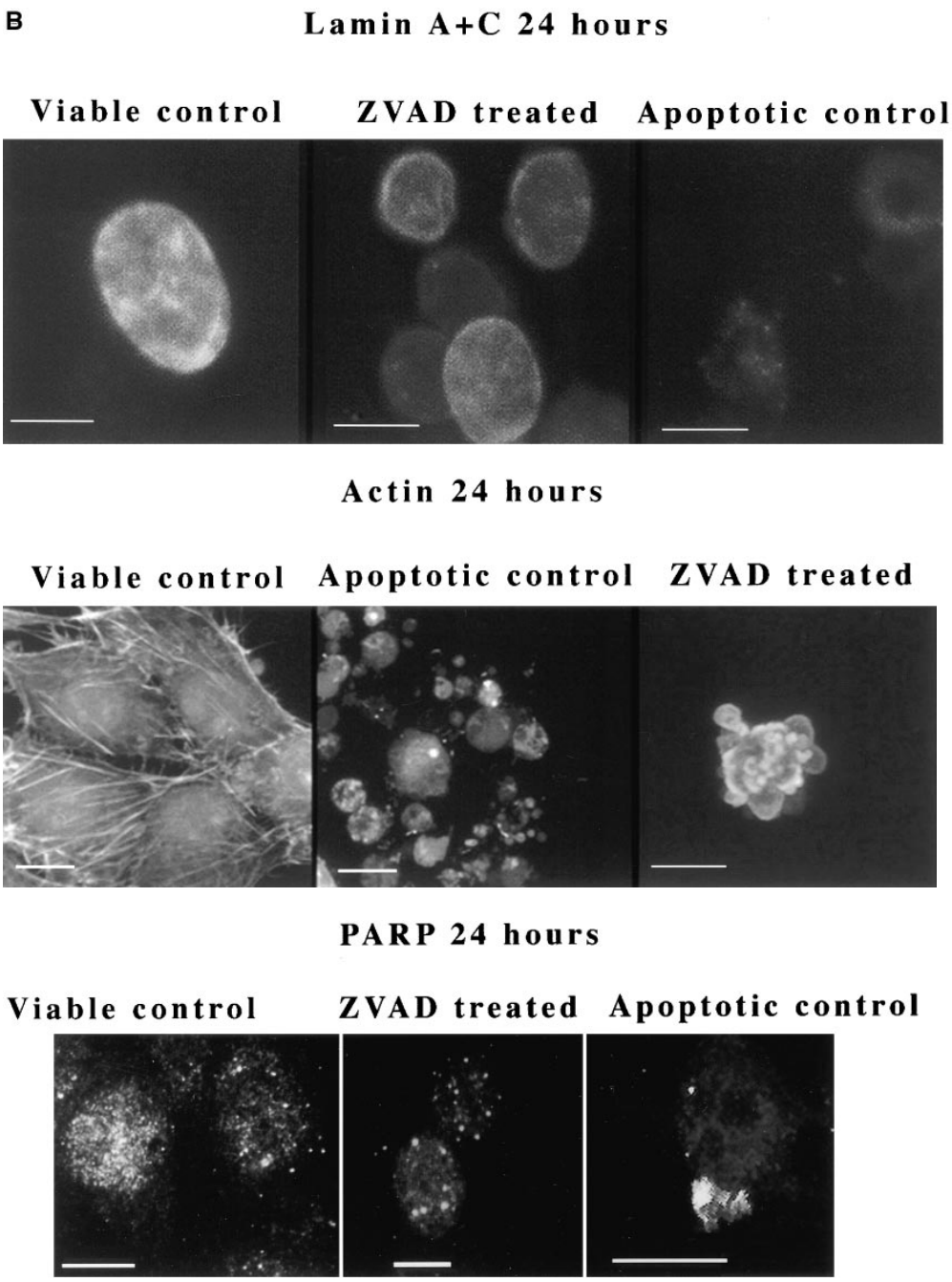


Figure 4. ZVAD.fmk inhibits cleavage of known substrates of ICE-related proteases in Rat-1/c-MycER™ cells expressing activated c-Myc in low serum. (*A*) Immunoblot analysis of cleavage of three known IRP substrates in Rat-1/c-MycER™ cells in which c-Myc has been activated in low serum in the presence or absence of ZVAD.fmk. (*i*) Inhibition of cleavage of lamins A and C by ZVAD.fmk. ZVAD.fmk treatment inhibits the appearance of the characteristic 46-kD IRP cleavage product in apoptosis (Oberhammer et al., 1994) at both 24 and 48 h. Lane *1*, time-0 cells; lane *2*, 24-h control cells; lane *3*, 24-h ZVAD.fmk-treated cells; lane *4*, 48-h control cells; lane *5*, 48-h ZVAD.fmk-treated cells. (*ii*) Inhibition of PARP cleavage by ZVAD.fmk. IRP cleavage of PARP produces a characteristic 85-kD fragment in apoptotic cells (Lazebnik et al., 1994). In non–ZVAD.fmk-treated Rat-1/c-MycER™ cells, the 85-kD PARP fragment is visible by 24 h and further increased at 48 h. In the ZVAD.fmk-treated cells, the 85-kD fragment is absent at 24 h and only faintly seen at 48 h. Lane *1*, time-0 control cells; lane *2*, time-0 ZVAD.fmk-treated cells; lane *3*, 24-h control cells; lane *4*, 24-h ZVAD.fmk-treated cells; lane *5*, 48-h control cells; lane *6*, 48-h ZVAD.fmk-treated cells. (*iii*) ZVAD.fmk inhibits actin cleavage in apoptotic cells, characterized by the appearance of a 15-kD fragment recognized by the antibody used. Appearance of this fragment is inhibited by ZVAD.fmk. Lane *1*, time-0 cells; lane *2*, 48-h control cells; lane *3*, 48-h ZVAD.fmk-treated cells; lane *4*, 96-h control cells; lane *5*, 96-h ZVAD.fmk-treated cells. (*B*) Confocal immunofluorescence microscopy of distribution of known IRP substrates. (*i*) Viable Rat-1/c-MycER™ cells stained with anti–lamin A+C antibody demonstrate characteristic lamin staining in the nuclear periphery. In apoptotic cells, such staining is absent because of degradation and dispersal of lamins. In contrast, ZVAD.fmk-treated blebbing cells exhibit near-normal lamin A+C staining when compared to controls. (*ii*) Rat-1/c-MycER™ cells stained with phalloidin-FITC to examine the distribution of actin. Viable cells exhibit normal actin fibers; apoptotic cells are condensed with no actin filaments visible. In ZVAD.fmk-treated cells, actin is clumped in blebs on the cell surface. Note that blebbing cells are markedly reduced in size compared with their viable counterparts. (*iii*) Both viable and blebbing Rat-1/c-MycER™ cells exhibit nuclear PARP staining, which is absent in apoptotic cells. Bars, 10 μm.

