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Cell-free reconstitution of Fas-, UV radiation- and ceramide-induced apoptosis

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Cell-free systems are valuable tools for the dissection of complex cellular processes. Here we show that cytoplasmic extracts from cells exposed to anti-Fas antibody or UV radiation contain an activity capable of reproducing morphological changes typical of apoptosis in nuclei added to these extracts, as well as internucleosomal cleavage of DNA and proteolysis of a protein known to be cleaved during the apoptosis of intact cells. Extracts from control cell populations were inactive in this respect. These effects were partly blocked by the addition of purified Bcl-2 protein or a competitive inhibitor peptide of interleukin-1β-converting enzyme to the extracts. Furthermore, apoptotic activity was induced in cytoplasmic extracts from untreated cells by the addition of ceramide, a lipid second messenger implicated recently in apoptosis signaling. These extracts should prove highly useful in the dissection of molecular events that occur during apoptosis.

Keywords: apoptosis/cell-free/ceramide/Fas/*Xenopus*

Introduction

Apoptosis, a mode of cell death characterized by distinct morphological features (Kerr *et al.*, 1972), accounts for the majority of programmed cell deaths observed in higher organisms (Kerr *et al.*, 1972; J.J.Cohen *et al.*, 1992; Raff, 1992). Many cell deaths that are not temporally programmed also proceed via apoptosis. For example, cell deaths that occur in the context of cytotoxic T lymphocyte killing (Duke *et al.*, 1983), the ligation of the tumor necrosis factor or Fas (CD95) receptors (Laster *et al.*, 1988; Trauth *et al.*, 1989; Yonehara *et al.*, 1989), after γ or UV irradiation (Sellins and Cohen, 1987; Martin and Cotter, 1991), infection by many viruses (Gregory *et al.*, 1991; Clem and Miller, 1993; Martin *et al.*, 1994a), exposure to a diverse array of cytotoxic drugs (Lennon *et al.*, 1991) and many other stimuli (Obeid *et al.*, 1993; Jarvis *et al.*, 1994), also exhibit the distinctive features of apoptosis.

Rapid progress has been made in defining gene products that can regulate the apoptosis 'machinery'. Significantly, several of the molecular regulators of apoptosis, such as Bcl-2 (Hockenbery *et al.*, 1990; Korsmeyer, 1992; Reed, 1995), Abl (Evans *et al.*, 1993; Bedi *et al.*, 1994; McGahon *et al.*, 1994), Myc (Evan *et al.*, 1992; Y.Shi *et al.*, 1992), p53 (Yonich-Rouach *et al.*, 1991; Clarke *et al.*, 1993; Lowe *et al.*, 1993) and Ras (Wyllie *et al.*, 1987; Gulbins *et al.*, 1995), are commonly mutated or expressed in a deregulated manner in many human malignancies (Williams and Smith, 1993; Martin and Green, 1995a), underscoring the importance of apoptosis in the maintenance of proliferating cell populations at equilibrium.

In contrast, knowledge of the molecular components of the death machinery itself remains limited. Recent studies have implicated proteases, particularly those that cleave at aspartate residues, as likely components of this machinery (L.Shi *et al.*, 1992; Kaufmann *et al.*, 1993; Miura *et al.*, 1993; Sarin *et al.*, 1993; Yuan *et al.*, 1993; Casciola-Rosen *et al.*, 1994; Fernandes-Alnemri *et al.*, 1994; Lazebnik *et al.*, 1994; Squier *et al.*, 1994; Boudreau *et al.*, 1995; Martin and Green, 1995b; Martin *et al.*, 1995; Voelkel-Johnson *et al.*, 1995). In addition, an endonuclease capable of cleaving DNA at internucleosomal sites is activated in many, but not all, forms of apoptosis (Wyllie, 1980; G.M.Cohen *et al.*, 1992; Oberhammer *et al.*, 1993). The identity and localization of this nuclease are still a matter of debate, because several candidate enzymes that exhibit this activity have been described (Peitsch *et al.*, 1994). Recent studies using cytoplasts, which are capable of undergoing apoptotic changes when exposed to anti-Fas antibodies or cytotoxic drugs, indicate that the death machinery is likely to be located primarily in the cytosol (Jacobson *et al.*, 1994; Schulze-Osthoff *et al.*, 1994). Coupled with other studies (Martin *et al.*, 1990), these data also indicate that the majority of apoptotic cell deaths do not require new gene expression.

Cell-free systems have proved to be valuable tools for the dissection of complex processes such as mitosis. Two such systems have been described recently for the study of apoptosis. In the first, cytoplasmic extracts from mitotic chicken hepatoma cells were found to induce chromatin condensation and DNA cleavage reminiscent of apoptosis in exogenously added nuclei (Lazebnik *et al.*, 1993). Furthermore, these extracts were also found to contain a protease activity capable of cleaving poly-(ADP-ribose) polymerase (Lazebnik *et al.*, 1994), a protein demonstrated previously to be cleaved during apoptosis (Kaufmann *et al.*, 1993). In a more recent study, cytoplasmic extracts of *Xenopus* eggs under certain conditions were found to induce apoptotic changes in nuclei added to these extracts (Newmeyer *et al.*, 1994). Remarkably, these events were inhibited by the addition of baculovirus-derived Bcl-2 protein to the extracts and also by the removal of a dense organelle fraction enriched in mitochondria.

A cell-free system for the study of human cell apoptosis,

similar to those described above, would be highly desirable. Here we describe such a system. Cytoplasmic extracts were prepared from several human cell types primed for apoptosis by either ligation of the Fas (CD95) molecule or a brief exposure to UVB radiation. These extracts were capable of inducing rapid and highly reproducible changes characteristic of apoptosis, such as DNA condensation and internucleosomal cleavage, in exogenously added nuclei from several sources. In contrast, extracts from control cell populations were largely inactive in this respect. Fas or UV-primed cell extracts also contained a protease activity capable of degrading fodrin (non-erythroid spectrin), a protein reported recently to be cleaved in many instances of apoptosis (Martin *et al.*, 1995). Cell-free apoptosis in this context was sensitive to the addition of purified recombinant human Bcl-2 protein or a substrate peptide for interleukin-1β-converting enzyme (ICE). Although cytoplasmic extracts from control cell populations did not induce apoptotic changes in exogenously added nuclei, upon the addition of synthetic ceramides to these extracts they now did so. Furthermore, the addition of synthetic ceramide to *Xenopus* egg extracts prevented from inducing apoptotic changes due to depletion of heavy membranes (Newmeyer *et al.*, 1994) overcame the requirement for the heavy membrane fraction.

