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Intracellular acidification induces apoptosis by stimulating ICE-like protease activity

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SUMMARY

ICE-like protease activation and DNA fragmentation are preceded by a decrease in intracellular pH (pH_i) during apoptosis in the IL-3 dependent cell line BAF3. Acidification occurs after 7 hours in cells deprived of IL-3 and after 4 hours when cells are treated with etoposide, close to the time of detection of ICE-like protease activity. Increasing extracellular pH reduces ICE-like protease activation and DNA fragmentation. Bcl-2 over-expression both delays acidification and inhibits ICE-like protease activation. Generation of a rapid intracellular pH decrease, using the ionophore nigericin, induces ICE-like protease activation and apoptosis. ZVAD, a cell permeable inhibitor of ICE-like proteases, does not affect acidification but inhibits apoptosis induced by IL-3 removal or nigericin treatment. These data suggest that intracellular acidification triggers apoptosis by directly or indirectly activating ICE-like proteases.

Key words: Interleukin-3, Intracellular pH, ICE protease, bcl-2, Apoptosis

INTRODUCTION

Growth and survival factors regulate programmed cell death (apoptosis) both during development and when controlling the turnover of adult cells (Raff, 1992). Studies on cells in culture have demonstrated that cells enter apoptosis in the absence of sufficient growth factors, particularly when cell cycle regulators such as c-myc or E2F are inappropriately expressed (Evan et al., 1992; Qin et al., 1994). Measurements of pH_i have shown a correlation between intracellular acidification and the onset of apoptosis. A population of cells with a low pH_i was observed in a T lymphocyte cell line deprived of the growth factor interleukin-2 (IL-2) (Li and Eastman, 1995; Rebollo et al., 1995), in neutrophils dying in the absence of survival factors (Gottlieb et al., 1995b), in HL60 and ML-1 cells treated with etoposide (Barry et al., 1993) and in Jurkat cells induced to enter apoptosis by u.v. radiation, cycloheximide or anti-Fas antibody (Gottlieb et al., 1996). The sub-population with lower pH appeared roughly at the time of onset of DNA fragmentation and when cells with low pH were sorted these were shown to have apoptotic morphology (Gottlieb et al., 1995b; Barry et al., 1993). Thus, acidification is a potential signal for the final, irreversible stages of cell death.

Genes that regulate apoptosis are highly conserved across evolution. Ced3, a *Caenorhabditis elegans* gene essential for cell death, is homologous to a family of mammalian cysteine proteases, the IL-1β converting enzyme (ICE)-like proteases (Yuan et al., 1993). Members of this family of proteases are believed to be critical for mammalian cell apoptosis because inhibitors of ICE-like proteases such as viral proteins or synthetic peptides can block apoptosis (Nicholson et al., 1995; Tewari and Dixit, 1995; Xue and Horvitz, 1995; Fearnhead et al., 1995). ICE-like proteases are synthesised as pro-enzymes and cleaved to form the active enzyme (Nicholson et al., 1995; Tewari et al., 1995b); in vitro this cleavage can be autocatalytic or catalysed by other ICE-like proteases (Kumar and Harvey, 1995) and in vivo there is evidence for an ICE-like proteolytic cascade (Enari et al., 1996). Fas or TNF receptor-induced apoptosis is triggered by recruitment of an ICE-like protease to the activated receptor (Boldin et al., 1996; Muzio et al., 1996). However, the mechanism by which the ICE-like protease cascade is activated during apoptosis following signals such as growth factor deprivation is not known.

The mechanism by which ICE-like proteases stimulate apoptosis is also not known but a number of abundant nuclear proteins have been shown to be their proteolytic substrates. CPP32/YAMA/apopain, TX, Nedd-2, Mch-3 and ICE-LAP6 have all been shown to cleave poly (ADP-ribose) polymerase (PARP) (Tewari et al., 1995b; Gu et al., 1995; Fernandes-Alnemri et al., 1995; Duan et al., 1996). Cleavage occurs during apoptosis at a tetrapeptide sequence identical to one of two sites in pro-IL1β cleaved by ICE (Lazebnik et al., 1994) and detection of specific PARP cleavage fragments can be used as an assay for ICE-like protease activation (Nicholson et al., 1995). Other nuclear proteins, including nuclear lamins (Lazebnik et al., 1995), histones, topoisomerases (Voelkel et al., 1995), U1 small ribonucleoprotein (Tewari et al., 1995a; Casciola-Rosen et al., 1994), the catalytic subunit of DNA dependent protein kinase (DNA-PKcs) (Song et al., 1996) and the cytoplasmic proteins actin (Mashima et al., 1995) and D4-GDI (a member of the rho superfamily) (Na et al., 1996), have all been shown to be cleaved by ICE-like proteases during apoptosis.

The bone marrow cell line, BAF3, is dependent on IL-3 for growth in culture and enters apoptosis on IL-3 removal (Rodriguez-Tarduchy et al., 1990). DNA fragmentation and loss of cell viability occur when IL-3 is removed, but only after a delay of at least 8 hours (Rodriguez-Tarduchy et al., 1990). New gene expression is not required for entry into apoptosis and IL-3 can rescue cells when readded close to the time of fragmentation (Rodriguez-Tarduchy et al., 1990; Collins et al., 1992). Apoptosis following factor removal is inhibited by overexpression of the bcl-2 protein, which allows cell survival, but does not promote cell growth (Marvel et al., 1993). Bcl-2 protein is associated with a variety of intracellular membranes and homodimerises and heterodimerises with a family of bcl-2 related proteins to inhibit apoptosis (Korsmeyer, 1995). Signalling events closely coupled to the IL-3 receptor, such as kinase activation, decrease within minutes of IL-3 removal from BAF3 cells (Marvel et al., 1993). Here we describe an intracellular signal, acidification, that occurs roughly at the time when the final, irreversible changes of apoptosis begin. In BAF3 cells, acidification stimulates apoptosis by activating an ICE-like protease(s).

MATERIALS AND METHODS

Cell culture and reagents

BAF3 cells (obtained from Dr Ronald Palacios, Basel Institute for Immunology) (Palacios and Steinmetz, 1985) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) and 5% WEHI-3B cell-conditioned medium which was used as a source of IL-3 throughout. BAF3 cells expressing human bcl-2 (clone Bcl15) were previously described (Marvel et al., 1993). Cells were grown at a density of 5×10^4 to 5×10^5 cells/ml at 37°C in a humidified atmosphere with 10% CO_2. To remove IL-3, cells were washed twice in DMEM at 37°C. pH_i was altered by incubating cells at 37°C in humidified air in serum-free simple medium at defined pH (140 mM NaCl, 5 mM KCl, 25 mM Tris/Hepes, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 5g/l D-glucose, pH adjusted at 37°C). Experiments with nigericin were performed in these defined media plus 5 mM or 130 mM KCl. Nigericin was obtained from Sigma, etoposide from Bristol-Myers Pharmaceuticals, ZVAD from Enzyme Systems Products, Dublin, USA. Bongkrekic acid was the gift of Professor J. A. Duine (Dept of Microbiology/Enzymology, Delft University of Technology, Netherlands).

