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Role of acid/base homeostasis in the suppression of apoptosis in haemopoietic cells by v-Abl protein tyrosine kinase

Quan Chen[1], Rod S. P. Benson[1], Anthony D. Whetton[3], Steven R. Brant[2], Mark Donowitz[2], Marshall H. Montrose[2], Caroline Dive[1] and Alastair J. M. Watson[4,*]

[1]Molecular Pharmacology Group, School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Road, Manchester M13 9PT, UK
[2]The Johns Hopkins University School of Medicine, Department of Gastroenterology, 918 Ross Research Building, 720 Rutland Avenue, Baltimore, MA 21205-2195, USA
[3]Leukaemia Research Fund Unit, Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, Sackville Street, Manchester M60 1QD, UK
[4]Department of Medicine, University of Manchester, G38 Stopford Building, Oxford Road, Manchester M13 9PT, UK

*Author for correspondence

SUMMARY

Removal of interleukin-3 from murine IC.DP pre-mast cells results in irreversible commitment to apoptosis within 18 hours. To identify early events necessary for the engagement of apoptosis we examined the regulation of intracellular pH (pH_i). IC.DP cells acidified 2 hours after removal of interleukin-3 (before discernible signs of apoptosis) and by 18 hours pH_i had decreased by 0.15 units. The acidification was due to both an increase in an acid-loading process which only occurs when intracellular pH is above 6.8 and a slight reduction in H^+ efflux via Na^+/H^+ exchange. Activation of a temperature sensitive mutant of v-Abl protein tyrosine kinase suppressed apoptosis of IC.DP cells in the absence of interleukin-3 but did not stimulate proliferation, and moreover prevented cellular acidification. Acidification of the cells by 0.2 units to pH 6.86 by complete inhibition of Na^+/H^+ exchange by 10 μM 5′-(*N*-methyl-*N*-isobutyl)-amiloride prevented the suppression of apoptosis by v-abl protein tyrosine kinase following IL 3 withdrawal. However in the presence of interleukin-3, addition of 10 μM 5′-(*N*-methyl-*N*-isobutyl)-amiloride only resulted in a fall of pH_i to 7.17. Apoptosis did not occur and the cells continued to proliferate. Thus, in this model intracellular pH must fall below a critical value for apoptosis to occur. Together these data point to a step in cytokine deprivation induced apoptosis (at least in some haemopoietic cell types) which is either enhanced by or dependent upon an acidic intracellular environment which is the result of an increase in acid loading and inhibition of Na^+/H^+ exchange activity. One of the mechanisms by which activation of v-Abl protein tyrosine kinase suppresses apoptosis is by prevention of intracellular acidification.

Key words: Apoptosis, Intracellular pH, Sodium hydrogen exchange, Interleukin-3, v-Abl protein tyrosine kinase

INTRODUCTION

In recent years it has been shown that apoptosis plays a critical role in adult tissue homeostasis and furthermore aberrant regulation of this process is implicated in several pathologies, including neoplasia and AIDS (reviewed by Thompson, 1995). Thus there is a clear need to be able to manipulate apoptosis for therapy and, not surprisingly, the molecular events involved in apoptosis are now the subject of intense scrutiny. After exposure of a cell to a potentially lethal stimulus, a precommitment phase occurs which is variable in duration, is reversible, and beyond which the cellular response is death by apoptosis or continued survival (Wood et al., 1994). The molecular events defining the irreversible commitment point to apoptosis are ill-defined and depend upon the genes and proteins expressed by the cell in question, including p53 (Lane, 1992), c-myc (Evan et al., 1992) and the rapidly expanding family of proteins homologous to Bcl-2 (Reed, 1994). Although many features of apoptosis have been well characterised, such as the non-random fragmentation of DNA and the degradation of substrates such as PARP and the nuclear lamins by apoptotic proteases (reviewed by Steller, 1995), early events in apoptotic pathway(s), for the most part, remain elusive.

There is accumulating evidence that a fall in intracellular pH (pH_i) occurs in cells which are committed to undergo apoptosis following growth factor deprivation (Gottlieb et al., 1995; Li and Eastman, 1995; Owen et al., 1993; Rajotte et al., 1992) or chemical insult (Gottlieb et al., 1996; Barbiero et al., 1995; Barry et al., 1993; Pérez-Sala et al., 1995). The mechanism of acidification is subject to debate. Under normal physiological conditions, Na^+/H^+ exchange acts to alkalinize the cell and its inhibition will result in acidification if there is continued generation of intracellular H^+. Some investigators have suggested that the acidification is the direct result of the inhibition of Na^+/H^+ exchange (Li and Eastman, 1995), but others have challenged this view (Pérez-Sala et al., 1995). However, these studies have not distinguished clearly whether acidification is

the result of a primary acid loading process or is secondary to failure of H^+ efflux through Na^+/H^+ exchange. Indeed so far no formal measurements of Na^+/H^+ exchange activity have been made in cells during the precommitment period of apoptosis, taking into account buffering capacity and other changes in acid/base homeostasis.

Regardless of the mechanisms of acidification, there is evidence implicating that it may be of functional importance in apoptosis (Owen et al., 1993; Pérez-Sala et al., 1995; Rajotte et al., 1992). In various haemopoietic cell types it has been demonstrated that suppression of apoptosis is concomitant with activation of Na^+/H^+ exchange and a subsequent increase in pH_i (Pérez-Sala et al., 1995; Rajotte et al., 1992). Prevention of cytoplasmic alkalinization using Na^+/H^+ exchange inhibitors abrogate the suppression of apoptosis (Rajotte et al., 1992). Furthermore recent structural analysis of Bcl-x_L, a suppressor of apoptosis has suggested it may be pH-sensitive membrane pore (Muchmore et al., 1996).