Results

Induction of morphological changes characteristic of apoptosis in human cell-free extracts

CEM, a T lymphoblastoid cell line, undergoes extensive apoptosis within 6–8 h of UVB irradiation, as do many other cell types (Martin and Cotter, 1991). To establish whether cytoplasmic extracts from UV-irradiated CEM cells could induce morphological changes characteristic of apoptosis in freshly added nuclei, cells were UV irradiated under conditions that reproducibly induced apoptosis in 80–95% of these cells within 8 h (see Materials and methods) and cytoplasmic extracts were made 2 h later. It is important to note that few cells in the irradiated cultures exhibited apoptotic features when extracts were prepared (data not shown).

The addition of either rat liver nuclei or CEM nuclei to extracts made from UV-irradiated cells resulted in the rapid destruction of the nuclei over a 60–120 min period in a manner strikingly similar to the nuclear changes that typify apoptosis in intact cells (Figure 1A and C). In contrast, cytoplasmic extracts prepared from untreated cell populations did not exhibit any apoptotic activity, with nuclei remaining intact and decondensed in these extracts for several hours (Figure 1A and C). Extracts prepared from cells immediately after UV irradiation did not induce apoptotic changes in exogenously added nuclei, indicating a requirement for some priming event that occurs during the 2 h incubation period (data not shown).

Cytoplasmic extracts were also made from Jurkat cells that had been exposed to anti-Fas IgM antibody (100 ng/ml) for 60 min. The ligation of the Fas molecule is well established as a potent stimulus for apoptosis in many cell types (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). As in previous experiments, extracts were made prior to the appearance of appreciable numbers of apoptotic cells in cultures exposed to anti-Fas. These extracts were also capable of inducing changes characteristic of apoptosis in rat liver and CEM nuclei, with similar kinetics to the changes observed in CEM cell extracts (Figure 1B and C).

Upon the addition of nuclei to either the UV- or Fas-primed extracts, filamentous meshes of cytoplasmic material assembled around the nuclei (Figure 2; 0 min), although this mesh also assembled in the absence of added nuclei (data not shown). Nuclei initially appeared to be decondensed, with the chromatin dispersed throughout the nuclear body, and nucleolar structures were apparent. Within 15–30 min of incubation in the extracts, the nuclear chromatin moved to the nuclear margins in crescent-shaped patches along the nuclear envelope (Figure 2; 15 and 30 min), which remained intact; they further condensed into many discrete spheres, highly characteristic of apoptosis, over the following 30–60 min (Figure 2). Nuclei completely disintegrated in these extracts after 3–4 h.

Internucleosomal DNA fragmentation in substrate nuclei

Because apoptosis is frequently accompanied by the cleavage of DNA at internucleosomal sites (Wyllie, 1980), we investigated whether nuclei added to UV- or Fas-primed cell extracts also suffered this type of chromatin destruction. Figure 3 illustrates that DNA from rat liver nuclei incubated in cytoplasmic extract from untreated cells remained unfragmented for several hours after addition to the extract. In contrast, chromatin was rapidly fragmented into integer multiples of ~200 bp within minutes of the addition of nuclei to UV- or Fas-primed extracts, and became progressively cleaved over the following 60 min (Figure 3; data not shown). Dilution of the apoptotic extract resulted in the delayed degradation of nuclei added to these extracts in a dose-dependent manner (Figure 3).

Proteolysis of α-fodrin in apoptotic extracts

Fodrin, a 240 kDa plasma membrane-associated cytoskeletal protein, is a homolog of spectrin and has been implicated in the maintenance of cell shape and plasma membrane lipid asymmetry (Williamson *et al.*, 1982; Glenney and Glenney, 1983). Recently, the fodrin α-subunit has been reported to undergo proteolysis during apoptosis initiated by a variety of stimuli, including Fas ligation, UV irradiation and ceramide treatment (Martin *et al.*, 1995). Therefore it was of considerable interest to determine whether the 'apoptotic' extracts described above contained a protease activity capable of effecting a similar cleavage of α-fodrin. Thus, control cell extract (as a source of intact fodrin) was incubated with either extract buffer alone or cell extract from Fas-treated cells for various periods of time, followed by the separation of extract proteins by SDS–PAGE and probing for α-fodrin by Western blotting. Figure 4 shows that α-fodrin remained intact in control cell extract for at least 2 h at 37°C. However, the addition of Fas-primed cell extract to the control cell extract resulted in the appearance of a 120 kDa α-fodrin cleavage product within 15 min, with fodrin becoming completely degraded in these extracts within 2 h (Figure 4).

Inhibition of cell-free apoptosis by Bcl-2 and an ICE substrate peptide

Many studies have established Bcl-2 as a potent apoptosis-repressor protein (Hockenbery *et al.*, 1990; Korsmeyer,