Cell cycle analysis

Cells (5×10^6) were pelleted, resuspended in 0.2 ml phosphate-buffered saline (PBS) and fixed by the addition of 1 ml ice-cold 70% ethanol/30% PBS. Fixed cells were pelleted, gently resuspended in PBS and incubated for 30 minutes at 37°C with 100 μg/ml RNase A and 40 μg/ml propidium iodide. The fluorescence of stained cells was analysed using the software LYSIS II on a FACScan (Becton and Dickinson). Apoptotic cells, identified by sub-G_1 DNA content, were quantitated as a percentage of total cells.

Intracellular pH measurements by FACScan

pH_i measurements were performed according to the method detailed by Rabinovitch and June (1990). Briefly, for the final 30 minutes of each experiment, 1×10^6 cells were incubated with 20 μM carboxy-SNARF-1-AM acetate (Calbiochem) in 200 μl of the experimental medium at 37°C. Loaded cells were then washed in electrolyte solution (5 mM Pipes/Tris, pH 7.2, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose), resuspended in fresh electrolyte solution and incubated at 37°C for 5 minutes before analysis on FACScan. Intracellular carboxy-SNARF-1 was excited at 488 nM and emission measured at 575 and 670 nM using linear amplifiers. pH_i values were determined by comparing mean 575/670 nM fluorescence ratio values to a calibration curve constructed by incubating carboxy-SNARF-1-AM-loaded BAF3 cells in a range of pH buffers in the presence of the ionophore nigericin at 2 μg/ml. pH calibration buffers were made by mixing 20 mM Pipes or 20 mM Tris in 130 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose to give a range of pH 5-8 at 37°C. To represent the fluorescence of each SNARF-1-AM labelled cell, a 2-dimensional dot plot with 575 nM fluorescence on the *x*-axis (SNARF-acid) and 670 nM fluorescence on the *y*-axis (SNARF-base) was displayed. On this plot, the distance of each cell from the origin is directly proportional to the amount of SNARF-1-AM loaded in the cell and the ratio of 575/670 nM fluorescence is a measure of pH_i.

Western blotting

Cells (5×10^5 per track) were lysed in gel sample buffer (186 mM Tris-HCl, pH 6.8, 6% SDS, 27.5% glycerol, 15% β-mercaptoethanol, 0.6% bromophenol blue), boiled and sonicated before size fractionation of proteins on 12.5% SDS-PAGE. This was to enable resolution of the 25 kDa ICE-like protease PARP cleavage product; intact PARP could not be quantitated. Proteins were transferred to nitro-cellulose membranes by electroblotting. Membranes were blocked overnight at 4°C with 5% milk powder in PBS before incubation at room temperature for 2 hours with anti-PARP antibody. The anti-PARP antibody used (anti-FII) was a polyclonal rabbit serum raised against a synthetic polypeptide corresponding to the second zinc finger of the DNA binding domain of human PARP (gift of Gilbert de Murcia, CNRS Illkirch, Strasbourg). This detects a 25 kDa fragment after PARP cleavage. HRP-conjugated goat anti-rabbit antibody and ECL (Amersham, UK) were used to detect anti-PARP antibody.

DNA fragmentation analysis by gel electrophoresis

In order to isolate apoptotic DNA fragments from treated cells, the method of Herrmann et al. (1994) was followed: 10^6 cells were washed in PBS and pelleted by centrifugation. Cell pellets were then lysed for 10 seconds with 50 μl lysis buffer (1% NP40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation at 1,300 ***g*** for 20 seconds, the supernatant was removed and extraction from the pellet repeated. Apoptotic DNA fragments were recovered from the supernatants by adding SDS to 1% and treating with 5 μg/ml RNase A for 2 hours at 56°C followed by digestion with proteinase K (2.5 μg/ml) for 2 hours at 37°C. DNA was precipitated with 0.5 vol. 10 M ammonium acetate and 2.5 vols ethanol, dissolved in gel loading buffer and separated by electrophoresis in 1.2% agarose gels.

Measurement of DNA fragmentation by [^{3}H]thymidine release

Cells (2×10^5 cells/ml) were incubated overnight with 1 μCi/ml of [^{3}H]thymidine (3,000 Ci/mMol, Amersham, UK) then washed twice before incubation for the time shown. Cells were harvested using a Dynatech cell harvester (Dynatech Labs Ltd Sussex, UK). [^{3}H]Thymidine remaining was measured by scintillation counting.

RESULTS

Fig. 1A shows FACScan scatter plots of BAF3 cells labelled with SNARF-1-AM (SemiNapthoRhodaFluor), a cell permeable probe which exhibits changes in fluorescence depending on pH_i (Bassnett et al., 1990). The plots are divided into regions R1 and R2 by a line that includes 90% control cells growing in IL-3 in R1. When the mean SNARF-acid/SNARF-base ratio of cell in R1 was compared to a calibration curve (see Materials and Methods) it was determined that cells in R1