Here we have exploited a cell system where we can readily manipulate the ability of a cell to undergo apoptosis by activating or inactivating a temperature sensitive (ts) mutant of v-Abl PTK to study the relationships between pH_i, Na^+/H^+ exchange and cell fate. Activated forms of the Abelson protein tyrosine kinase (Bcr/Abl and v-Abl) can potently suppress apoptosis in haemopoietic cells induced by withdrawal of IL-3 or exposure to various DNA damaging drugs (Evans et al., 1993; Chapman et al., 1995;). Haemopoietic IC.DP cells protected from death via activation of v-Abl PTK survive but do not proliferate (Evans et al., 1993), thus providing an ideal system to study specifically survival signalling. In this study we investigated the mechanism(s) of cellular acidification in IC.DP cells deprived of IL-3 and specifically we examined events occurring in the reversible precommitment period prior to the appearance of apoptotic cells. We also examined the effect of v-Abl PTK activation on acid/base homeostasis and whether maintenance of pH_i above 6.9 plays a role in the suppression of apoptosis by v-Abl PTK.

MATERIALS AND METHODS

Chemicals and solutions

2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acid (BCECF acid) and its acetoxymethyl ester (BCECF-AM), 5-(and-6)-carboxy-SNARF-1 acetoxymethyl ester (SNARF-AM), nigericin, Hoechst 33342, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) and H_2-DIDS were purchased from Molecular Probe Inc (Eugene, OR, USA). 5′ (*N*-methyl-*N*-isobutyl)-amiloride (NMIA) was from RBI (Natick, MA, USA). Iodoacetic acid, bafilomycin A, proteinase K, RNase A, phenylmethylsulphonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin were obtained from Sigma Chemical (Poole, UK). DNA molecular mass markers were from Bio-Rad Laboratories Ltd (Hemel Hampstead, UK).

For experiments in which intracellular pH (pH_i) was measured, cells were placed in a medium ('bicarbonate medium') containing: 113 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM Hepes, 23.8 mM $NaHCO_3$, 10 mM glucose, pH 7.35. In experiments where acid-loading and Na^+/H^+ exchange was measured cells were placed in a medium ('Na medium') containing: 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.75 mM Na_2HPO_4, 0.25 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes, titrated to pH 7.30 at 37°C with NaOH. In some experiments, a Na^+-free medium was used in which Na^+ was replaced by tetramethylammonium (TMA). Intracellular pH was calibrated in a nigericin/high K^+ medium containing: 75 mM KCl, 45 mM K gluconate, 15 mM Na gluconate, 1 mM $CaCl_2$, 1 mM $MgSO_4$-$7H_2O$, 20 mM Hepes/TMA and 10 μM nigericin and titrated to the desired pH with 0.1 N HCl (Watson et al., 1991, 1992).

Cell culture

The haemopoietic cell line (IC2.9) is an IL-3 dependent murine premast cell line which has been stably transfected with a ts mutant of v-Abl to generate the IC.DP cell line (Kipreos et al., 1987) (both cell lines are mycoplasma free). The v-Abl encoded tyrosine kinase (v-Abl PTK) is active at 32°C but inactive at 39°C. IC.DP and IC2.9 murine mast cells were cultured as previously described (Evans et al., 1993). IL-3 withdrawal-mediated induction of apoptosis was performed as follows: cells were resuspended in Fischers medium containing only glutamine and antibiotics, after two washes in this medium v-Abl PTK was inactivated by incubating the IC.DP cells for 2 hours at 39°C (Owen et al., 1993). Then cells were transferred to the desired experimental conditions (either 32°C or 39°C). This time point is referred to as 0 hours in the text and figures. IC2.9 cells were treated using an identical protocol and were included to control for temperature effects.

Measurement of apoptosis

The percentage of cells exhibiting apoptotic morphology was determined by fluorescence microscopy using Hoechst 33342 (10 μM in PBS). To confirm that apoptosis was the mode of death in our experiments, DNA integrity was analysed by conventional agarose gel electrophoresis (Smith et al., 1989).

Analysis of the accumulation of BCECF in viable and apoptotic cells by flow cytometry

BCECF-AM (3 μl, 1 mM) was added to IC.DP cells at room temperature (with v-Abl PTK inactivated) 30 hours after removal of IL-3. Cells were immediately analysed with respect to forward and orthogonal light scatter, green fluorescence (530±30 nm) and time using a Becton Dickinson FACs Vantage instrument (BD, San Jose, CA, USA). The instrument was set to excite at 250 mW using the 488 nm line of an Enterprise laser (Coherent, Inc., California, USA) and 50,000 cells were analysed continuously over 10 minutes. Data were analysed using Lysis II software and a Hewlett Packard 32 consort system. Cell subpopulations corresponding to apoptotic and viable cells were gated with respect to forward versus orthogonal light scatter and their green fluorescence profile was determined (Dive et al., 1992).