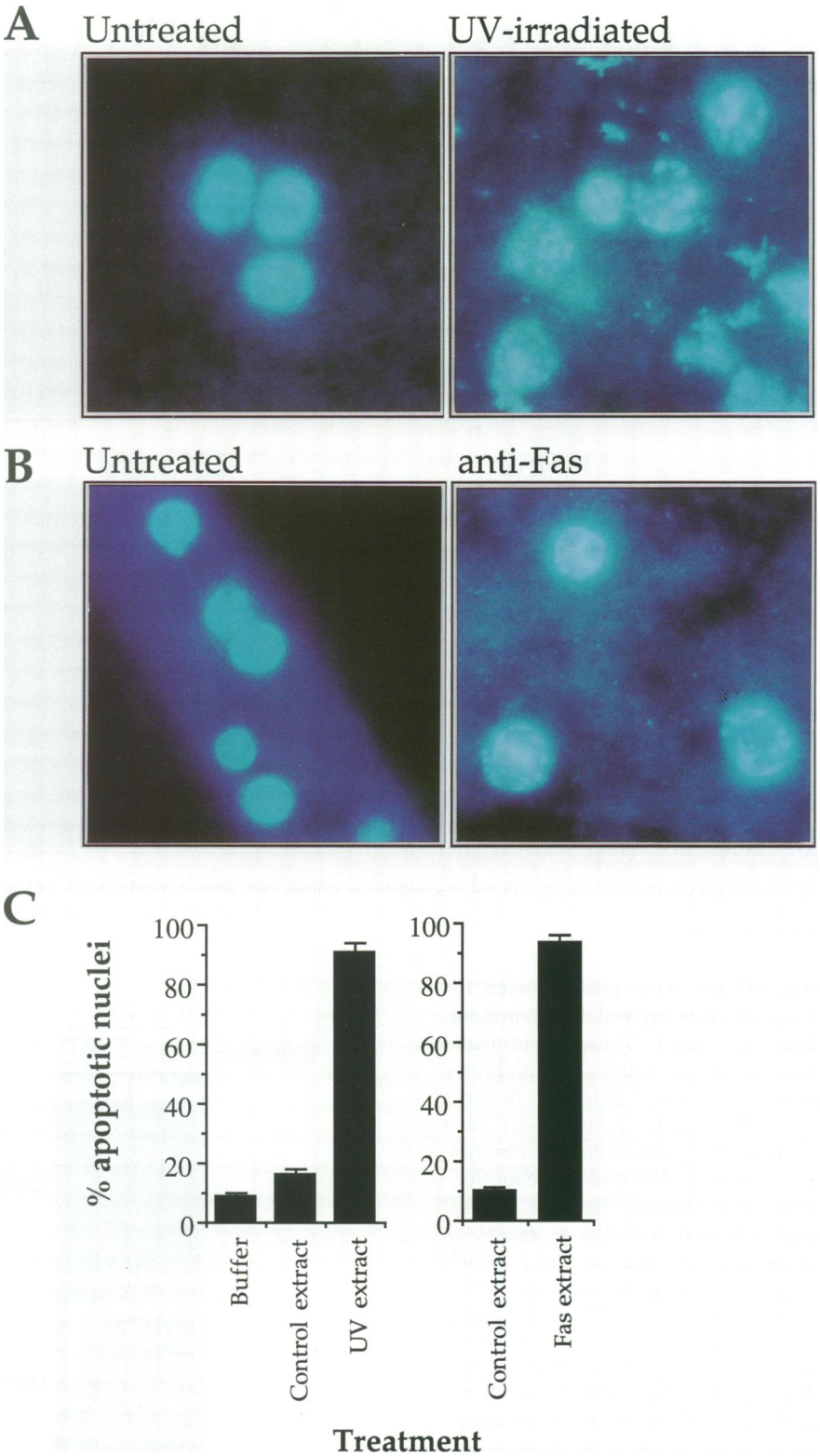

Fig. 1. Cell-free apoptosis in UV-irradiated or anti-Fas-treated cell extracts. (**A**) Rat liver nuclei were incubated for 2 h in cytoplasmic extracts from untreated CEM cells or CEM cells that were UV irradiated for 10 min, followed by incubation for 2 h at 37°C prior to the preparation of extract. (**B**) Rat liver nuclei were incubated for 2 h in cytoplasmic extracts from untreated Jurkat cells or Jurkat cells exposed to 100 ng/ml anti-Fas antibody for 60 min at 37°C prior to the preparation of extract. (**C**) Quantitation of apoptotic changes in rat liver nuclei after a 2 h incubation in the presence of extract buffer alone; cytoplasmic extract from untreated CEM (left panel) or Jurkat (right panel) cells (control extract); or extract from the same cells exposed to either UV radiation or anti-Fas IgM, as described above. A minimum of 350 nuclei were scored under each condition. Results are representative of four to six independent experiments.

1992; Miyashita and Reed, 1993; Reed, 1995). A previous study, using cytoplasmic extracts from *Xenopus* eggs, also showed that Bcl-2 is capable of exerting its inhibitory effects in a cell-free apoptosis system (Newmeyer *et al.*, 1994). To determine whether this was also the case in cytoplasmic extracts from UV-irradiated CEM and anti-