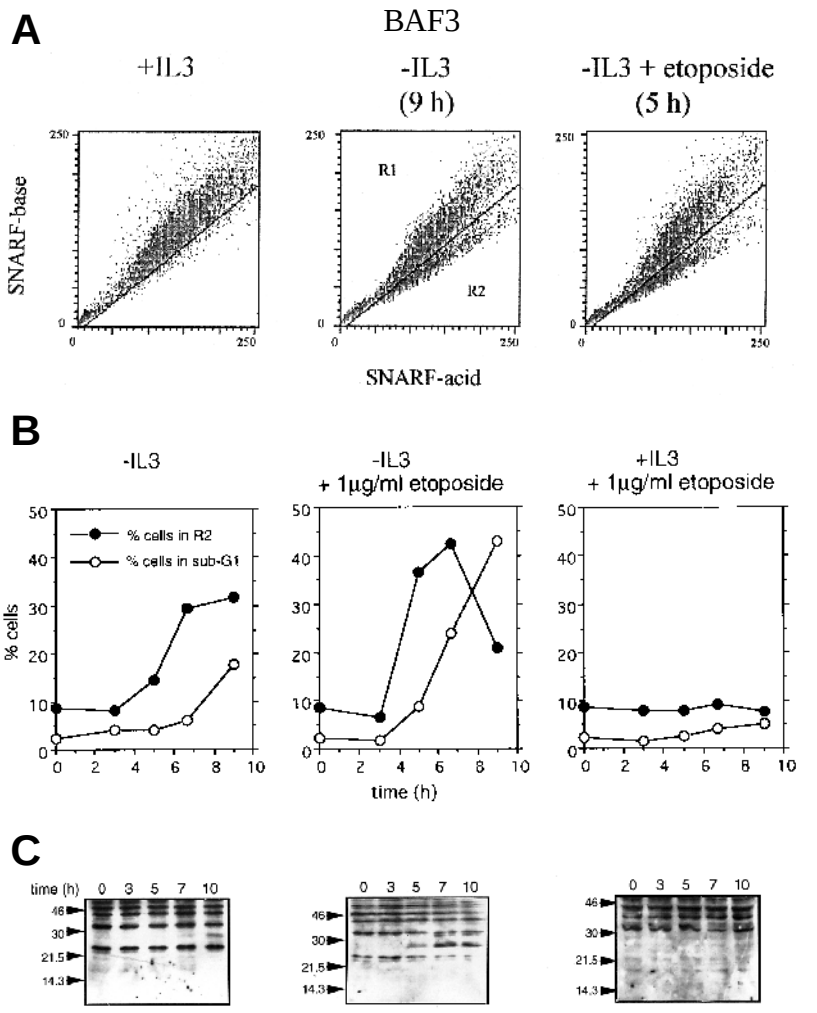

Fig. 1. PARP cleavage and DNA fragmentation are preceded by a decrease in intracellular pH. (A) BAF3 cells, labelled with SNARF-1 for the final 30 minutes of the treatment indicated, were displayed on a two-dimensional scatter plot according to fluorescence intensity at 575 nM (SNARF-acid) and 670 nM (SNARF-base). Representative scatter plots from 1 of 3 separate experiments, where region R1 included 90% of cells in the presence of IL-3, are shown. Comparison with a calibration curve performed in parallel (see Materials and Methods) demonstrated that cells in R1 had an intracellular pH of 7.30±0.06 (mean ± s.e., 3 experiments). R2 defines cells in which the acid form of SNARF-1-AM predominates; their pH was measured as 6.90±0.03 (mean ± s.e., 3 experiments). 9 hours after the removal of IL-3 28.8±2.8% (mean ± s.e., 3 experiments) of cells were in R2. After 5 hours treatment with 1 μg/ml etoposide in the absence of IL-3, 30.6±6.1% (mean ± s.e., 3 experiments) of cells were in R2. (B) Parallel samples of cells were taken for analysis of intracellular pH and cell cycle distribution at the indicated times after treatment. The percentage of cells in R2 and sub-G_1 were determined using LYSIS II software. Results are representative of 3 separate experiments. (C) Whole cell extracts were prepared from parallel samples of cells to those displayed in B. After SDS-PAGE proteins were transferred to nitrocellulose and probed with polyclonal anti-FII antibody, which detected a 25 kDa PARP-cleavage product.

have a mean pH of 7.3. Fig. 1A shows that when apoptosis was induced by IL-3 removal or etoposide treatment in the absence of IL-3, a discrete population of cells appeared in R2. Cells in R2 had a pH 0.4 units lower than those in R1. Cells with low pH had maintained their membrane integrity as determined by PI exclusion (data not shown). Fig. 1B shows that the drop in pH preceded entry of cells into apoptosis, defined by the appearance of cells in a population with sub-G_1 DNA content. At 7 hours after IL-3 removal 30% of cells had decreased pH. This proportion of cells with low pH was observed approximately 4.5 hours after cells were treated with 1 μg/ml etoposide in the absence of IL-3, which accelerated the appearance of apoptotic cells (Collins et al., 1992; and Fig. 1B). IL-3 can protect BAF3 cells from apoptosis induced by DNA damaging agents (Collins et al., 1992). Fig. 1B shows that when cells were treated with etoposide in the presence of IL-3, which inhibits apoptosis (Collins et al., 1992), no cells were found in the low pH population confirming that the appearance of cells with a low pH correlates with the onset of apoptosis.

Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) occurs during apoptosis (Kaufmann et al., 1993); it is among the substrates of ICE-like proteases (Fernandes-Alnemri et al., 1995; Gu et al., 1995; Tewari et al., 1995b). As a marker for ICE-like protease activity, proteolytic cleavage of PARP was measured by probing western blots with the anti-FII antibody, which detects a 25 kDa cleavage fragment of PARP (Kaufmann et al., 1993). Fig. 1C shows that PARP cleavage occurred in BAF3 cells when apoptosis was triggered by IL-3 removal or etoposide treatment in the absence of IL-3. The 25 kDa PARP cleavage fragment was detected 7 hours after factor removal. When apoptotic death was accelerated by the addition of etoposide, the 25 kDa PARP cleavage fragment first appeared after 5 hours; this cleavage was inhibited by the presence of IL-3. This confirms previous observations that PARP cleavage occurs at a similar time to the onset of DNA fragmentation (Kaufmann et al., 1993) and demonstrates that, in BAF3 cells, PARP cleavage is first detected after intracellular acidification.

We therefore examined the effect of altering extracellular pH, using serum-free simple media without CO_2/HCO_3, buffered at pH 7.3 or 7.9, on the onset of PARP cleavage and DNA fragmentation. After a 4 hour incubation in these media, in the presence of 1 μM etoposide and the absence of IL-3, a single population with average pH_i 7.7 was observed when cells were incubated in media at pH 7.9. PARP cleavage was induced more rapidly in these defined media than in the more physiological tissue culture medium with serum (compare Figs 2A and 1C) and long-term viability was difficult to achieve. However, PARP cleavage induced by etoposide was clearly reduced by increasing the pH of the external medium to pH 7.9

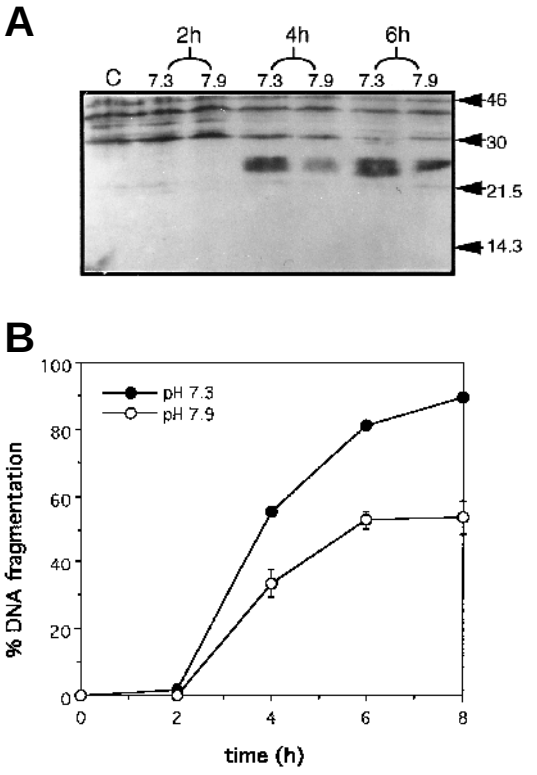

Fig. 2. Raising extracellular pH delays apoptosis. (A) Cells treated with 1 μg/ml etoposide in the absence of IL-3 were incubated in serum-free simple buffered medium at pH 7.3 and 7.9 (see Materials and Methods). Samples were prepared at the indicated times for PARP cleavage analysis by western blot. Calculation of the relative intensity of the 25 kDa band by densitometry, showed that at 4 hours the amount of PARP cleavage was 2.4-fold less in cell extract from cells incubated at pH 7.9 compared to that from cells incubated at pH 7.3. Note that apoptosis occurs more rapidly in these defined media than in the more physiological tissue culture medium with serum which has been used elsewhere unless stated. (B) DNA fragmentation was quantitated by measurement of release of [^{3}H]thymidine labelled DNA, from cells incubated in serum-free simple buffered media at pH 7.3 and 7.9, with 1 μg/ml etoposide in the absence of IL-3. Release was expressed as a % of total DNA; each point represents the mean ± s.e. of triplicates.