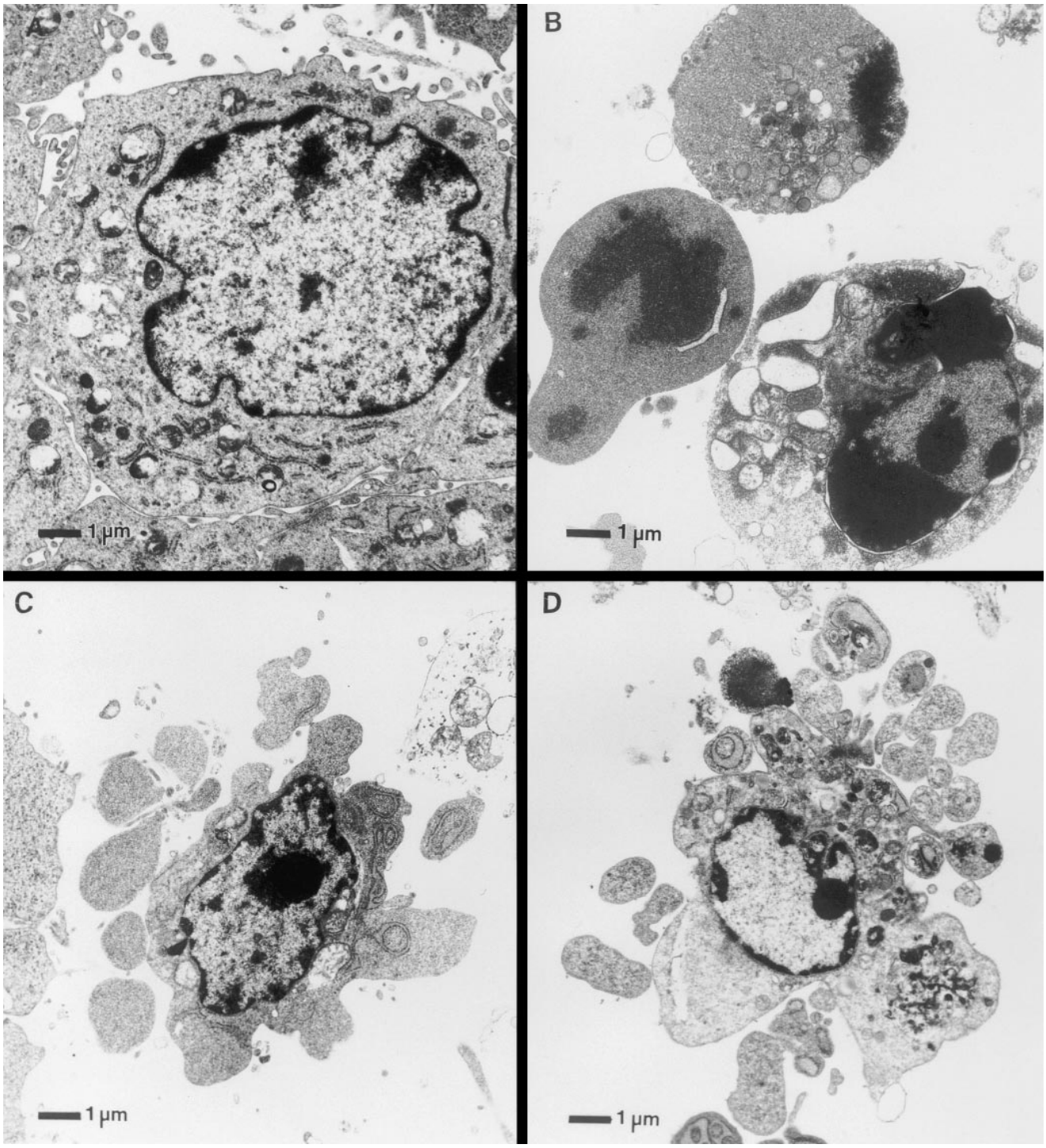


Figure 5. Electron microscopic analysis of apoptosis in serum-deprived Rat-1/c-MycER™ cells in the presence and absence of ZVAD.fmk. Electron microscopical analysis of individual Rat-1/c-MycER™ cells undergoing 4-OHT–induced apoptosis in low serum. (*A*) Normal, viable Rat-1/c-MycER™ cells. (*B*) A typical apoptotic cell. (*C* and *D*) Early and late stages, respectively, of the morphological changes where apoptosis is induced in the presence of ZVAD.fmk, showing dramatic cytoplasmic blebbing but absence of chromatin condensation. Bars, 1 μm.