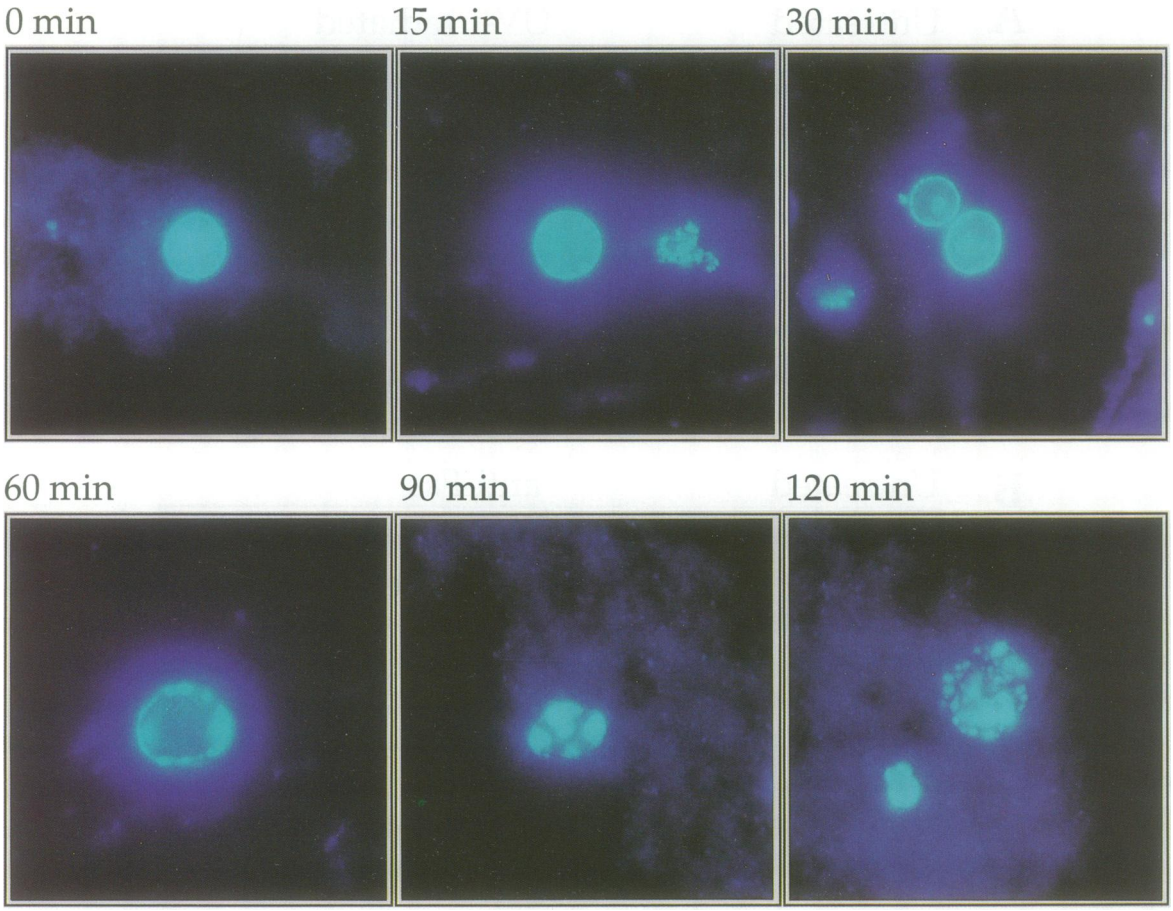

Fig. 2. Time course of apoptotic changes in rat liver nuclei exposed to cytoplasmic extract from anti-Fas-treated cells. Jurkat cells were cultured in the presence of anti-Fas antibody under the same conditions as described in Figure 1 prior to the preparation of cytoplasmic extract. Similar results were obtained in six independent experiments.

Fas-treated Jurkat cells, extracts were pre-incubated for 30 min with purified baculovirus-derived Bcl-2 protein, followed by the addition of nuclei. These experiments revealed that Bcl-2 had a partial, but significant, inhibitory effect on the appearance of nuclei with apoptotic features in these extracts (Figure 5). Similar results were also generated with Bcl-2-containing lysates from baculovirus-infected *sf*9 cells, whereas control *sf*9 lysates were ineffective in this regard (data not shown). Because several studies have implicated ICE and ICE-like proteases as apoptosis-effector molecules (Miura *et al.*, 1993; Fernandes-Alnemri *et al.*, 1994; Gagliardini *et al.*, 1994; Wang *et al.*, 1994; Boudreau *et al.*, 1995), including apoptosis due to Fas ligation (Tewari and Dixit, 1995; Kuida *et al.*, 1995), we also investigated whether an ICE competitor peptide (Tyr–Val–Ala–Asp–AFC) could delay or block apoptosis in cell-free extracts. Figure 5 illustrates that the ICE competitor peptide significantly inhibited the apoptotic changes in cytoplasmic extracts from both UV-irradiated and anti-Fas antibody-treated cells. These results are in agreement with recent findings generated in a chicken hepatoma cell-derived cytoplasmic extract using similar peptides (Lazebnik *et al.*, 1994).

Ceramide promotes apoptotic activity in control cell extracts

Apoptosis induced by either UV irradiation or Fas ligation proceeds in the absence of a requirement for new protein

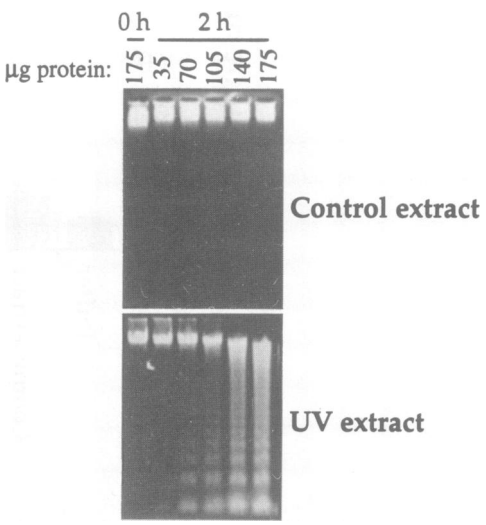

Fig. 3. Internucleosomal cleavage of DNA in nuclei exposed to UV-irradiated CEM cell extract. Rat liver nuclei (2×10^5) in a 25 μl reaction volume were incubated in the presence of the indicated amounts of cytoplasmic extracts from either untreated (control extract) or UV-irradiated CEM cells incubated for 2 h prior to the preparation of extract. Data are representative of four separate experiments with similar results. Similar data were also generated with cytoplasmic extracts from anti-Fas IgM-treated Jurkat cells.

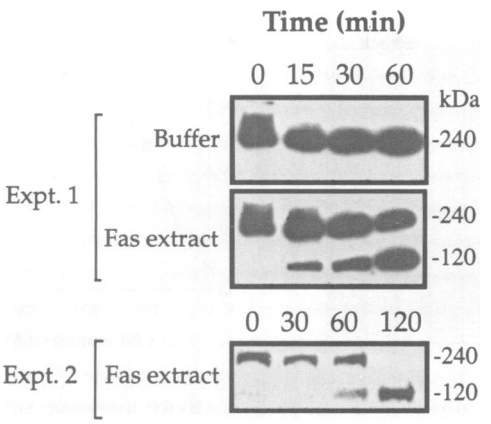

Fig. 4. Proteolytic activity in anti-Fas-treated cell extracts. Cytoplasmic extract (100 μg) from untreated Jurkat cells was incubated at 37°C with either cell extract buffer alone or 20 μg cytoplasmic extract from anti-Fas antibody-treated Jurkat cells (see the legend to Figure 1) for the indicated time periods. Portions of each extract (50 μg) were then separated by SDS–PAGE, followed by Western blotting and probing with an anti-α-fodrin monoclonal antibody (1622; Chemicon International). Data shown are representative of four independent experiments.