(Fig. 2A). DNA fragmentation was quantitated by measuring release of [^{3}H]thymidine labelled DNA. Fig. 2B shows that cells incubated at pH 7.9 showed a 2-fold reduction in fragmentation compared to incubation at pH 7.3. This suggests that the pH drop is one of the signals which contributes to activation of ICE-like protease(s) and DNA fragmentation.

BAF3 cells over-expressing human bcl-2 protein (Bcl15) survive up to 7 days in the absence of IL-3 and accumulate in the G_1 phase of the cell cycle after 24 hours (Marvel et al., 1993). Fig. 3A shows Bcl15 cells labelled with SNARF-1-AM, with R1 again drawn to include 90% of control cells. Bcl15 cells growing in IL-3 had a pH_i of 7.3. Although no cells became apoptotic on IL-3 removal, cells did appear in the low pH population R2. However, the timing of the pH drop was different to that in BAF3 cells. Fig. 3A shows no shift at

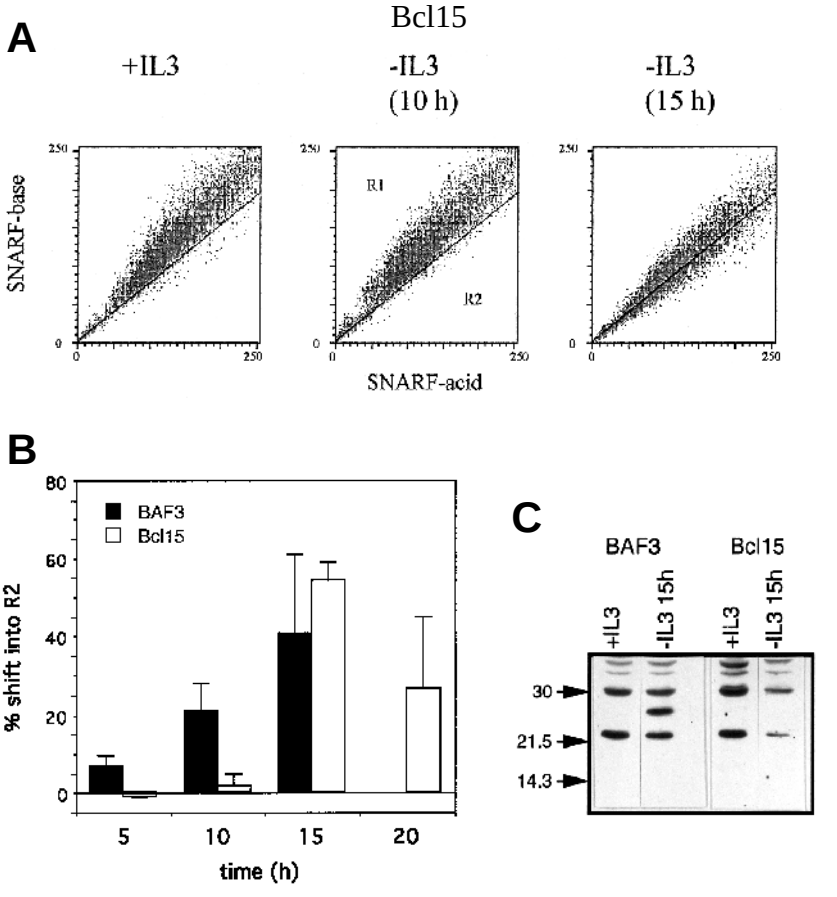

Fig. 3. Cells over-expressing bcl-2 (Bcl15) show a delayed pH decrease when deprived of IL-3. (A) Scatter plots of SNARF-1-AM labelled Bcl15 cells in the presence of IL-3, or 10 and 15 hours after IL-3 removal. Plots are representative of 4 separate experiments. Cells in R1 had an intracellular pH of 7.30±0.09 (mean ± s.e., 4 experiments) whereas those in R2 had a pH of 6.94±0.18. The % of cells in R2 in the experiment shown was: +IL-3= 8.1%, –IL-3 10 hours = 9.8%, –IL-3 15 hours = 45.9%.(B) The percentage shift of cells into R2 after IL-3 removal was determined for BAF3 and Bcl15 cells. Mean shifts into R2 ± s.e. of 4 experiments are shown. (C) Whole cell extracts from BAF3 and Bcl15 cells, either incubated in IL-3 or deprived of IL-3 for 15 hours, were analysed for PARP cleavage.

10 hours in the Bcl15 cells whereas at 15 hours a high proportion of cells were seen in R2. The percentage shift of parental BAF3 or Bcl15 cells into R2 with time is compared in Fig. 3B. Although BAF3 cells began to show a sub-population with a low pH at 5 hours increasing to a maximum of 40% at 15 hours, Bcl15 cells showed no shift at 5 or 10 hours but at 15 hours 55% of cells had a pH of 0.4 units less than control cells. G_1-arrested cells stayed in this low pH population at 20 hours after IL-3 removal (Fig. 3B). A similar delay in pH decrease was observed when 2 parental cell clones (BO6 and BO4) were compared to 2 Bcl-2 over-expressing cell clones (Bcl14 and Bcl15) (data not shown). Proteolytic cleavage of PARP was also analysed; Fig. 3C shows that no PARP cleavage was observed 15 hours after IL-3 removal in Bcl15 cells and PARP cleavage was not observed in further experiments 48 hours after IL-3 removal (data not shown). Therefore, delayed intracellular acidification occurs in the presence of Bcl-2 but this is not sufficient to activate ICE-like protease(s) and apoptosis.