Measurement of intracellular pH using BCECF and conventional spectrofluorometry

Intracellular pH was measured by spectrofluorometry as described previously (Watson et al., 1991, 1992). Briefly, following incubation at either 32°C or 39°C for the desired time, cells (1×10^7) were incubated with BCECF-AM (5-10 μM) for 10 minutes at 37°C in Na^+ medium (pH 7.4). Cell samples were washed once in Na^+ medium and resuspended at 10×10^7 cells/ml in ice-cold Na^+ medium. Samples of 10-30 μl were added to 2.5 ml Na^+ or bicarbonate medium, brought to 37°C in a quartz cuvette with constant stirring and the BCECF fluorescence intensity was measured in a computer controlled Perkin Elmer LS-50 spectrofluorometer (excitation 440±10 and 500±10 nm, emission 530±10 nm) (Perkin Elmer Ltd, PE, Beaconsfield, UK). Intracellular pH was calibrated as described previously. The whole manoeuvre was completed within 30 minutes of the temperature switch from 32°C to 37°C during which the v-Abl PTK activity is maintained (Owen et al., 1993).

Measurement of intracellular buffering capacity for H^+

Cells loaded with BCECF were acidified to various pH_i within the range 6.2 to 7.2 by exposure to 15-45 mM NH_4Cl for varying durations. These acidified cells were washed with a large volume of TMA medium and 0.5-15 mM NH_4Cl was added to induce a rapid

intracellular alkalinization of 0.15-0.3 pH units. The buffering capacity ($\beta_i=[\Delta H_i^+]/\Delta pH$) was calculated as described previously (Watson et al., 1991, 1992).

Measurement of H^+ efflux kinetics

Cells loaded with BCECF were pulsed with 30-45 mM NH_4Cl for 5-15 minutes in Na^+ medium and washed in a large volume of ice-cold TMA medium. This manoeuvre resulted in acidification of the cells. The cells were then added to Na^+ medium at 37°C and the recovery in pH_i measured by spectrofluorometry was performed as described above. The rate of Na^+ dependent alkalinization was obtained by calculating the first-order derivative of the Na^+-dependent pH recovery trace (Watson and Montrose, 1994; Watson et al., 1991). Net H^+ efflux rates (μM H^+/second) were then determined by multiplying the rate of change in pH_i by the cellular buffering capacity at the corresponding pH values. Alkalinization was ascribed positive values. Under some experimental conditions, there was an endogenous acidification process which occurred simultaneously with the Na^+-dependent alkalinization process (see results below). The rate of this acidification process was measured following complete blockade of Na^+/H^+ exchange with 10 μM NMIA by multiplying the rate of change in pH by cellular H^+ buffering capacity at the corresponding pH value. Total H^+ efflux rates were calculated by subtraction of the acidification rates (negative values) from the net H^+ efflux rates (positive values) and at corresponding values of pH_i.

Measurement of intracellular pH by flow cytometry using carboxy SNARF-1

Because the fluorescence properties of BCECF are not ideal for flow cytometric studies we employed carboxy-SNARF-1 to measure pH_i. IC.DP cells (10^7/ml) were incubated with carboxy-SNARF-1-AM (10 μM) for 10 minutes at 37°C then washed and prepulsed with NH_4Cl (30 mM) for 10 minutes in Na^+ medium at 37°C, washed and resuspended in TMA medium. Cells were then placed in Na^+ medium to initiate a Na^+-dependent pH_i recovery and immediately analysed with continuous time as an additional FCM parameter. The 488 nm laser line was used to excite carboxy-SNARF-1 at 250 mW and fluorescence was measured at 575±30 nm (FL575) and 630±22 nm (FL630) (Rothe and Valet, 1988) in addition to forward and orthogonal light scatter for 10,000 cells per sample. FL575/FL630 was converted to pH_i and plotted against time. Any apoptotic cells were excluded from the analysis on the basis of their altered forward and orthogonal light scatter profiles. High K^+ medium containing nigericin (10 μM) was used to calibrate pH_i and the ratio of FL630/FL575 was converted to pH_i according to the calibration curve (Rothe and Valet, 1988). Calibration of pH_i was performed for every experiment.

Detection by western blot of cellular protein tyrosine phosphorylation following manipulation of ts v-Abl PTK

At specific time points after temperature switch to activate or inactivate v-Abl PTK, 1×10^7 cells were harvested, washed in PBS (pH 7.4) and lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na_3VO_4 and 50 mM NaF. Cellular proteins were separated by 8% SDS-PAGE and 100 μg of protein from total cell lysates were loaded per sample. Tyrosine phosphorylation of total cellular proteins was analysed by western blotting using mouse monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc, Lake Placid, NY) following the protocol provided by the manufacturer. Tyrosine phosphorylated protein was detected using the ECL system (Amersham Life Science, Amersham, UK).

Detection of *NHE* gene expression by RT-PCR

Total RNA was extracted from IC.DP cells and human kidney cells (Maniatis et al., 1989). Total RNA (6.25 μg) was reverse transcribed, with the use of random hexamer primers, into cDNA with the superscript preamplification system for first strand cDNA synthesis kit (Gibco) according to the manufacturer's recommendations in a 20 μl reaction volume. To detect the presence of human *NHE1* or *NHE3* cDNA, PCR amplification was performed for 35 cycles, each cycle consisting of 94°C (50 seconds), 58°C (45 seconds), and 71°C (2 minutes) on 2 μl of each reverse transcription (RT) product with human NHE1 cDNA primer pair B19 (5′-CAAGAGAC-GAAGCGCTCCATCAACG-3′; nucleotides 1,587-1,611) and B20 (5′-ATCTGGTTCCAGGCTTCCTCGTAGG-3′; nucleotides 2,050-2,026) (Brant et al., 1995). For analysis, 15 μl of PCR product was separated on an ethidium bromide-stained 1.4% agarose gel. Confirmation that the PCR products encoded mouse NHE1 cDNA was determined by direct sequencing of the PCR products amplified from 18 hours v-Abl PTK inactive amplified samples as described (Brandt et al., 1995).