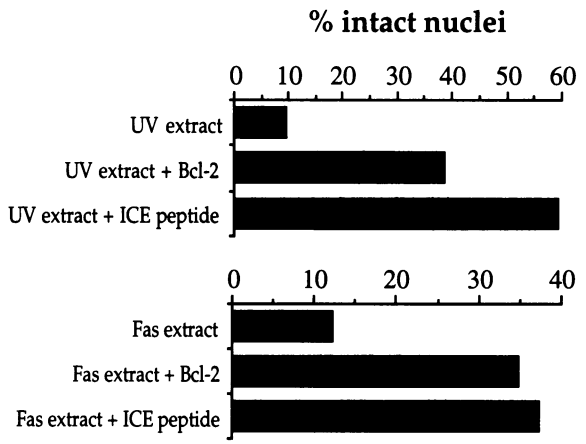

Fig. 5. Inhibition of cell-free apoptosis by Bcl-2 protein and a substrate peptide for ICE. Cytoplasmic extracts from either UV-irradiated CEM cells (top) or Fas-treated Jurkat cells (bottom) were incubated for 2 h in the presence or absence of 1 ng/ml purified recombinant Bcl-2 protein (baculovirus-derived) or 100 μM ICE substrate peptide (Tyr–Val–Ala–Asp–AFC; Enzyme Systems Products). A minimum of three fields of nuclei was scored under each condition, with a minimum of 100 nuclei scored per field. The error between fields was <5% of the mean. Data shown are representative of two independent experiments with similar results.

expression (Yonehara *et al.*, 1989; Martin *et al.*, 1990; Martin and Cotter, 1991). Thus the ability of these agents to induce an apoptotic activity detectable in cell extracts is likely to occur as a consequence of changes in the cellular biochemistry rather than the expression of new genes. Therefore we sought to identify agents capable of mimicking the signals induced in intact cells by UV irradiation or ligation of the Fas molecule, leading to the generation of apoptosis-promoting activity.

Ceramide is a lipid second messenger that is produced as a consequence of the activation of the sphingomyelin pathway (Kolesnick and Golde, 1994). This pathway is engaged under a number of conditions, including the ligation of Fas (Cifone *et al.*, 1994; Gulbins *et al.*, 1995) and exposure to UV radiation (R.N.Kolesnick, unpublished observations). In addition, synthetic ceramides are capable of inducing apoptosis (Obeid *et al.*, 1993; Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994), leading to the idea that intracellular ceramide accumulation is an important messenger in some signaling pathways leading to apoptosis (Gulbins *et al.*, 1995; Hannun and Obeid, 1995; Kolesnick and Fuks, 1995). Therefore we considered the possibility that ceramide could potentially substitute for/mimic signals induced by UV irradiation or Fas ligation, and thus trigger the apoptotic process in cytoplasmic extracts from untreated cells. As illustrated in Figure 6, this was found to be the case.

Rat liver or HeLa nuclei incubated for several hours in the presence of synthetic ceramide in cell extract buffer alone did not exhibit any apoptotic changes (Figure 6A), and nor did nuclei cultured in cytoplasmic extracts from untreated cells in the absence of ceramide, as before (Figure 6B). In contrast, the exposure of nuclei to synthetic ceramide in the presence of cytoplasmic extract resulted in a significant increase in apoptotic nuclei in these extracts (Figure 6C–E). This effect was seen in CEM cell extracts as well as extracts from myelomonocytic U937 cells, and was dependent upon the dose of ceramide added (Figure 6F). Apoptotic changes were observed as early as 30 min after the addition of ceramide, and peak apoptosis >60% was observed in some experiments (data not shown).

Ceramide induces apoptotic effects in nuclei formed de novo, and bypasses a requirement for a heavy membrane fraction

Extracts prepared from *Xenopus* eggs support the *de novo* formation of nuclei upon the addition of sperm DNA. Under certain conditions, these nuclei subsequently undergo profound apoptotic changes and disintegrate within a few hours (Newmeyer *et al.*, 1994). As reported previously, the destruction of nuclei in this system is dependent upon the presence of a heavy membrane fraction enriched in mitochondria in these extracts (Newmeyer *et al.*, 1994). Based on our observations described above, we also examined the effects of synthetic ceramide in this cell-free system.

First we re-confirmed that the removal of heavy membranes from *Xenopus* egg extracts allowed the formation of nuclei without supporting subsequent apoptotic changes. Nuclei formed in membrane-depleted extracts at the same frequency as in the presence of the heavy membrane fraction, but did not undergo apoptosis (Figure 7A). The addition of the heavy membrane fraction (present at 1/100 volume of the egg extract) supported apoptotic changes and the destruction of nuclei (Figure 7A).

Strikingly, the requirement for a heavy membrane fraction was effectively bypassed by the addition of synthetic ceramide to the extract (Figure 7B). Fractionated extracts supported the formation of nuclei, but in the presence of ceramide these nuclei then underwent apoptotic changes. This effect was dose dependent, and the changes observed were indistinguishable from those seen in the presence of the mitochondrial fraction (results not shown). In contrast, dihydro-C2-ceramide, which lacks signaling function, did not induce apoptotic changes in these extracts (Figure 7). Thus, ceramide appears to be a potent stimulus for apoptosis in diverse cell-free systems.