In order to determine whether a decrease in intracellular pH could activate ICE proteases and DNA fragmentation, we used the ionophore nigericin. Since nigericin is an H^+/K^+ exchanger, it produces the equilibrium $[H_i^+]/[H_o^+]=[K_i^+]/[K_o^+]$. As $[K_i^+]$ is approximately 130 mM, a rapid equilibration of pH_i with pH_o occurs when cells are incubated in medium containing 130 mM K^+ and 5 μg/ml nigericin in the absence of CO_2/HCO_3. After incubation for 10 minutes under these conditions, flow cytometry demonstrated that the mean pH_i was 6.3 when pH_o was 6.5 and 7.4 when pH_o was 7.2 (data not shown). Fig. 4A shows that rapid PARP cleavage (within 60 minutes) was induced by nigericin, but only when cells were incubated in 130 mM K^+ at pH 6.5. PARP cleavage was not induced by nigericin after 60 minutes when cells were incubated in 5 mM K^+ at pH 6.5 (Fig. 4A) or 130 mM K^+ at pH 7.2 (data not shown). A small amount of PARP cleavage was induced by nigericin after 90 minutes in 130 mM K^+ at pH 7.2, but again the effect of nigericin at pH 6.5 in 130 mM K^+ was greater (Fig. 4B). Nigericin could affect the mitochondrial pH gradient as well as plasma membrane H^+/K^+ equilibration. We were therefore concerned that it might be triggering the mitochondrial permeability transition (PT) which has recently been linked with induction of apoptosis (Zamzami et al., 1996), as a change in mitochondrial membrane potential can be a cause of PT (Bernardi et al., 1993). Bongkrekic acid (BA) is a specific ligand for the mitochondrial adenine nucleotide translocator (ANT) that is thought to be responsible for the PT. BA prevents the PT and inhibits apoptosis induced by many agents whose primary site of action is mitochondria (Zamzami et al., 1996). Fig. 4C shows that the induction of PARP cleavage by nigericin was not inhibited by 100 μM BA. Fig. 4D shows that DNA fragmentation was also induced rapidly by nigericin in 130 mM K^+ at pH 6.5 but not at pH 7.2. Again some slower fragmentation was induced by nigericin in 130 mM K^+ at pH 7.2 (Fig. 4D) in agreement with the slower ICE-like protease activation (Fig. 4B). As it was not possible to measure intracellular pH after 90 minutes exposure to nigericin, due to fragility of the cells, we cannot determine whether these late effects in pH 7.2 medium involve cytoplasmic acidification or reflect an alternative pathway of ICE-like protease activation. Fragmentation was not induced by nigericin in 5 mM K^+ at pH 6.5 (data not shown). These data

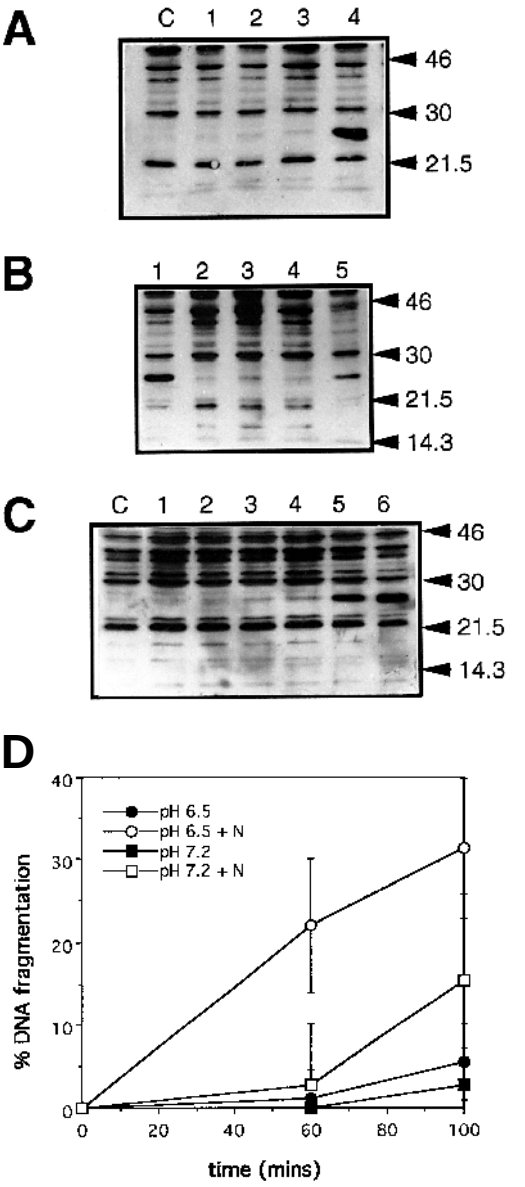

Fig. 4. Rapid intracellular acidification induces ICE-like protease activity and DNA fragmentation. Samples were prepared for analysis of PARP cleavage by western blot. (A) Whole cell extracts were prepared from samples of cells incubated in serum-free simple buffered medium at pH 6.5 after *t*=0 minutes (C), or *t*=60 minutes (1-4) in 5 mM KCl (1), 5 mM KCl + 5 μg/ml nigericin (2), 130 mM KCl (3) or 130 mM KCl + 5 μg/ml nigericin (4). (B) Cell extracts were prepared from cells after 90 minutes incubation in serum-free simple buffered media with 130 mM KCl at pH 6.5 in the presence of 5 μg/ml nigericin (1) or pH 7.2 (2-5) in 5 mM KCl (2), 5 mM KCl + 5 μg/ml nigericin (3), 130 mM KCl (4) or 130 mM KCl + 5 μg/ml nigericin (5). (C) Whole cell extracts were prepared from samples of cells incubated in serum-free simple buffered medium at pH 6.5 with 130 mM KCl after *t*=0 minutes (C), *t*=15 minutes (1-3) or *t*=30 minutes (4-6); no addition (1 and 4), + 5 μg/ml nigericin (2 and 5), + 5 μg/ml nigericin in the presence of 100 μM bongkrekic acid (3 and 6). (D) Cells were incubated for the time shown in serum-free simple buffered medium plus 130 mM KCl, at the pH shown, in the presence or absence of 5 μg/ml nigericin (N). % DNA fragmentation was determined by release of [^{3}H]thymidine-labelled DNA. Release was expressed as a % of total DNA; each point represents the mean ± s.e. of triplicates.