This is consistent with the notion that Bcl-2, serum and survival factors like IGF-I signaling act *upstream* of the onset of membrane blebbing, effectively reducing the probability that the apoptotic program will be initiated in any individual cell. In complete contrast, ZVAD.fmk acts downstream of membrane blebbing but before the later stages of apoptosis. To investigate further the temporal relationship between serum survival factors and ZVAD.fmk inhibition of apoptosis, we induced blebbing in ZVAD.fmk-treated Rat-1/c-MycER™ cells by addition of

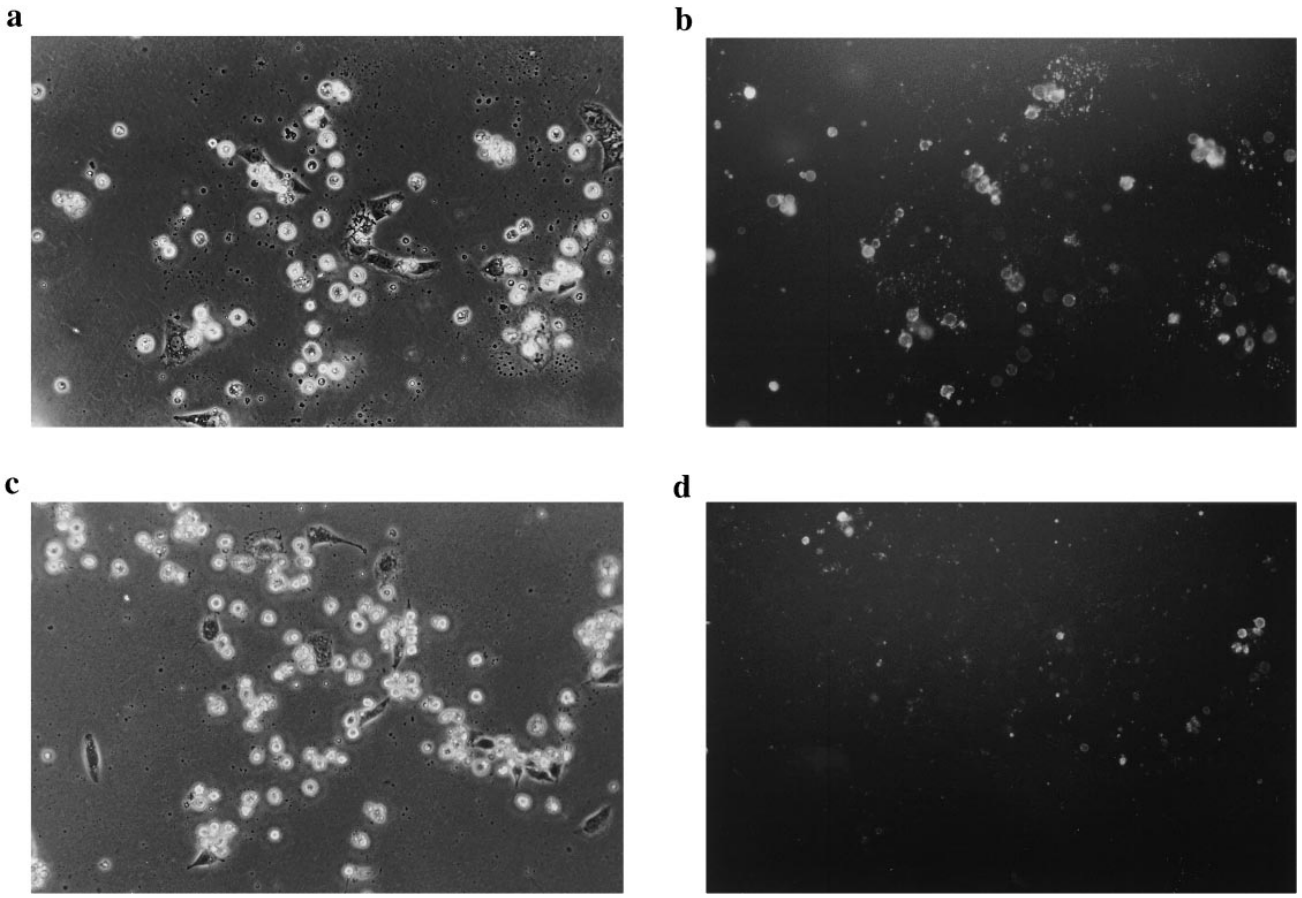


Figure 6. Expression of cell surface phosphatidyl serine. ZVAD.fmk delays the emergence of annexin V binding. Serum-deprived Rat-1/c-MycER™ cells undergoing c-Myc–induced apoptosis in the presence or absence of ZVAD.fmk were examined by phase-contrast microscopy to determine their morphologies (*a* and *c*). FITC-labeled annexin V was then added to the culture dishes to a final concentration of 2.5 μg/ml, and the same cells were examined by fluorescence microscopy (*b* and *d*). In the absence of ZVAD.fmk, apoptotic cells stain with annexin V (*a* and *b*). In contrast, ZVAD.fmk-treated blebbing cells do not bind annexin V (*c* and *d*).

4-OHT in the absence of serum and then examined whether readdition of serum could reverse the blebbing phenotype and allow cell recovery. Cultures were observed by time-lapse videomicroscopy. At the time serum was added, some cells within the population had just begun to bleb, whereas others had been blebbing for hours. In none of >500 cells examined was there any reversal of blebbing, and all cells that had initiated blebbing eventually went on to die. A study of a representative population is depicted in Fig. 8. From examination of time-lapse video data, it was also apparent that ZVAD.fmk-treated blebbing cells do not proliferate. Of some 1,000 cells examined, no division was ever observed of any cell that had started blebbing. However, this is not due to a cytostatic effect of ZVAD.fmk *per se* since nonapoptotic cells in ZVAD.fmk-treated cultures divide at normal rates, as judged by time-lapse videomicroscopy (see Note Added in Proof for Web Information) and DNA profile (Fig. 3 *B*). Thus, we conclude the onset of blebbing is coincident with loss of clonagenic potential. Interestingly, cells that had not yet initiated blebbing at the time of serum readdition remained viable, never blebbed, and, in the presence of ZVAD.fmk, proliferated to eventually repopulate the culture (not shown). Thus, readdition of survival factors before the onset of membrane blebbing can completely protect both the viability and proliferative potential of cells.

Finally, we asked whether ZVAD.fmk-blocked, blebbing cells might be rescued by removal of 4-OHT from the growth medium, thus inactivating c-Myc protein, which, in the Rat-1/c-MycER™ cells, is dependent upon the continuous presence of 4-OHT (Littlewood et al., 1995). Of some 500 cells examined, we have observed no recovery of any ZVAD.fmk-treated blebbing cell upon removal of 4-OHT (data not shown), affirming the commitment of these cells to eventual death.