Statistical analysis

Where applicable the data are presented as the mean ± s.e.m. unless indicated. Comparison of means was performed by Student's unpaired *t*-test. A probability of 0.05% or less was considered significant.

RESULTS

Validation of spectrofluorometric analysis of BCECF fluorescence in heterogeneous cell samples

In agreement with previous work (Evans et al., 1993), IC2.9 cells and IC.DP cells in the absence of v-Abl PTK activation underwent apoptosis when IL-3 was withdrawn. In order to determine the contribution that overtly apoptotic cells make to the average BCECF fluorescence of a sample measured by fluorometry, we assessed BCECF fluorescence in viable and apoptotic cells by flow cytometry. At 30 hours after the removal of IL-3 from IC.DP cells in the absence of v-Abl PTK activity there was 37% apoptotic cells based upon altered light scatter characteristics (Fig. 1A). Of the two subpopulations identified, R1 contained viable cells and R2 contained apoptotic cells (this assessment was based on microscopic analysis of cells after sorting for condensed and fragmented chromatin; data not shown). When IC.DP cells in these conditions were exposed to BCECF-AM and the resultant BCECF fluorescence measured with time, two subpopulations were detected. One subpopulation exhibited increased BCECF fluorescence with time and the other did not (Fig. 1B). By gating R1 and R2 as described above we observed that only R1 cells accumulated BCECF. In the fluorometric experiments described below the percentage of apoptotic cells is small (<5%). Therefore, this minor population of apoptotic cells (equivalent to R2 cells) does not have a major effect on the sample average BCECF fluorescence that is measured in the fluorometer.

The effects of v-Abl PTK activity on the kinetics of apoptosis and changes in pH_i following withdrawal of IL-3

The onset of apoptosis in IC2.9 and IC.DP cultures at 39°C occurred 16-18 hours after withdrawal of IL-3 and activation of v-Abl PTK by incubation at 32°C potently suppressed apoptosis following removal of IL-3 (Fig. 2A).

In cells deprived of IL-3 where v-Abl PTK was active, there was a rise in pH_i which was apparent by 2 hours and by 18 hours had increased from pH 7.04±0.01 (n=20) to 7.23±0.03

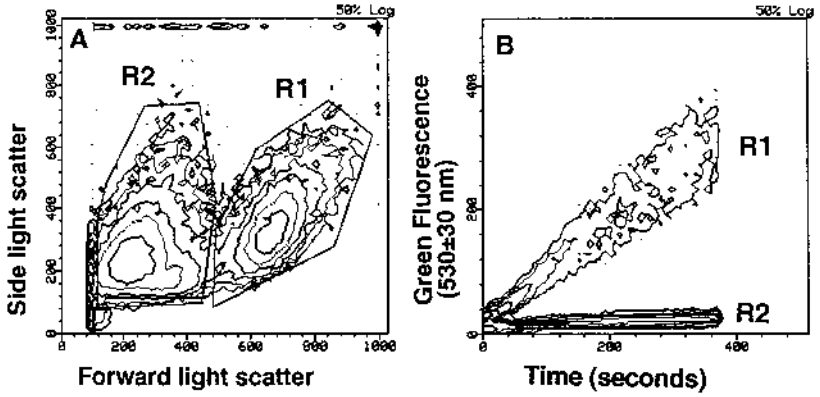

Fig. 1. Flow cytometric analysis of the accumulation of BCECF into viable and apoptotic IC.DP cells after exposure to BCECF-AM. (A) Forward light scatter (cell size) and orthogonal light scatter (cell granularity) were measured in IC.DP cells (v-Abl PTK inactive) for 30 hours following IL-3 withdrawal. Cell sorting experiments demonstrated (data not shown) that the cells in region R1 are viable IC.DP cells whereas the cells in region R2 are apoptotic IC.DP cells. (B) BCECF-AM (10 μM) was added to IC.DP cells and green fluorescence at 530±30 nm was analysed immediately at room temperature by flow cytometry as described in Materials and Methods. Electronic gating analysis demonstrated that only viable cells in region R1 have BCECF fluorescence. Results are from a typical experiment.

(*n*=18) (Fig. 2B). A similar rise in pH_i was observed when IC.DP cells (irrespective of v-Abl PTK activation status) (Fig. 2C) or IC2.9 cells were cultured in the presence of IL-3 (data not shown). Conversely, in cells deprived of IL-3 with inactive v-Abl PTK there is a fall in pH_i from pH 7.04±0.01 to 6.88±0.01 (*n*=20) at 18 hours prior to development of morphological changes indicative of apoptosis (Fig. 2B). In IC2.9 cells, pH_i decreased following withdrawal of IL-3 irrespective of incubation at 32°C or 39°C (data not shown). Because there were substantial changes in pH_i at 18 hours and because at this time point, the proportion of cells already undergoing apoptosis was small (<5%), the 18 hour time point was chosen for subsequent detailed analysis of the effects of v-Abl PTK and IL-3 upon acid/base homeostasis.