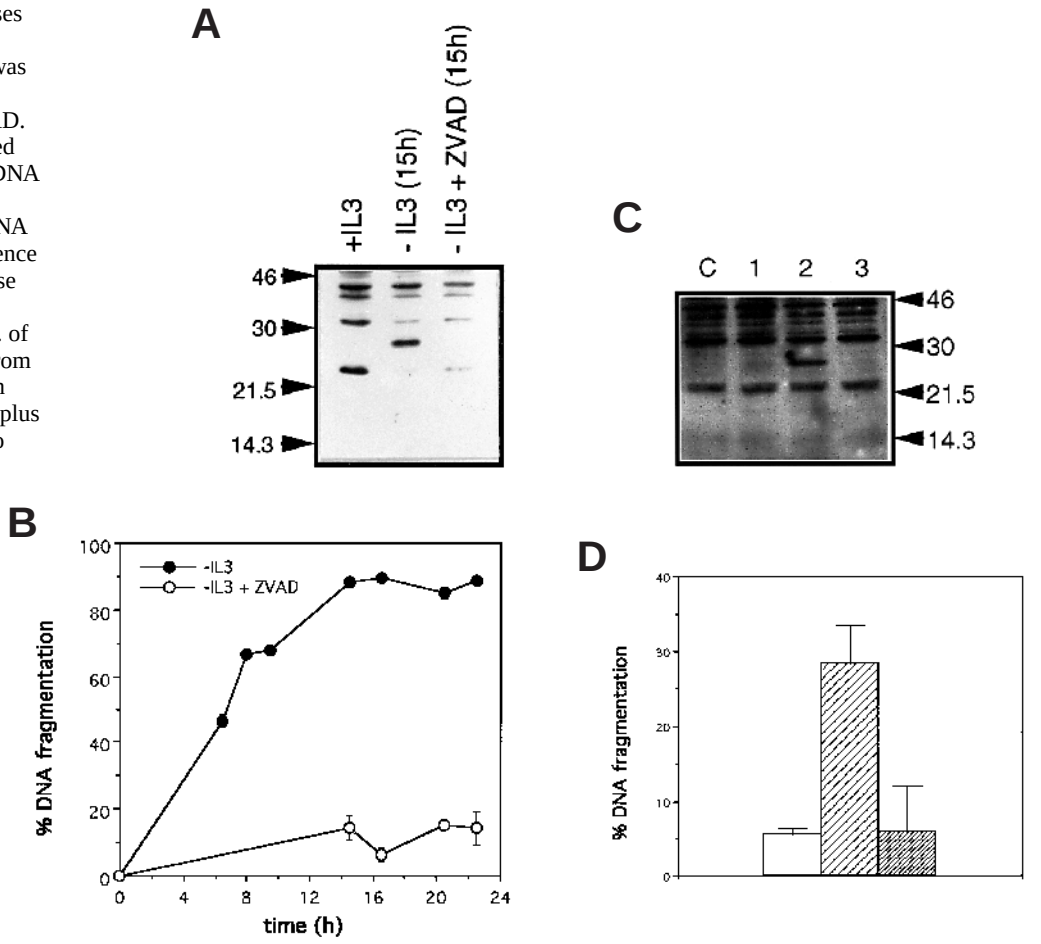

Fig. 5. Inhibition of ICE-like proteases blocks apoptosis induced by intracellular acidification. (A) IL-3 was removed from BAF3 cells in the presence or absence of 400 μM ZVAD. After 15 hours samples were prepared for analysis of PARP cleavage. (B) DNA fragmentation was determined by release of [^{3}H]thymidine-labelled DNA in cells deprived of IL-3, in the presence or absence of 400 μM ZVAD. Release was expressed as a % of total DNA; each point represents the mean ± s.e. of triplicates. (C) Whole cell extracts from cells at t=0 (C) or after 30 minutes in serum-free simple buffered medium plus 130 mM KCl at pH 6.5 (1-3) with no further addition (1), + 5 μg/ml nigericin (2), + 5 μg/ml nigericin + 400 μM ZVAD (3) were analysed for PARP cleavage by western blotting. (D) DNA fragmentation was determined by [^{3}H]thymidine release from labelled cells in serum-free simple buffered medium at pH 6.5 with 130 mM KCl (□), or 130 mM KCl + 5 μg/ml nigericin (▨), or 130 mM KCl + 5 μg/ml nigericin in the presence of 400 μM ZVAD (▨). Release after 60 minutes is expressed as a % of total DNA; each point represents the mean ± s.e. of triplicates.

demonstrate that PARP cleavage and fragmentation were most strongly induced by nigericin under conditions where a decrease in pH_i was induced. Therefore the mechanism of induction of apoptosis by nigericin is most probably to decrease intracellular pH.

The cell permeable peptide inhibitor benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone (ZVAD) is a general inhibitor of ICE-like proteases (Fletcher et al., 1995). Here ZVAD was used to determine the role of ICE-like protease activity in the induction of apoptosis by intracellular acidification. When BAF3 cells were deprived of IL-3 for 15 hours in the presence of 400 μM ZVAD, proteolytic cleavage of PARP was blocked (Fig. 5A). DNA fragmentation and loss of cell viability following IL-3 removal were also inhibited by ZVAD (Fig. 5B and Table 1). ZVAD did not affect the decrease in pH_i observed in cells deprived of IL-3. Table 1 shows that in the presence of ZVAD, cells were maintained in the low pH population 15 hours after IL-3 removal. Furthermore, when pH_i was decreased directly by incubation with nigericin, the presence of ZVAD did not affect pH_i (data not shown) but did inhibit PARP cleavage induced by low pH (Fig. 5C). Again, when ICE-like proteases were inhibited, DNA fragmentation induced by nigericin was also blocked (Fig. 5D). These data demonstrate that activation of an ICE-like protease is necessary for the induction of apoptosis by intracellular acidification.

Table 1. Inhibition of apoptosis by ZVAD does not block intracellular acidification

	% Viability	% Shift into R2
+IL3	100	0
–IL3	36.0 (±2.0)	40.8 (±20.2)
+IL3 + 400 μM ZVAD	98.2 (±1.8)	0.4
–IL3 + 400 μM ZVAD	88.5 (±4.4)	58.8 (±5.6)

Cells incubated for 15 hours in the presence or absence of IL-3 and in the presence or absence of 400 μM ZVAD as indicated, were either labelled with SNARF-1-AM to measure intracellular pH in viable cells (as in Fig. 1A) or stained with trypan blue and counted on a haemocytometer to calculate viable cells. Mean ± s.e. of 3 separate experiments is shown.

DISCUSSION

Apoptosis can be induced in IL-3 dependent BAF3 cells by removal of growth factor and treatment with the DNA damaging agent etoposide. A drop in intracellular pH is observed prior to DNA fragmentation when apoptosis is induced by these signals. Proteolytic cleavage of PARP is observed indicating that an ICE-like protease(s) is activated. When PARP cleavage is inhibited by the cell permeable ICE-like protease inhibitor ZVAD, the drop in intracellular pH is still observed but DNA fragmentation and cell death are inhibited. When a rapid intracellular pH drop is induced by nigericin, ICE-like protease activity and DNA fragmentation

are rapidly stimulated. The ICE-like proteolytic activity induced by low pH can be inhibited by ZVAD and DNA fragmentation is then blocked. This suggests that a drop in pH is part of a signalling pathway preceding ICE-like proteolytic events and that ICE-like protease activation is required for this drop in pH to stimulate DNA fragmentation. A different conclusion was drawn by Meisenholder et al. (1996), who showed that ZVAD blocked acidifcation induced by Fas ligation and therefore suggested that acidification was downstream of ICE-like protease activation. However, the ICE-like protease which forms part of the activated Fas receptor complex is inhibited by ZVAD (Muzio et al., 1996). It is therefore possible that acidification following Fas ligation may be involved in activating ICE-like proteases further downstream in the pathway, as it does in BAF3 cells. It also remains possible that ICE-like proteases which are not inhibited by ZVAD are activated prior to acidification in BAF3 cells.