Discussion

A cell suicide program appears to have arisen early in metazoan evolution and been substantially conserved. Two of the three principal genes directly involved in control and execution of ontogenetic cell death in the developmentally invariant nematode worm *C. elegans*, *ced*-9 and *ced*-3, encode polypeptides that have multiple mammalian homologues: the Bcl-2 (Hengartner and Horvitz, 1994) and ICE-related cysteine protease (Xue and Horvitz, 1995) families, respectively. Both the Bcl-2 and IRP families are implicated in vertebrate cell suicide, a process

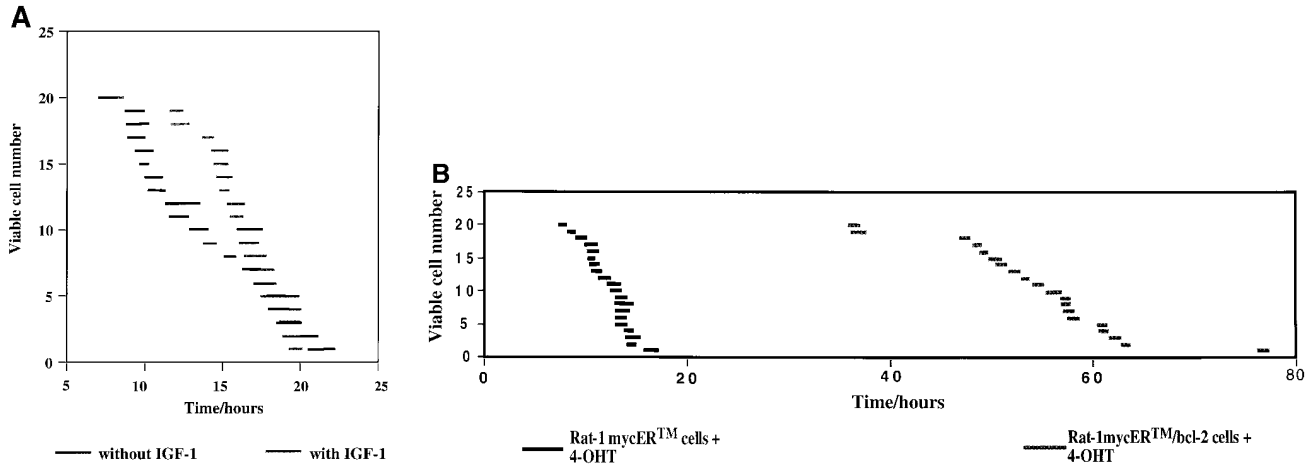


Figure 7. IGF-I and Bcl-2 delay the initiation of the apoptotic program but not its execution. Serum-deprived Rat-1/c-MycER™ cells were treated with 100 nM 4-OHT to activate c-Myc, and this was followed by time-lapse videomicroscopy. Onset of apoptosis was scored at the start of blebbing. End points of cell death were scored at the point of cell detachment, and the time between these two is represented by the length of the horizontal line. (*A*) Apoptotic events in the presence or absence of IGF-1. The effect of IGF-1 on the kinetics of Myc-induced apoptosis. (*B*) The effect of Bcl-2 expression on the kinetics of Myc-induced apoptosis. Apoptotic events in the presence or absence of co-expressed Bcl-2.

whose morphological manifestation is apoptosis. In contrast to *C. elegans*, however, cell suicide in vertebrates is influenced by many diverse factors that include extracellular signals, genotoxic and physical trauma, anoxia, oncogene expression, and immune killing. How all of these differing agents impact upon the underlying cell death mechanism is unclear. Specifically, it is unclear which of the factors that modulate vertebrate cell death are part of the actual execution machinery—the mechanism that physically dismantles the cell—and which are merely regulatory mechanisms that govern the engagement of the execution process.

The best candidates for components of the execution machinery of vertebrate apoptosis are the Ced-3 homologues, the ICE-related proteases. Consistent with this, many diverse studies implicate IRPs in apoptosis induced by insults such as growth factor deprivation (Milligan et al., 1995; Nicholson et al., 1995), loss of contact with extracellular matrix (Boudreau et al., 1995), Fas/TNF (Enari et al., 1995; Los et al., 1995; Duan et al., 1996*a*; Schlegel et al., 1996), and cytotoxic T cell killing (Darmon et al., 1995). We have therefore investigated the requirement of IRPs in mammalian apoptosis using the IRP inhibitors ZVAD.fmk and BD.fmk. ZVAD.fmk and BD.fmk are particularly useful inhibitors for studying IRP action in intact cells because their intrinsic hydrophobicity permits their entry into cells, whereupon intracellular esterases convert the aspartyl methyl ester to aspartate to generate irreversible inhibitors of IRPs. Both ZVAD.fmk and BD.fmk have been shown to be effective inhibitors of apoptosis in intact cells with broad reactivities amongst tested ICE family proteases (Fearnhead et al., 1995; Slee et al., 1996). In our studies, we observed no differences in effect between ZVAD.fmk and BD.fmk. Consequently, most of the data we show is confined to studies with just one of these inhibitors, ZVAD.fmk.

We investigated the ability of ZVAD.fmk to inhibit apoptosis triggered by diverse promoters of apoptosis: c-Myc and E1A oncogene expression, DNA damage induced by the topoisomerase II inhibitor etoposide, and ectopic expression of the killer member of the Bcl-2 family Bak. In all cases, ZVAD.fmk provided substantial protection from apoptosis that correlated with inhibition of cleavage of the known IRP substrates, actin, lamins, and PARP. Thus, oncogenes, p53 (after DNA damage), and pro-apoptotic members of the Bcl-2 family all induce apoptosis via a ZVAD.fmk-sensitive activity, most probably one or more IRPs. These findings extend the critical role of IRPs in mammalian apoptosis to include stimuli of fundamental importance in the genesis and progression of neoplasia.