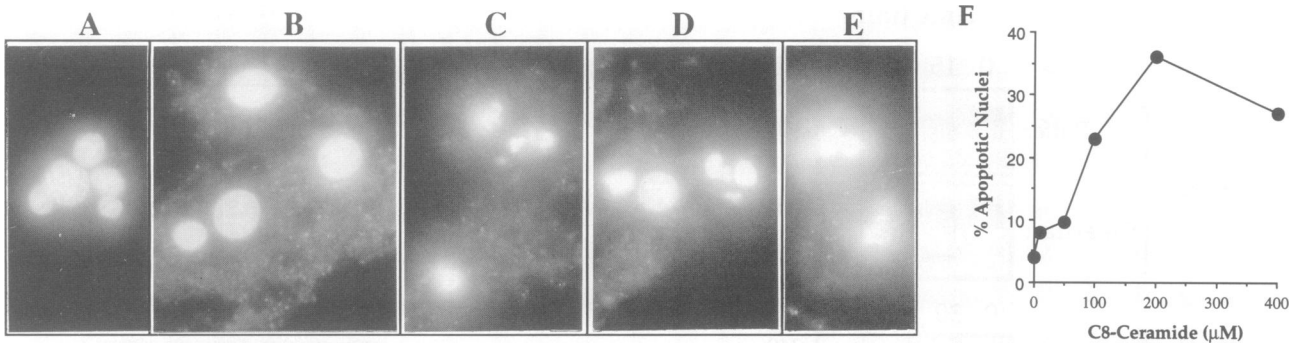

Fig. 6. Synthetic ceramides induce apoptotic activity in control extracts. (**A**) The majority of rat liver nuclei incubated with 40 μM C2-ceramide for 4 h have the same appearance as untreated nuclei (results not shown). The frequency of 'degenerate' nuclei was 21%. (**B**) Rat liver nuclei cultured for 4 h with CEM cell extract alone (without ceramide). The frequency of 'degenerate' nuclei was 25%. (**C–E**) Rat liver nuclei cultured for 4 h in CEM cell extract containing 40 μM C2-ceramide exhibit condensation and fragmentation. The frequency of these nuclear structures was 41.2%. (**F**) Apoptosis of HeLa cell nuclei incubated for 30 min in cytoplasmic extract from U937 cells in response to the indicated doses of C8-ceramide. Dimethylsulfoxide (2%) was added to the control (0). A minimum of 400 nuclei was counted for each group. Photographs (A–E) are from a single experiment. (F) contains data from a different experiment. Results shown are representative of five independent experiments.

Discussion

The studies presented here represent approaches to the *in vitro* reconstitution of the biochemical events mediating apoptosis induced by Fas ligation, UV radiation and ceramide. Extracts prepared from cells that were induced to undergo apoptosis following a brief exposure to UV light or ligation of the Fas molecule contain an activity that causes profound changes in added nuclei, characteristic of those seen during apoptosis in intact cells (Figure 1). These changes are associated with oligonucleosomal DNA fragmentation (Figure 3), an event that accompanies apoptosis in many cell types (Wyllie, 1980) including those used in our study. Thus, extracts from cells induced to undergo apoptosis (but in which no obvious apoptotic changes are yet apparent) produced apoptotic effects in normal nuclei. This is consistent with observations in cytoplasts (Jacobson *et al.*, 1994; Schulze-Osthoff *et al.*, 1994) and other cell-free systems (Lazebnik *et al.*, 1993; Newmeyer *et al.*, 1994), suggesting that while impacting on the nucleus, the apoptotic program is predominantly extranuclear.

The nuclear changes induced by 'apoptotic' extracts were gradual and displayed a reproducible progression of intermediate forms (Figure 2). Within a few minutes of the addition to 'apoptotic' extracts, chromatin was observed to condense along the nuclear envelope (margination), leaving a clearly visible nucleolus (Figure 2) which subsequently moved to the periphery. A similar sequence of events has been described previously for nuclei undergoing apoptosis in intact cells. Upon further incubation in the extracts, the accumulation of condensed chromatin into a few clusters dominates the nucleus, giving it a fragmented appearance. Ultimately, the nucleus dissociates, and these clusters disperse. It is likely that these morphological changes occur, at least in part, as a result of DNA cleavage (Arends *et al.*, 1990), although such chromatin condensation in apoptosis can occur without the generation of internucleosomal DNA cleavage (G.M.Cohen *et al.*, 1992; Oberhammer *et al.*, 1993). In one elegant study, some of these morphological events were very rapidly induced in isolated nuclei by exposure to bacterial endonuclease (Arends *et al.*, 1990), suggesting that the activation of a nuclease in our system may account for some of the observed effects. The difference in kinetics between our system and that employing the bacterial endonuclease (1 h versus 10 min) indicates that it is not simply the presence of a nuclease in the 'apoptotic' extracts that produces these effects. Instead, we consider it likely that

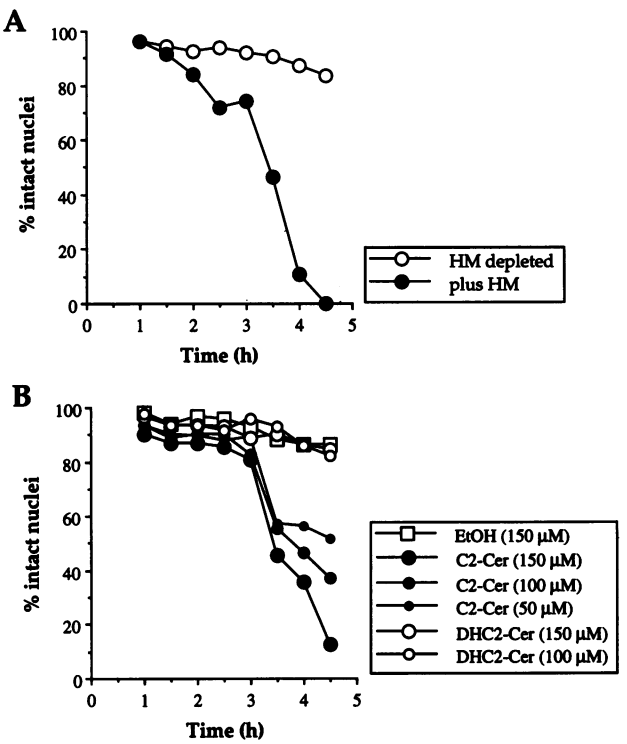

Fig. 7. Ceramide bypasses the requirement for a heavy membrane component for apoptotic effects in *Xenopus* egg extracts. Apoptotic extracts were prepared from *Xenopus* eggs and the heavy membrane fraction was removed as described previously (Newmeyer *et al.*, 1994). The addition of frog sperm chromatin to these extracts resulted in the *de novo* formation of nuclei (Newmeyer and Wilson, 1991). C2-Ceramide, at the indicated concentrations, was added at time 0, and nuclei were examined at intervals thereafter. (**A**) Effect of the addition of a heavy membrane fraction (1/100 dilution) on cell-free apoptosis. (**B**) Effect of the addition of C2-ceramide or dihydro-C2-ceramide on cell-free apoptosis. Similar results were obtained in three independent experiments.

these extracts contain an activity that functions to stimulate an endogenous endonuclease to cleave DNA.