pH is regulated in cells mainly by the activity of Na^+/H^+ antiport and H+ translocating ATPases. The mechanism for the pH decrease observed during apoptosis following growth factor withdrawal has been suggested to be by disregulation of ion transporters. GCSF delays programmed cell death in neutrophils by up-regulating the vacuolar H^+ATPase (Gottlieb et al., 1995b). Steel factor and GM-CSF, shown to inhibit cell death in myeloid cell lines, induce a rapid and sustained alkalisation of intracellular pH (Caceres-Cortes et al., 1994; Rajotte et al., 1992). The inhibitory activity of these factors is abrogated by inactivation of the Na^+/H^+ antiport (Caceres-Cortes et al., 1994). Similarly, inhibition of the antiport by amiloride derivatives has been shown to inhibit the protective effect of IL-3 in BAF3 cells (data not shown). Stimulation of ion transporter activity by the addition of phorbol esters has been shown to block cell death and intracellular acidification in IL2 deprived CTLL-2 (Rebollo et al., 1995). Of the other transmembrane channels, the cystic fibrosis transmembrane conductance regulator (CTFR) has been proposed to play a role in pHi regulation during apoptosis. The drop in pH preceding apoptosis observed in cycloheximide-treated cells expressing wild-type CTFR is blocked when CTFR activity is inhibited either by expression of the mutant CTFR (delF508) or by diphenylamine carboxylate (Gottlieb et al., 1996).

BAF3 cells over-expressing human Bcl-2 do not undergo apoptosis when IL-3 is removed, instead they arrest in G_1. These G_1 arrested cells have an acid pH_i, which has been described in other cell types deprived of growth factors (Moolenaar, 1986). The detection of a single population of cells with low pH when apoptosis was inhibited by Bcl-2 or ZVAD, as opposed to a sub-population with low pH when IL-3 is removed, is probably because the IL-3 deprived BAF3 cells rapidly lose viability after entering the low pH population. Strikingly, Bcl-2 also delayed acidification upon IL-3 removal. Similarily, Bcl-2 and its family member Mcl-1 have been shown to partially inhibit intracellular acidification in CHO cells treated with staurosporine (Reynolds et al., 1996) and to block acidification following anti-Fas or cycloheximide treatment of Jurkat cells (Meisenholder et al., 1996). Consistent with its localisation in intra-cellular membranes (Krajewski et al., 1993), Bcl-2 has been proposed to act by regulating ion fluxes. For example, Ca^{2+} re-partitioning on IL-3 removal is inhibited by Bcl-2 (Baffy et al., 1993). Because of the widespread intra-cellular distribution of SNARF-1-AM (Seksek et al., 1991), we cannot determine whether Bcl-2 is affecting the sub-cellular location of the pH change as well as its timing. The slow pH decrease observed in Bcl-2 over-expressing cells may fail to trigger apoptosis because it does not occur in the relevant sub-cellular compartment. While the final cleavages of the ICE-like protease cascade, such as that of PARP, presumably occur in the nucleus, the activity triggered by low pH may occur in a compartment where the pH can be regulated by Bcl-2. Indeed, when a rapid and general pH decrease was induced by nigericin, Bcl-2 was unable to block ICE-like protease activation and apoptosis (data not shown). We did consider that the inhibition of mitochondrial PT by Bcl-2 (Zamzami et al., 1996) might be relevant, as low pH could be a trigger of PT (Zoratti and Szabo, 1995). However, bongkrekic acid, a specific inhibitor of PT, failed to inhibit apoptosis induced by growth factor removal or etoposide in BAF3 cells (data not shown). It is clear that Bcl-2 is either inhibiting ICE-like protease activation or ICE-like protease activity in BAF3 cells deprived of IL-3. This is also the case in Jurkat cells, where transfection of Bcl-2 or BclX inhibits the generation of YAMA/ICE-LAP3 following FAS or TNF stimulation (Chinnaiyan et al., 1996).

The mechanism by which a pH drop might activate downstream effector pathways in apoptosis has not previously been described. A pH dependent endonuclease has been identified in a number of apoptotic systems and been proposed to be the primary target of the pH decrease (Gottlieb et al., 1995a, 1996; Barry and Eastman, 1993). Endonuclease activity can be induced in isolated nuclei of BAF3 cells by dropping pH below 7.0 and highly purified nuclease activity from BAF3 nuclei is activated by decreasing pH (Collins et al., 1996). However, the data presented here argue that the primary target for low pH triggering of apoptosis in intact cells is ICE-like protease(s) as, when ICE-like protease activation is blocked by ZVAD, nuclease activation following a pH decrease does not occur. In vitro studies have previously suggested that proteases activated during apoptosis are required to stimulate DNA fragmentation in isolated nuclei (Nicholson et al., 1995; Lazebnik et al., 1994) and ZVAD has been reported to inhibit DNA fragmentation induced in intact cells by a variety of signals (Fearnhead et al., 1995). Whether low pH directly activates one or more of the ICE-like proteases, or an upstream component of the pathway, remains to be determined. In vitro measurement of ICE-like protease activity has thus far used a high concentration of recombinant protein at neutral pH (Xue and Horvitz, 1995). The fall in pH required to trigger apoptosis is relatively small, which argues that the component activated by low pH has a very sharp pH dependence to prevent its accidental activation.

REFERENCES

Baffy, G., Miyashita, T., Williamson, J. R. and Reed, J. C. (1993). Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J. Biol. Chem.* **268**, 6511-6519.

Barry, M. A. and Eastman, A. (1993). Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch. Biochem. Biophys.* **300**, 440-450.

Barry, M. A., Reynolds, J. E. and Eastman, A. (1993). Etoposide induced apoptosis in human HL60 cells is associated with intracellular acidification. *Cancer Res.* **53**, 2349-2357.

Bassnett, S., Reinisch, L. and Beebe, D. C. (1990). Intracellular pH measurement using single excitation-dual emission fluorescence ratios. *Am. J. Physiol*. **258**, 171-178.

Bernardi, P., Veronese, P. and Petronilli, V. (1993). Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore, *J. Biol. Chem.* **268**, 1005-1010.

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

Caceres-Cortes, J., Rajotte, D., Dumouchel, J., Haddad, P. and Hoang, T. (1994). Product of the steel locus suppresses apoptosis in hemopoietic cells. Comparison with pathways activated by granulocyte macrophage colony-stimulating factor. *J. Biol. Chem.* **269**, 12084-12091.

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

Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G. and Dixit, V. M. (1996). Molecular ordering of the cell death pathway: Bcl-2 and Bcl-X_L function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.* **271**, 4573-4576.

Collins, M. K., Marvel, J., Malde, P. and Lopez-Rivas, A. (1992). Interleukin 3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. *J. Exp. Med.* **176**, 1043-1051.

Collins, M. K. L., Furlong, I. J., Malde, P., Ascaso, R., Oliver, F. J. and Lopez-Rivas, A. (1996). An apoptotic endonuclease that can be activated by either decreasing pH or addition of calcium. *J. Cell Sci.* **109**, 2393-2399.