However, detailed time-lapse videomicroscopic analysis of cells protected by ZVAD.fmk revealed an unexpected and novel phenotype. An early manifestation of apoptosis in most cells is the onset of vigorous membrane blebbing. Membrane blebbing is a well-described characteristic of apoptosis in many cell types that, in the normal course of apoptosis in fibroblastic cells, is rapidly (20–60 min) followed by cell shrinkage, chromatin condensation, and nuclear and cellular fragmentation. Surprisingly, in cultures of ZVAD.fmk-treated cells induced to initiate apoptosis by the action of c-Myc, E1A, or Bak or the genotoxic agent etoposide, we found that the onset of membrane blebbing was not delayed by the presence of ZVAD.fmk in the culture medium. However, whereas untreated blebbing apoptotic cells rapidly proceed through the latter stages of chromatin condensation and cell fragmentation, ZVAD.fmk-treated cells continue to bleb vigorously for greatly extended periods of time, typically several days. Such blebbing cells exhibit few characteristics of apoptotic cells: cell nuclei remain intact with little evidence of chromatin condensation or nuclear fragmentation, plasma membrane integrity and mitochondrial function are preserved, and phosphatidylserine is not expressed on the cell surface. Thus, ZVAD.fmk appears not to inhibit the onset of membrane blebbing, although it effectively suppresses classical late-stage apoptotic events.

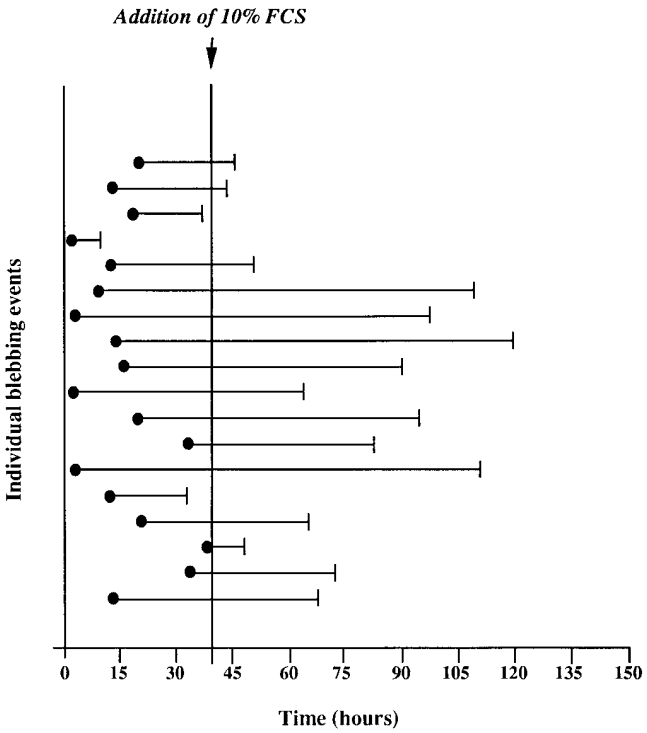


Figure 8. Serum survival factors do not rescue ZVAD.fmk-blocked apoptotic cells once they have initiated blebbing. Apoptosis was induced in serum-deprived Rat-1/c-MycER™ fibroblasts by addition of 4-OHT, and cells were observed by time-lapse videomicroscopy. 40 h after addition of 4-OHT, FCS was added back to the growth medium to a final concentration of 10%. Cells that had initiated membrane blebbing were then followed to determine their fates. The figure shows a representative study of 18 independent cells fates. Initiation of membrane blebbing (●) and death of the cell (|) were determined as before, and the time interval between the two is given by the horizontal line. All cells that initiated membrane blebbing before serum readdition eventually died. In contrast, all observed cells that had not yet initiated blebbing at the time of serum readdition survived and went on to divide (not shown).

Moreover, although ZVAD.fmk substantially ameliorates the pro-apoptotic effects of c-Myc, DNA damage, and Bak in Rat-1 fibroblasts, most cells nonetheless do eventually detach from the substratum. Such cells exhibit some chromatin condensation (although far less than in normal late-stage apoptotic cells) but preserve intact nuclear lamina; they also have extensively fragmented and vacuolated cytoplasm. Eventually, however, late-stage ZVAD.fmk-treated cells lose their integrity and take up vital dyes—indisputable evidence of their ultimate death. We conclude that late-stage ZVAD.fmk-treated cells undergo a delayed "partial" apoptotic process, although a more detailed characterization of these late-stage cells will be required to establish their precise phenotype. We have investigated whether the delay to cell death afforded by ZVAD.fmk might be extended either by addition of higher initial concentrations of ZVAD.fmk to the growth medium or by its repeated readdition. However, neither alters the kinetics of cell death within Rat-1 cell cultures. It is difficult to assess the significance of this observation because of uncertainty as to the effective intracellular concentration of ZVAD.fmk administered to culture medium, or of the inhibitor's half-life in culture media or within cells. Nonetheless, it is possible that a continued pro-apoptotic stimulus eventually overrides the ZVAD.fmk block or bypasses the ZVAD.fmk-sensitive components of the apoptotic program.