Bcl-2, which blocks apoptosis under a variety of conditions (Hockenbery *et al.*, 1990; Korsmeyer, 1992; Miyashita and Reed, 1993; Reed, 1995), at least partially interferes with the nuclear changes seen in this system (Figure 5), which lends further support to the idea that the observed effects are representative of an apoptotic process. This effect was observed using both purified baculovirus-derived Bcl-2 protein, as well as Bcl-2-containing *sf*9 cell lysates. This finding is similar to previous observations that both purified recombinant Bcl-2 (D.D.Newmeyer *et al.*, unpublished observations) and a Bcl-2-containing *sf*9 lysate (Newmeyer *et al.*, 1994) block apoptotic effects in a *Xenopus* egg cell-free system. Clearly, both systems will provide powerful tools for the analysis of the function of Bcl-2 and other apoptosis-repressor proteins.

Recently we have reported that the proteolytic cleavage of α-fodrin (non-erythroid spectrin) accompanies apoptosis induced by UV irradiation, ceramide, staurosporine or the ligation of Fas in intact cells (Martin *et al.*, 1995). Cytoplasmic extracts of UV-irradiated or anti-Fas antibody-treated cells similarly contain a fodrin-cleaving protease (Figure 4), which may be calpain I (Martin *et al.*, 1995). Calpain activation has been observed during certain instances of apoptosis (Squier *et al.*, 1994), and calpain inhibitors interfere with apoptosis in some systems (Sarin *et al.*, 1993). The role of fodrin in the cytoskeleton and its perceived role in controling cell shape suggest that its cleavage may be partly responsible for the plasma membrane changes that take place during apoptosis (Martin *et al.*, 1995).

Other proteases that have been implicated in apoptosis are ICE and its related family members (Miura *et al.*, 1993; Yuan *et al.*, 1993; Fernandes-Alnemri *et al.*, 1994; Wang *et al.*, 1994; Kuida *et al.*, 1995). ICE is a homolog of the Ced-3 protein of *Caenorhabditis elegans*, which is required for the programmed cell deaths that are seen during the development of this worm (Yuan *et al.*, 1993). Furthermore, the ectopic expression of ICE and other ICE family proteases have been found to induce apoptosis in mammalian cells (Miura *et al.*, 1993; for a review see Martin and Green, 1995b). Crm A, a cowpox virus-derived ICE inhibitory protein, blocks apoptosis in some systems (Gagliardini *et al.*, 1994), including that induced by the ligation of Fas (Tewari and Dixit, 1995). Animals with targeted disruption of the ICE gene show normal development (Li *et al.*, 1995) but may have a defect in Fas-induced apoptosis (Kuida *et al.*, 1995). This is consistent with our observation that a peptide competitive inhibitor of ICE function partially interferes with apoptosis induced by extracts of UV-irradiated or anti-Fas antibody-treated cells (Figure 5). Such peptides have also been shown to interfere with apoptosis in the chicken hepatoma cell-free system (Lazebnik *et al.*, 1994).

Ceramide, a lipid second-messenger product of the sphingomyelin pathway (Kolesnick and Golde, 1994), has been shown to trigger a ceramide-activated kinase (Joseph *et al.*, 1993), a phosphatase (Dobrowsky and Hannun, 1992) and the Vav proto-oncogene product (Gulbins *et al.*, 1994). Ceramide is unique among lipids in its ability to induce apoptosis in a number of different cell lines (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Gulbins *et al.*, 1995). This has raised the interesting possibility that the sphingomyelin pathway and other routes to the production of ceramide may have important functions in the induction of apoptosis. The sphingomyelin pathway is rapidly induced by tumor necrosis factor (Obeid *et al.*, 1993; Jarvis *et al.*, 1994), the ligation of Fas (Cifone *et al.*, 1994; Gulbins *et al.*, 1995), γ radiation (Haimovitz-Friedman *et al.*, 1994) and UV radiation (R.N.Kolesnick, unpublished observations), all of which are capable of inducing apoptosis. The cytotoxic drug daunorubicin similarly induces apoptosis via the intracellular generation of ceramide, although this appears to be as a consequence of the induction of ceramide synthase (Bose *et al.*, 1995). Thus, intracellular ceramide accumulation may occur in different forms of apoptosis via different routes.

Therefore we considered the possibility that ceramide might mimic the action of UV radiation or Fas ligation in inducing apoptotic activity *in vitro*. The addition of synthetic ceramide to cytoplasmic extracts of untreated cells caused apoptotic changes in added nuclei (Figure 6), which were not observed if nuclei were exposed to ceramide in the absence of cell extract. This suggests not only that ceramide can serve as an apoptosis-inducing signal, but that the activation of the apoptotic pathway can occur entirely *in vitro*. However, it should be noted that the morphological changes in mammalian nuclei induced by the addition of ceramide to normal human cell extracts were not as dramatic as those observed when extracts from UV- or anti-Fas antibody-primed cells were employed (compare Figure 2 with Figure 6). Thus, it is possible that some parts of the apoptotic pathway fail to be engaged under the conditions of this ceramide-induced *in vitro* system.

Another cell-free system for the analysis of apoptosis was described recently (Newmeyer *et al.*, 1994) which involves the use of extracts from *Xenopus* eggs. The addition of sperm DNA to these extracts leads to the *de novo* formation of functional nuclei, which subsequently undergo apoptotic changes. The generation of this 'apoptotic' extract appears to depend critically upon the regimen of hormone injections and the time at which the eggs are laid, because not all extracts support apoptosis. We examined the effect of synthetic ceramide on a non-apoptotic or control extract and observed that ceramide induced profound apoptotic changes in sperm nuclei with kinetics very similar to those shown in Figure 7 (results not shown).

Apoptosis in the *Xenopus* egg extract system depends upon the presence of a heavy membrane fraction that is enriched in mitochondria (Newmeyer *et al.*, 1994). This fraction need not come from apoptotic cells, and can be provided by a variety of sources of heavy membranes (D.D.Newmeyer and D.M.Farschon, unpublished observations). This requirement is shown in Figure 7A. We observed that the effect of ceramide in this system bypasses the need for this heavy membrane fraction (Figure 7B). It is possible that this fraction is simply a source of ceramide or sphingomyelin which becomes hydrolyzed to ceramide because of the action of a sphingomyelinase in the extract. It is also possible that ceramide induces an apoptotic pathway that proceeds independently of the requirement for heavy membranes. If so, then this suggests

that there may be fundamentally different routes to apoptosis that nevertheless culminate in the same effects.