Na^+/H^+ exchange activity and cellular acid loading prior to apoptosis and suppression of apoptosis by v-abl PTK

Intracellular pH of IC.DP cells not undergoing apoptosis is determined by an alkalinization process counterbalanced by an acid-loading process. Following acidification by transient exposure to NH_4Cl of IC.DP cells in the presence of IL-3 there is a Na^+-dependent recovery to a steady-state of pH_i 7.17±0.04 (*n*=5) which can be completely inhibited by the addition of 10 μM NMIA (Fig. 3). These data demonstrate that, in the absence of extracellular HCO_3/CO_2, alkalinization is completely accounted for by Na^+/H^+ exchange. If 10 μM NMIA is added when pH_i is higher than pH 6.8, rather than yielding a stable pH_i, cellular acidification is observed (Fig. 3). This demonstrates there is also an acid-loading process which is only active when pH_i is greater than about 6.8 and counters the activity of Na^+/H^+ exchange.

Next, we compared pH_i recovery from an NH_4Cl-induced acid load with or without activation of v-Abl PTK and in the absence of IL 3. At 18 hours, prior to overt apoptosis induced by the withdrawal of IL-3 there was a small reduction in the rate of alkalinization and a larger reduction in the steady-state pH_i following recovery from an acid load in the cells with v-abl PTK active compared to cells with v-abl PTK inactive (Fig. 3B) consistent with the net intracellular acidification seen (Fig. 2B).

The activity of the acid-loading process and Na^+/H^+ exchange were quantified to determine whether cellular acidification during apoptosis is due to a decrease in Na^+/H^+ exchange activity, an increase in acid-loading or a combination of the two. Induction of apoptosis by withdrawal of IL-3 for 18 hours significantly increased the rate of acid-loading (Fig. 4A) with the pH_i at which acid-loading process reaches zero

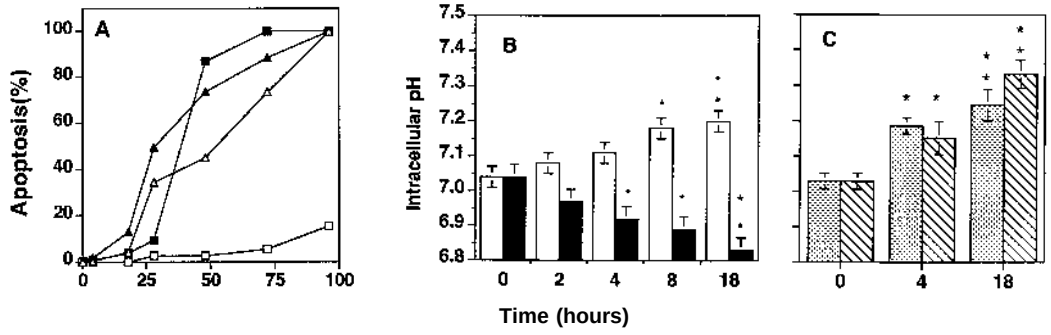

Fig. 2. (A) Kinetics of apoptosis for IC2.9 cells (triangles) and IC.DP cells (squares) following withdrawal of IL-3. Cells in log phase of growth were washed and incubated in Fischer's medium in an atmosphere of 5% CO_2 with inactive v-Abl PTK for 2-3 hours. v-Abl PTK was then either activated by temperature switch (open symbols) or maintained in an inactive state (closed symbols) and IL-3 was removed from the cell cultures. The percentage apoptosis was determined by fluorescence microscopy after nuclear staining with Hoechst 333342. Results are the mean value of 3 separate experiments. (B) Kinetics of the change in pH_i after withdrawal of IL-3 from IC.DP cells with v-Abl PTK active (open bars) or inactive (closed bars). (C) Kinetics of the change in pH_i in IC.DP cells cultured in the presence of IL-3 with either v-Abl PTK active (dotted bars) or inactive (striped bars). pH_i was measured at 37°C as described in Materials and Methods. pH_i values are the mean value of at least 18 replicate measurements made during 3 independent experiments. **P*< 0.05, ***P*<0.01 compared to values at 0 hours.