Duan, H., Orth, K., Chinnaiyan, Poirier, G., Froelich, C., He, W.-W. and Dixit, V. (1996). 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.

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

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128.

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

Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G., Earnshaw, W. C., Litwack, G. and Alnemri, E. S. (1995). Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55**, 6045-6052.

Fletcher, D., Agarwal, L., Chapman, K., Chin, J., Egger, L., Limjuco, G., Luell, S., MacIntyre, D., Peterson, E., Thornberry, N. and Kostura, M. (1995). A synthetic inhibitor of interleukin-1beta converting enzyme prevents endotoxin-induced interleukin-1beta production in vitro and in vivo. *J. Interferon Cytokine Res.* **15**, 243-248.

Gottlieb, R. A., Giesing, H. A., Engler, R. L. and Babior, B. M. (1995a). The acid deoxyribonuclease of neutrophils: a possible participant in apoptosis-associated genome destruction. *Blood* **86**, 2414-2418.

Gottlieb, R. A., Giesing, H. A., Zhu, J. Y., Engler, R. L. and Babior, B. M. (1995b). Cell acidification in apoptosis: GCSF delays programed cell death in neutrophils by up-regulating the vacuolar H^+-ATPase. *Proc. Nat. Acad. Sci. USA* **92**, 5965-5968.

Gottlieb, R. A. and Dosanjh, A. (1996). Mutant cystic fibrosis transmembrane conductance regulator inhibits acidification and apoptosis in C127 cells: Possible relevance to cystic fibrosis. *Proc. Nat. Acad. Sci. USA* **93**, 3587-3591.

Gottlieb, R. A., Nordberg, J., Skowronski, E. and Babior, B. M. (1996). Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification, *Proc. Nat. Acad. Sci. USA* **93**, 654-658.

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

Herrmann, M., Lorenz, H. M., Voll, R., Grunke, M., Woith, W. and Kalden, J. R. (1994). A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucl. Acids Res.* **22**, 5506-5507.

Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E. and Poirier, G. G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* **53**, 3976-3985.

Korsmeyer, S. J. (1995). Regulators of cell death. *Trends Genet.* **11**, 101-105.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W. and Reed, J. C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res.* **53**, 4701-4714.

Kumar, S. and Harvey, N. (1995). Role of multiple cellular proteases in the execution of programmed cell death. *FEBS Lett.* **375**, 169-173.

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

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

Li, J. and Eastman, A. (1995). Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na(+)/H(+)-antiport. *J. Biol. Chem.* **270**, 3203-3211.

Marvel, J., Perkins, G. R., Lopez-Rivas, A. and Collins, M. K. L. (1993). Bcl-2 over-expression in murine bone marrow cells induces refractoriness to interleukin-3 stimulation of proliferation. *Oncogene* **9**, 1117-1122.

Mashima, T., Naito, M., Fujita, N., Noguchi, K. and Tsuruo, T. (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.

Meisenholder, G., Martin, S., Green, D., Nordberg, J., Babior, B. and Gottlieb, R. (1996). Events in apoptosis: intracellular acidification is downstream of protease activation and Bcl-2 protection. *J. Biol. Chem.* **271**, 16260-16262.

Moolenaar, W. (1986). Effects of growth factors on intracellular pH regulation. *Annu. Rev. Physiol.* **48**, 363-376.

Muzio, M., Chinnaiyan, A., Kischkel, F., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J., Zhang, M., Gentz, R., Mann, M., Krammer, P., Peter, M. and Dixit, V. (1996). FLICE, a novel FADD-homologous ICE/Ced3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85**, 817-827.

Na, S., Chuang, T., Cunningham, A., Turi, T., Hanke, J., Bokoch, G. and Danley, D. (1996). D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. *J. Biol. Chem.* **271**, 11209-11213.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43.

Palacios, R. and Steinmetz, M. (1985). IL3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration and generate B lymphocytes in vivo. *Cell* **41**, 727-734.

Qin, X., Livingston, D., Kaelin, W. and Adams, P. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Nat. Acad. Sci. USA* **91**, 10918-10922.

Rabinovitch, P. S. and June, C. H. (1990). *Flow Cytometry; A Practical Approach*. pp. 178-185. IRL Press.

Raff, M. C. (1992). Social controls on cell survival and cell death. *Nature* **356**, 397-400.

Rajotte, D., Haddad, P., Haman, A., Cragoe, E. J. and Hoang, T. (1992). Role of protein kinase C and the Na+/H+ antiporter in suppression of apoptosis by GMCSF and IL3. *J. Biol. Chem.* **267**, 9980-9987.

Rebollo, A., Gomez, J., Martinez, A., Lastres, P., Silva, A. and Perez-Sala, D. (1995). Apoptosis induced by IL-2 withdrawal is associated with an intracellular acidification. *Exp. Cell Res.* **218**, 581-585.

Reynolds, J. E., Li, J., Craig, R. W. and Eastman, A. (1996). BCL-2 and MCL-1 expression in Chinese hamster ovary cells inhibits intracellular acidification and apoptosis induced by staurosporine. *Exp. Cell Res.* **225**, 430-436.

Rodriguez-Tarduchy, G., Collins, M. and Lopez-Rivas, A. (1990). Regulation of apoptosis in interleukin-3-dependent hemopoietic cells by interleukin-3 and calcium ionophores. *EMBO J.* **9**, 2997-3002.

Seksek, O., Henry, T. N., Sureau, F. and Bolard, J. (1991). SNARF-1 as an intracellular pH indicator in laser microspectrofluorometry: a critical assessment *Anal. Biochem.* **193**, 49-54.

Song, Q., Lees-Miller, S., Kumar, S., Zhang, N., Chan, D., Smith, G., Jackson, S., Alnemri, E., Litwack, G., Khanna, K. and Lavin, M. (1996). DNA-dependent protein kinase catalytic subunit as a target for an ICE-like protease in apoptosis *EMBO J.* **15**, 3238-3246.

Tewari, M., Beidler, D. R. and Dixit, V. M. (1995a). CrmA-inhibitable cleavage of the 70-kDa protein component of the U1 small nuclear

ribonucleoprotein during Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* **270**, 18738-18741.

Tewari, M. and Dixit, V. M. (1995). Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. *J. Biol. Chem.* **270**, 3255-3260.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S. and Dixit, V. M. (1995b). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801-809.

Voelkel, J. C., Entingh, A. J., Wold, W. S., Gooding, L. R. and Laster, S. M. (1995). Activation of intracellular proteases is an early event in TNF-induced apoptosis. *J. Immunol.* **154**, 1707-1716.

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

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

Zamzami, N., Susin, S., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M. and Kroemer, G. (1996). Mitochondrial control of nuclear apoptosis. *J. Exp. Med.* **183**, 1533-1544.

Zoratti, M. and Szabo, I. (1995). The mitochondrial permeability transition. *Biochim. Biophys. Acta* **1241**, 139-176.

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