In conclusion, we have described a versatile cell-free system which effectively reconstitutes some of the changes associated with apoptosis (nuclear condensation and fragmentation, DNA cleavage, fodrin proteolysis) and permits the analysis of apoptosis induced in cells by UV radiation and ligation of the Fas molecule. Furthermore, we expect that the system can be adapted to most forms of apoptosis induction. In addition, the system allows the analysis of induction of apoptosis *in vitro* by the lipid second messenger ceramide. Its inhibition by Bcl-2 and an ICE substrate peptide provides further insights into the process, and demonstrates the potential for a detailed analysis of the mechanisms of action of pro- and anti-apoptotic molecules in this system. Finally, our observations add further strong support to the idea of apoptosis as a cytoplasmic phenomenon, because the cell-free apoptotic activity was completely independent of the presence (as assessed by fodrin proteolysis) or the source of nuclei.

Materials and methods

Induction of apoptosis

Cells were induced to undergo apoptosis either by irradiation for 10 min on a UVB transilluminator, as described previously (Martin and Cotter, 1991), or by exposure to 100 ng/ml anti-Fas mAb (CH-11) for 60 min.

Preparation of cytoplasmic extracts from human cell lines

Cytoplasmic extracts were prepared essentially as described by Lazebnik *et al.* (1993), with modifications. Briefly, human CEM, Jurkat or U937 cells were incubated for various time periods under conditions designed to induce apoptosis (see Results) and were then pelleted at 200 *g*. Cells were washed twice with 50 ml PBS, pH 7.2, followed by a single wash with 5 ml of cell extract buffer [CEB; 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM cytochalasin B and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were pelleted and the supernatant aspirated. Then they were transferred to a 2 ml glass Dounce homogenizer in the remaining droplet of buffer. Cells were allowed to swell by the addition of an equal volume of CEB to the volume occupied by the packed cell pellet (~100 μl per 10^8 cells), followed by incubation on ice for 20 min. Cells were lysed gently with 20 strokes of a B-type pestle. Lysis was monitored by staining a small aliquot of the lysate with trypan blue and examining under the light microscope. The cell lysate was then transferred to a 1 ml Eppendorf tube and centrifuged at 4°C for 15 min at 14 000 *g*. The clear cytosol was carefully removed without disturbing the nuclear pellet and then diluted to 7.5–15.0 mg/ml with extract dilution buffer (EDB; 10 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine and 50 μg/ml creatine kinase). Extracts were either used immediately or frozen at –80°C for later use. Extracts stored in this way were found to lose little of their apoptosis-inducing activity.

Preparation of cytoplasmic extracts from Xenopus eggs

Cytoplasmic extracts were prepared and depleted of heavy membranes, as described previously (Newmeyer *et al.*, 1994).

Preparation of nuclei

Rat liver nuclei were prepared as described previously (Newmeyer and Wilson, 1991; Newmeyer *et al.*, 1994). CEM and HeLa nuclei were prepared as follows. Cells were harvested by centrifugation at 200 *g* for 10 min, followed by three 50 ml washes in PBS, pH 7.2, and one 15 ml wash with nuclei isolation buffer (NB; 10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B and 1 mM PMSF); they were resuspended in 10 volumes of this buffer. Cells were allowed to swell on ice for 20 min, followed by gentle lysis with a Dounce homogenizer. Liberated nuclei were then layered over 30% sucrose in NB and centrifuged at 800 *g* for 10 min, followed by washing in NB and resuspension in nuclei storage buffer (10 mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF and 50% glycerol) at 2×10^8 nuclei/ml. Nuclei were then stored at –80°C in 20 μl aliquots until required.

Reconstitution of the cell-free extract

Cell-free reactions (25 μl) comprised 20 μl of cytoplasmic extract (7.5–15.0 mg/ml protein), 1 μl (2×10^5) of either rat liver, CEM or HeLa nuclei from frozen stocks, and 4 μl of EDB, recombinant proteins or synthetic peptides diluted in this buffer. Extracts were then incubated at 37°C for various time periods.

Quantitation of cell-free apoptosis

At the time points indicated in the text, a 3.5 μl aliquot of the extract was removed and stained with 1.5 μl of a 10 μM solution of Hoescht 33342 in formalin on a glass slide. A coverslip was then placed on the slide, and slides were examined by fluorescence microscopy. Nuclei were scored as apoptotic if they exhibited margination and condensation of the chromatin similar to that observed in normal apoptotic cells (Kerr *et al.*, 1972; Martin *et al.*, 1994b). In general, apoptotic changes proceeded with remarkable synchrony in these extracts, as reported previously for extracts from chicken hepatoma cells (Lazebnik *et al.*, 1993). Apoptotic nuclei were easily distinguishable from normal nuclei (Figure 1). For the quantitation of apoptosis, a minimum of 150 nuclei from five to ten fields were evaluated for the changes described above.

Isolation and electrophoresis of DNA

After incubation in the cell-free extracts for various time periods, nuclei were lysed in TE buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA) containing 0.5% sodium lauroyl sarkosyl and 0.5 mg/ml proteinase K. Digestion was continued for 1–3 h at 50°C, followed by the addition of RNase A to 0.1 mg/ml and further incubation for 1 h. Running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol) was then added in a 1:6 ratio of dye:sample, and DNA preparations were electrophoresed in 1.5% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 4 V per cm of gel. DNA was visualized by ethidium bromide staining.

Electrophoresis and Western blotting of proteins

Cell extracts were assayed for protein content using the Bio-Rad microassay. Prior to loading on the gel, bromophenol blue dye was added to each sample (0.002% final concentration), equal amounts of total protein were loaded per lane and proteins were separated under reducing conditions for 2 h at 70 V in 4–15% gradient SDS–polyacrylamide gels. Separated proteins were then Western blotted at 150 mA overnight. Blots were blocked for 1 h in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dried milk, and then probed for 2 h with an appropriate dilution of the primary antibody diluted in the same buffer, washed for 1 h in several changes of TBST and probed for a further 1 h with a peroxidase-coupled secondary antibody (Amersham, UK). Bound antibody was detected by enhanced chemiluminescence (Amersham).

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Note added in proof

After submission of this paper, Chow *et al.* (1995) (*FEBS Lett.*, **364**, 134–138) reported a similar Fas-primed cell-free system.