Why should apoptotic cells exhibit prolonged blebbing when treated with ZVAD.fmk? One possibility is that the blebbing we observe in ZVAD.fmk-treated cells is different from the normal blebbing that accompanies apoptosis and has nothing to do with apoptosis. Three factors argue against this. By time-lapse videomicroscopy and light and electron microscopy, the blebbing observed in the presence of ZVAD.fmk appears identical to that seen in control cells undergoing normal apoptosis. In addition, the onset of blebbing in ZVAD.fmk-treated cell populations is identical to that in control populations; it merely continues for far longer as it is not accompanied by cell and nuclear fragmentation. Finally, time-lapse videomicroscopy shows that blebbing ZVAD.fmk-treated cells adhere to live cells in the same way as true apoptotic bodies (Whyte, M., N. McCarthy, and G.I. Evan, unpublished data) (see Note Added in Proof for Web Information), indicating that at least some of the pro-phagocytic processes triggered during normal apoptosis (although not surface display of phosphatidylserine) also occur in ZVAD.fmk-blocked cells. A second possibility is that ZVAD.fmk fails to prevent membrane blebbing because blebbing is not part of the basal apoptotic program but merely a dispensable epiphenomenon that commonly accompanies the process. However, the notion that membrane blebbing is not part of apoptosis is difficult to reconcile with our time-lapse videomicroscopic data, which show that once blebbing is initiated, it is not reversed by the action of antiapoptotic cytokines or by removal of the original pro-apoptotic trigger. Thus, blebbing appears to be concomitant with commitment to death. We also dislike this explanation because it seeks to explain the ZVAD.fmk phenotype by redefining apoptosis so as to exclude a characteristic of the process that is so unique and ubiquitous as to give the phenomenon its name. A third possibility is that membrane blebbing is part of the *bona fide* apoptotic process but triggered by IRPs that are not inhibited by ZVAD.fmk. This possibility needs to be investigated using a range of IRP inhibitors with differing spectrums of specificity. However, at present the possibility remains that membrane blebbing is triggered by a completely different mechanism that might not involve IRP action at all.

The phenotype of continuous membrane blebbing that we observe in ZVAD.fmk-treated apoptotic cells appears to contradict several reports that indicate that chemical and viral IRP inhibitors can afford long term protection against apoptosis. For example, peptide and viral inhibitors of IRPs protect motor neurons against apoptosis after factor withdrawal (Gagliardini et al., 1994; Martinou et al., 1995; Tewari et al., 1995), protect cells from Fas and TNF-induced killing (Beidler et al., 1995; Enari et al., 1995, 1996; Los et al., 1995), and block developmental apoptosis in *Drosophila* (Hay et al., 1995) and *C. elegans* (Xue and Horvitz, 1995). In some of these instances, it is possible that the protected cells have nonetheless initiated a membrane blebbing program that is not easily discernible using

static imaging techniques. Furthermore, it is possible that blebbing cells remain capable of sustaining some of the functions of normal living cells within the soma, at least temporarily. In other situations, cell death may be triggered by directly activating or recruiting IRPs, in which case inhibiting IRP activity might be sufficient to prevent completely all manifestations of apoptosis. For example, the DEVD-specific IRP CPP32β is directly cleaved and activated by the cytotoxic T cell granule serine esterase Granzyme B and both the CD95 and TNF-R1 cytotoxic signaling pathways directly recruit (and presumably activate) the IRP FLICE/MACH via their "Death Domains" (Boldin et al., 1996; Muzio et al., 1996). By inference, the *Drosophila* Reaper, another Death-Domain protein, may act similarly to recruit directly downstream IRPs during developmental apoptosis in the fly. In the cases of oncogene deregulation, DNA damage/p53, and Bak, our data indicate that all these triggers of apoptosis act on some upstream regulator that can independently activate both IRPs and membrane blebbing.

By time-lapse videomicroscopic analysis, we observe a clear difference in the anti-apoptotic actions of the IRP inhibitor ZVAD.fmk, on the one hand, and the well-characterized antiapoptotic factors Bcl-2 and IGF-I, on the other. The actions of both Bcl-2 and IGF-I are to lower the probability of initiation of the apoptotic program, but neither has any effect on the kinetics of each apoptotic event (as defined as the time from onset of blebbing to cell fragmentation) once initiated. In complete contrast, ZVAD.fmk does not inhibit initiation of the apoptotic program, as judged by the onset of membrane blebbing, but prolongs each individual apoptotic event by inhibiting the cellular fragmentation that normally rapidly ensues. Importantly, cells that have initiated membrane blebbing but are blocked in completion of the apoptotic program by ZVAD.fmk cannot be rescued by the action of antiapoptotic survival factors and thus appear to be beyond a point of commitment to die. In addition, ZVAD.fmk-blocked blebbing cells do not divide; they have no clonagenic potential and from the stance of their neoplastic potential may therefore be considered as "genetically dead." The clear implication from this is that, unlike more upstream inhibitors of apoptosis like Bcl-2 and survival factors, IRP inhibitors (whether viral or chemical) will not act as generic carcinogens because they inhibit the apoptotic program downstream of an irreversible point where replicative potential is lost.

In conclusion, we have shown that oncogene deregulation, DNA damage, and expression of the Bcl-2 family member Bak all induce apoptosis in mammalian fibroblasts via a ZVAD.fmk-inhibitable mechanism, probably one or more IRP. Nonetheless, time-lapse videomicroscopic analysis clearly distinguishes between the antiapoptotic actions of Bcl-2 or the survival factor IGF-I on the one hand and IRP inhibition on the other. Both Bcl-2 and IGF-I signaling act to suppress initiation of the apoptotic program, yet have no effect on the kinetics of each apoptotic event. In contrast, ZVAD.fmk has no effect on initiation of apoptosis, as determined by membrane blebbing, but acts to arrest each apoptotic program before completion. This observation raises the intriguing possibility that membrane blebbing is a discrete subprogram operating during mammalian apoptosis that can lead to cell death via an IRP-independent mechanism. If true, this will constrain the potential therapeutic use of IRP inhibitors. However, such speculations can only be validated by the development of specific inhibitors for each IRP and a more detailed understanding of the intracellular targets and kinetics of these enzymes.

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Note Added in Proof. Quick-Time movies showing c-Myc–induced apoptosis in the absence of serum (Movie One) and c-Myc–induced apoptosis in the absence of serum and the presence of 100 μM zVAD.fmk, the blebbing phenotype (Movie Two), are available from the following Web site address: http://www.icnet.uk/axp/bcn/

References

Beidler, D., M. Tewari, P. Friesen, G. Poirier, and V. Dixit. 1995. The baculovirus p35 protein inhibits Fas- and Tumour Necrosis Factor-induced apoptosis. *J. Biol. Chem.* 270:16526–16528.

Boldin, M., T. Goncharov, Y. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell.* 85:803–815.