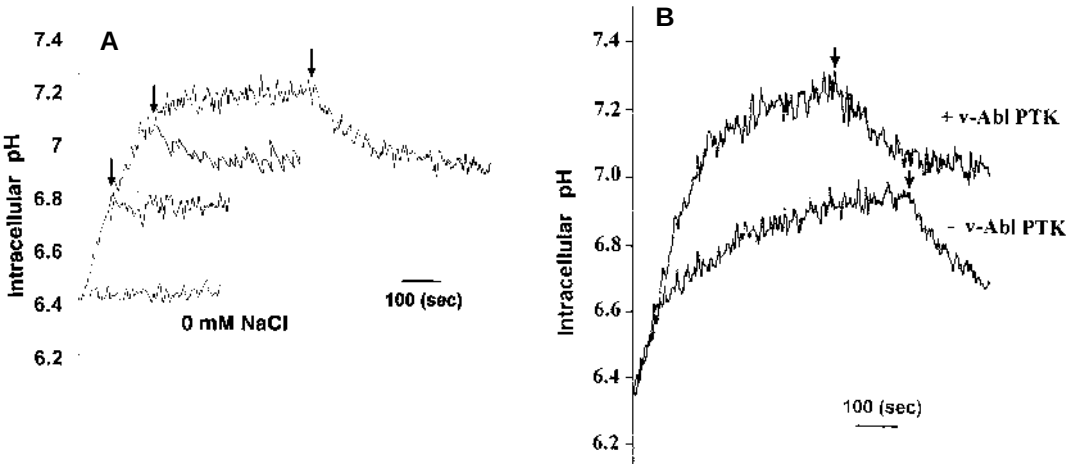

Fig. 3. (A) Characterisation of pH_i recovery in IC.DP cells following imposition of an acid load by transient exposure to NH_4Cl. The composite diagram demonstrates pH_i recovery in IC.DP cells after exposure to 30 mM NH_4Cl for 15 minutes in the presence of IL-3. Cells were suspended in either Na^+-containing or Na^+-free medium (TMA medium) and pH_i was monitored. Arrows indicate where in separate experiments 10 μM NMIA was added. The horizontal bar represents 100 seconds. Results are from an experiment typical of 3 repeats. (B) Comparison of pH_i recovery in IC.DP cells with v-Abl PTK inactivated for 18 hours in the presence or absence of IL-3. Prior to the start of recorded trace, cells were acidified by transient exposure to 30-45 mM NH_4Cl for 15 minutes. Measurement was initiated when cells were added to Na^+-containing medium. Data are representative of at least 10 experiments.

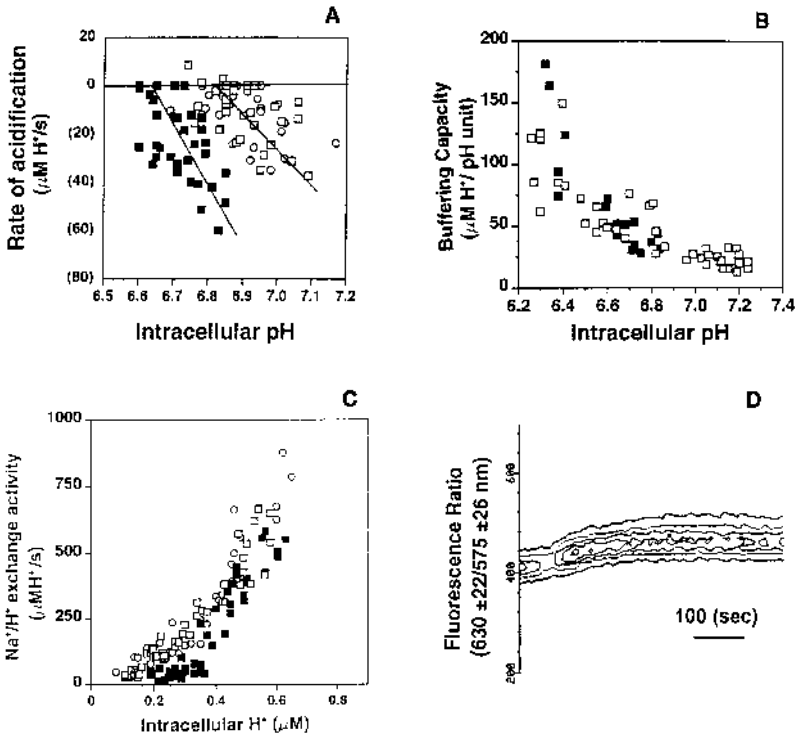

Fig. 4. (A) Effects of Abl PTK activity on the acidification rate of IC.DP cells in the absence of Na^+/H^+ exchange. The effects of v-Abl PTK activation on the acidification rate following blockade of Na^+/H^+ exchange with 10 μM NMIA. IC.DP cells were washed and incubated with Abl PTK inactive for 2 hours prior to further incubation for 18 hours in the presence of IL-3 (open circles) or absence of IL-3 (closed squares) or with Abl PTK activated in the absence of IL-3 (open squares). The rate of acidification was measured and calculated as indicated in Materials and Methods and Results. Each point shown represents a single measurement. All measurements were performed within 30 minutes at 37°C. (B) Activation of v-Abl PTK in IC.DP cells does not affect cellular buffering capacity. Comparison of the buffering capacity of IC.DP cells for H^+ with v-Abl PTK either active (open squares) or inactive (closed squares). Buffering capacity was determined as indicated in Materials and Methods. Regression lines from 6.4 to 7.2 and 6.2 to 6.4 were used to provide a value for buffering capacity for subsequent calculation of proton efflux rates and kinetics of acidification. All the measurements were performed at 37°C. Data are shown for 62 independent experiments. (C) The effects of v-Abl PTK activation on Na^+/H^+ exchange activity in IC.DP cells. Cells were washed and incubated with v-Abl PTK inactivated for 2 hours prior to further incubation for 18 hours in the presence (open circles) or absence (closed squares) of IL-3 or with v-Abl PTK activated in the absence of IL-3 (open squares). Na^+/H^+ exchange activity was measured and calculated as described in the Materials and Methods and Results. The data were collected and calculated from at least 15 typical experiments to measure pH_i recovery of the first-order derivative. All measurements were performed within 30 minutes at 37°C. (D) Effect of Abl PTK activity on the recovery from an imposed acid load in IC.DP cells. pH_i recovery in IC.DP cells with v-Abl PTK inactive 18 hours following IL-3 withdrawal. SNARF-loaded cells were acidified as described in Materials and Methods and then suspended in Na^+-containing medium to initiate an Na^+-dependent pH_i recovery. Cells were analysed immediately by flow cytometry to measure forward and orthogonal light scatter simultaneously with the ratio of emitted fluorescence intensity FL630/FL575 and time. The instrument was configured as described in Materials and Methods. Measurements were made at 37°C using a circulating water bath. Cell debris and any apoptotic cells were excluded by electronic gating based upon light scatter profiles. The 2-dimensional contour plots of fluorescence ratio versus time are representative of 5 independent experiments.