Boudreau, N., C.J. Sympson, Z. Werb, and M.J. Bissell. 1995. Suppression of ice and apoptosis in mammary epithelial-cells by extracellular-matrix. *Science (Wash. DC).* 267:891–893.

Brancolini, C., M. Benedetti, and C. Schneider. 1995. Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:5179–5190.

Braselmann, S., P. Graninger, and M. Busslinger. 1993. A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proc. Natl. Acad. Sci. USA.* 90:1657–1661.

Casciola Rosen, L.A., D.K. Miller, G.J. Anhalt, and A. Rosen. 1994. Specific cleavage of the 70-kD protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* 269:30757–30760.

Casciola Rosen, L.A., G.J. Anhalt, and A. Rosen. 1995. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* 182:1625–1634.

Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, N.K. Van, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, et al. 1992. Molecular cloning of the interleukin-1 β converting enzyme. *Science (Wash. DC).* 256:97–100.

Chittenden, T., E. Harrington, R. O'Connor, G. Evan, and B. Guild. 1995. Induction of apoptosis by the Bcl-2 homologue Bak. *Nature (Lond.).* 374:733–736.

Darmon, A.J., D.W. Nicholson, and R.C. Bleackley. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature (Lond.).* 377:446–448.

Debbas, M., and E. White. 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.* 7:546–554.

Duan, H., A.M. Chinnaiyan, P.L. Hudson, J.P. Wing, W.W. He, and V.M. Dixit. 1996*a.* ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* 271:1621–1625.

Duan, H., K. Orth, A.M. Chinnaiyan, G.G. Poirier, C.J. Froelich, W.W. He, and V.M. Dixit. 1996*b.* ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J. Biol. Chem.* 271:16720–16724.

Ellis, H.M., and H.R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans. Cell.* 44:817–829.

Emoto, Y., Y. Manome, G. Meinhardt, H. Kisaki, S. Kharbanda, M. Robertson, T. Ghayur, W.W. Wong, R. Kamen, R. Weichselbaum, et al. 1995. Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:6148–6156.

Enari, M., H. Hug, and S. Nagata. 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature (Lond.).* 375:78–81.

Enari, M., R. Talanian, W. Wong, and S. Nagata. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature (Lond.).* 380:723–726.

Evan, G., A. Wyllie, C. Gilbert, T. Littlewood, H. Land, M. Brooks, C. Waters, L. Penn, and D. Hancock. 1992. Induction of apoptosis in fibroblasts by c-*myc* protein. *Cell.* 63:119–125.

Fadok, V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:2207–2216.

Fanidi, A., E. Harrington, and G. Evan. 1992. Cooperative interaction between c-*myc* and *bcl*-2 proto-oncogenes. *Nature (Lond.).* 359:554–556.

Faucheu, C., A. Diu, A.W. Chan, A.M. Blanchet, C. Miossec, F. Herve, V. Collard Dutilleul, Y. Gu, R.A. Aldape, J.A. Lippke, et al. 1995. A novel human protease similar to the interleukin-1 β converting enzyme induces apoptosis in transfected cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1914–1922.

Fearnhead, H.O., D. Dinsdale, and G.M. Cohen. 1995. An interleukin-1 β-converting enzyme-like protease is a common mediator of apoptosis in thymocytes. *FEBS Lett.* 375:283–288.

Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1β-converting enzyme. *J. Biol. Chem.* 269:30761–30764.

Fernandes-Alnemri, T., G. Litwack, and E. Alnemri. 1995*a.* Mch2, a new member of the apoptotic ced-3/ICE cysteine protease gene family. *Cancer Res.* 55:2737–2742.

Fernandes-Alnemri, T., A. Takahashi, R. Armstrong, J. Krebs, L. Fritz, K. Tomaselli, L. Wang, Z. Yu, C. Croce, G. Salveson et al. 1995*b.* Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* 55: 6045–6052.

Fletcher, D.S., L. Agarwal, K.T. Chapman, J. Chin, L.A. Egger, G. Limjuco, S. Luell, D.E. MacIntyre, E.P. Peterson, N.A. Thornberry, et al. 1995. A synthetic inhibitor of interleukin-1 β converting enzyme prevents endotoxin-induced interleukin-1 β production in vitro and in vivo. *J. Interferon Cytokine Res.* 15:243–248.

Gagliardini, V., P.A. Fernandez, R.K. Lee, H.C. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by the crmA gene. *Science (Wash. DC).* 263:826–828.

Gu, Y., C. Sarnecki, R.A. Aldape, D.J. Livingston, and M.S. Su. 1995*a.* Cleavage of poly(ADP-ribose) polymerase by interleukin-1 β converting enzyme and its homologs TX and Nedd-2. *J. Biol. Chem.* 270:18715–18718.

Gu, Y., J. Wu, C. Faucheu, J.L. Lalanne, A. Diu, D.J. Livingston, and M.S. Su. 1995*b.* Interleukin-1 β converting enzyme requires oligomerization for activity of processed forms in vivo. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1923–1931.

Harrington, E., A. Fanidi, M. Bennett, and G. Evan. 1994. Modulation of Myc-induced apoptosis by specific cytokines. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3286–3295.

Hay, B.A., D.A. Wassarman, and G.M. Rubin. 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell.* 83:1253–1262.

Hengartner, M., and H. Horvitz. 1994. *C. elegans* cell survival gene *ced*-9 encodes a functional homolog of the mammalian proto-oncogene *bcl*-2. *Cell.* 76:665–676.

Kamens, J., M. Paskind, M. Hugunin, R.V. Talanian, H. Allen, D. Banach, N. Bump, M. Hackett, C.G. Johnston, P. Li, et al. 1995. Identification and characterization of ICH-2, a novel member of the interleukin-1 β-converting enzyme family of cysteine proteases. *J. Biol. Chem.* 270:15250–15256.

Koopman, G., C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals, and M.H. van Oers. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 84:1415–1420.

Kuida, K., J.A. Lippke, G. Ku, M.W. Harding, D.J. Livingston, M.-S. Su, and R.A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1-β converting-enzyme. *Science (Wash. DC).* 267:2000–2003.

Kumar, S., M. Kinoshita, M. Noda, N. Copeland, and N. Jenkins. 1994. Induction of apoptosis by the *Nedd2* gene, which encodes a protein similar to the *Caenorhabditis elegans* cell death gene *ced*-3 and the mammalian IL-1β-converting enzyme. *Genes Dev.* 8:1613–1626.

Lamarre, D., B. Talbot, Y. Leduc, S. Muller, and G. Poirier. 1986. Production and characterization of monoclonal antibodies specific for the functional domains of poly(ADP-ribose) polymerase. *Biochem. Cell. Biol.* 64:368–376.

Lazebnik, Y., S. Kaufmann, S. Desnoyers, G. Poirier, and W. Earnshaw. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346–347.

Lazebnik, Y.A., A. Takahashi, G. Poirier, S.H. Kaufmann, and W. Earnshaw. 1995*a.* Characterization of the execution phase of apoptosis *in vitro* using extracts from condemned-phase cells. *J. Cell. Sci.* 19:41–49.

Lazebnik, Y.A., A. Takahashi, R.D. Moir, R.D. Goldman, G.G. Poirier, S.H. Kaufmann, and W.C. Earnshaw. 1995*b.* Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. *Proc. Natl. Acad. Sci. USA.* 92:9042–9046.

Littlewood, T., D. Hancock, P. Danielian, M. Parker, and G. Evan. 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.* 23:1686–1690.

Los, M., M. Van de Craen, L.C. Penning, H. Schenk, M. Westendorp, P.A. Baeuerle, W. Droge, P.H. Krammer, W. Fiers, and K. Schulze Osthoff. 1995. Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. *Nature (Lond.).* 375:81–83.

Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell.* 74:957–967.

Martinou, I., P. Fernandez, M. Missotten, E. White, B. Allet, R. Sadoul, and J. Martinou. 1995. Viral protein E1B 19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. *J. Cell. Biol.* 128:201–208.

Mashima, T., M. Naito, N. Fujita, K. Noguchi, and T. Tsuruo. 1995. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem. Biophys. Res. Commun.* 217:1185–1192.

Milligan, C.E., D. Prevette, H. Yaginuma, S. Homma, C. Cardwell, L.C. Fritz, K.J. Tomaselli, R.W. Oppenheim, and L.M. Schwartz. 1995. Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. *Neuron.* 15:385–393.

Mosmant, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65: 55–63.

Munday, N.A., J.P. Vaillancourt, A. Ali, F.J. Casano, D.K. Miller, S.M. Molineaux, T.T. Yamin, V.L. Yu, and D.W. Nicholson. 1995. Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. *J. Biol. Chem.* 270:15870–15876.

Muzio, M., A. Chinnaiyan, F. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. Bretz, M. Zhang, R. Gentz, et al. 1996. FLICE, a novel FADD homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. *Cell.* 85:817–827.

Nicholson, D., A. Ali, N. Thornberry, J. Vaillancourt, C. Ding, M. Gallant, Y. Gareau, P. Griffin, M. Labelle, Y. Lazebnik, et al. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature (Lond.).* 375:37–43.

Oberhammer, F.A., K. Hochegger, G. Froschl, R. Tiefenbacher, and M. Pavelka. 1994. Chromatin condensation during apoptosis is accompanied by degradation of lamin A + B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* 126:827–837.

Orth, K., A.M. Chinnaiyan, M. Garg, C.J. Froelich, and V.M. Dixit. 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* 271:16443–16446.

Rao, L., M. Debbas, P. Sabbatini, D. Hockenberry, S. Korsmeyer, and E. White. 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA.* 89: 7742–7746.

Schlegel, J., I. Peters, S. Orrenius, D.K. Miller, N.A. Thornberry, T.T. Yamin, and D.W. Nicholson. 1996. CPP32/apopain is a key interleukin 1 β converting enzyme-like protease involved in Fas-mediated apoptosis. *J. Biol. Chem.* 271:1841–1844.

Slee, E., H. Zhu, S. Chow, M. MacFarlane, D. Nicholson, and G. Cohen. 1996. Benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethylketone (ZVAD.fmk) inhibits apoptosis by blocking the processing of CPP32. *Biochem. J.* 315:21–24.

Takahashi, A., E. Alnemri, Y. Lazebnik, T. Fernades-Alnemri, G. Litwack, R. Moir, R. Goldman, G. Poirier, S. Kaufmann, and W. Earnshaw. 1996. Cleavage of lamin A by Mch2α but not CPP32: multiple ICE-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl. Acad. Sci. USA.* 93:8395–8400.

Tewari, M., L. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. Beidler, G. Poirer, G. Salvesen, and V. Dixit. 1995. Yama/CPP32β, a mammalian homolog of CED-3 is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-Ribose) polymerase. *Cell.* 81:801–809.

Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature (Lond.).* 356:768–774.

Walker, N., R. Talanian, K. Brady, L. Dang, N. Bump, C. Ferenz, S. Franklin, T. Ghayur, M. Hacket, L. Hammill, et al. 1994. Crystal structure of the cysteine protease interleukin-1 β-converting enzyme: a (p20/p10)2 homodimer. *Cell.* 78:343–352.

Wang, L., M. Miura, L. Bergeron, H. Zhu, and J. Yuan. 1994. Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell.* 78:739–750.

Wang, X., J.T. Pai, E.A. Wiedenfeld, J.C. Medina, C.A. Slaughter, J.L. Goldstein, and M.S. Brown. 1995. Purification of an interleukin-1 β converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. *J. Biol. Chem.* 270:18044–18050.

Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.).* 284:555–556.

Xue, D., and H.R. Horvitz. 1995. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature (Lond.).* 377:248–251.

Yuan, J.Y., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell-death gene *ced*-3 encodes a protein similar to mammalian interleukin-1 β converting enzyme. *Cell.* 75:641–652.