falling from 6.92±0.02 in viable cells to 6.63±0.05 (n=14) in cells precommitted to apoptosis. This increase in acid-loading during apoptosis is not due to a change in buffering capacity as there was no detectable difference in the buffering capacity of cells with or without the activation of v-Abl PTK following withdrawal of IL-3 (Fig. 4B). Analysis of Na^+/H^+ exchange activity showed there is a small reduction in Na^+/H^+ exchange activity in cells pre-committed to apoptosis (inactive v-Abl

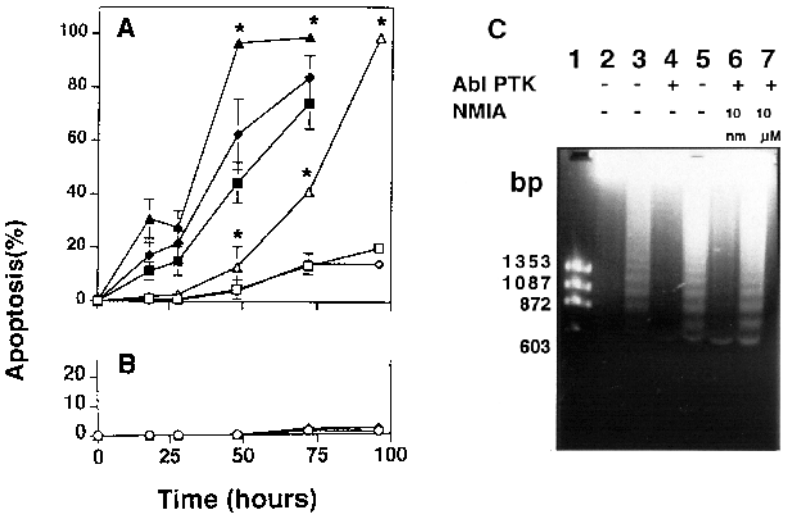

Fig. 5. Inhibition of Na^+/H^+ exchange restores an apoptotic response to the deprivation of IL-3 in IC.DP cells with activated v-Abl PTK. (A) Kinetics of apoptosis in IC.DP cells following withdrawal of IL-3 at time zero with v-Abl PTK either active (open symbols) or inactive (closed symbols). Cells were washed and exposed to either NMIA (10 μM, triangles), amiloride (10 μM, diamonds) or vehicle control (squares). Apoptosis was assessed by Hoechst 333342 staining of nuclear morphology and fluorescence microscopy. The data are mean values of 3 separate experiments ± s.e.m. Error bars are omitted when they are contained within the symbols. Comparisons are between NMIA or amiloride conditions and the corresponding temperature control. **P*<0.05. (B) As for A except that IL-3 was present in all cell samples. (C) Cell samples were prepared 48 hours after activation or inactivation of v-Abl PTK and DNA integrity was examined by agarose gel electrophoresis as described in Materials and Methods. Lane 1, DNA marker, øX174 DNA-*Hae*III digest molecular mass standard (Gibco); lane 2, IC.DP cells with v-Abl PTK inactive in the presence of IL-3; lane 3, IC2.9 cells (no v-Abl PTK) in the absence of IL-3; lane 4, IC.DP cells with v Abl PTK active (without IL-3); lane 5, IC.DP cells with v-Abl PTK inactive (without IL-3); lane 6, IC.DP cells with v-Abl PTK active (without IL-3), and continuously exposed to 10 nM NMIA; lane 7, IC.DP cells with v-Abl PTK active (without IL-3), and continuously exposed to 10 μM NMIA.

PTK without IL-3) compared to normal cells (v-Abl PTK inactive but in the presence of IL-3), (n=15, Fig. 4C). Flow cytometric analysis of recovery in pH_i from an acid load showed that this change in Na^+/H^+ exchange activity was not due to the appearance of subpopulations which do not regulate pH_i (Fig. 4D). Like BCECF, overtly apoptotic cells do not accumulate carboxy-SNARF-1 when exposed to carboxy-SNARF-1-AM (data not shown). Activation of v-Abl PTK in the absence of IL-3 restored both acid-loading and Na^+/H^+ exchange activity to that of cells without v-Abl PTK but in the presence of IL-3 (Fig. 4A and C). Analysis of mRNA by reverse transcription-polymerase chain reaction indicated that IC.DP cells express the NHE-1 isoform of Na^+/H^+ exchanger even though v-abl PTK is inactive for 18 hours (data not shown) (Brant et al., 1995). Overall, these results suggest that the decrease in pH_i seen prior to apoptosis following withdrawal of IL-3 are due to both an increase in an acid-loading process and slight decrease in Na^+/H^+ exchange activity.

Acidification prevents suppression of apoptosis by v-Abl PTK

Having demonstrated that cells in the precommitment phase of apoptosis become acidic (starting 2 hours after removal of IL-3) and that v-Abl PTK prevents this acidification, we investigated whether changes in pH_i play a functional role in the regulation of apoptosis and the protective action of v-Abl PTK. We reasoned that if Na^+/H^+ exchange activity was blocked with NMIA then the acidification process would act unopposed resulting in deep cellular acidification and the potentiation of apoptosis. To explore this hypothesis, we cultured the cells in the presence of 10 μM NMIA for up to 96 hours and assessed apoptosis by Hoechst 33342 staining of nuclear morphology. These studies were performed using cells cultured in the presence of 5% CO_2 and in medium containing bicarbonate. In the absence of IL-3, 10 μM NMIA both prevents the suppression of apoptosis by v-Abl PTK and accelerates the onset of apoptosis when v-Abl PTK is inactive (Fig. 5A). The effects of NMIA were mirrored by changes in pH_i. In the absence of IL-3, exposure of IC.DP cells to 10 μM NMIA for 18 hours resulted in an acidification of about 0.25 pH units down to pH 6.86 (Fig. 6A). Similar results were found when the cells were exposed to 50 μM EIPA, another analog of amiloride (data not shown). In the presence of IL-3, NMIA (10 μM) did not induce apoptosis (Fig. 5B) demonstrating that 10 μM NMIA is not intrinsically toxic and had no effect on IL-3 stimulation of cell proliferation (data not shown). Again acidification occurred but intracellular pH only fell from 7.25 to 7.17. This suggest that for apoptosis to occur pH_i must fall below a critical value of approximately 6.9. Western blotting showed that NMIA had no effect on the ability of v-Abl PTK to induce tyrosine phosphorylation (Fig. 6B).

We further characterised the effect of NMIA on levels of apoptosis by examination of its effects on non-random cleavage of DNA. Internucleosomal DNA fragmentation was observed 48 hours after withdrawal of IL-3 and is inhibited by activation of v-Abl PTK (Fig. 5C). NMIA (10 μM) blocked the v-Abl PTK suppression of internucleosomal DNA cleavage confirming the results obtained by examination of cellular morphology above (Fig. 5A). NMIA at a concentration of 10 nM, which inhibits Na^+/H^+ exchange by 35%, had no effect on DNA cleavage (Fig. 5C).

To determine whether the action of NMIA in cells with Abl PTK active and in the absence of IL-3 was related to its inhibition of Na^+/H^+ exchange, an equimolar concentration of amiloride, the parental analogue of NMIA, was used as a control. At a concentration of 10 μM, amiloride is a threefold less potent inhibitor of Na^+/H^+ exchange in IC.DP cells (data not shown) and does not cause acidification (Fig. 6A). At this concentration, amiloride does not restore an apoptotic response in the presence of v-Abl PTK activity and had no significant effect on apoptosis when v-Abl PTK was inactive (Fig. 5A). Together these data suggest that NMIA prevents the suppression of apoptosis through its effects on intracellular pH rather than other mechanisms.

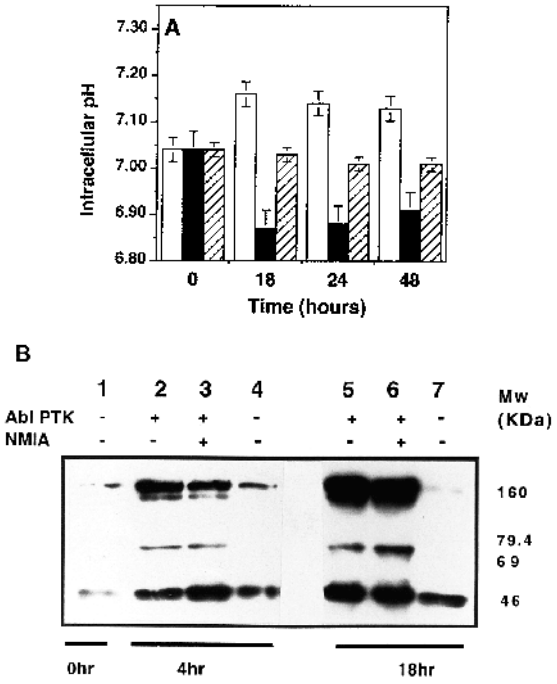

Fig. 6. (A) Effects of the inhibition of Na^+/H^+ exchange on intracellular pH in IC.DP cells in the presence of v-Abl PTK activity without IL-3. The kinetics of changes in pH_i of IC.DP cells with v-Abl PTK active but without IL-3 incubated in Fischer's medium in an atmosphere of 5% CO_2 and continuously exposed to NMIA (10 μM, solid bars), amiloride (10 μM, hatched bars) or vehicle control (open bars). The pH_i was measured as described in Materials and Methods. Data shown are the mean of 5 independent repeat experiments ± s.e.m. (B) Inhibition of Na^+/H^+ exchange does not affect v-Abl PTK induction of cellular protein tyrosine phosphorylation. Detection of cellular protein tyrosine phosphorylation in IC.DP cells in the absence of IL-3 by western blotting with anti-phosphotyrosine antibody. Lane 1, 0 hours; lane 2, v-Abl PTK active for 4 hours without NMIA; lane 3, v-Abl PTK active for 4 hours with 10 μM NMIA; lane 4, v-Abl PTK inactive for 4 hours NMIA; lane 5, v-Abl PTK active for 18 hours without NMIA; lane 6, v-Abl PTK active for 18 hours with 10 μM NMIA; lane 7, v-Abl PTK inactive for 18 hours without NMIA. 100 μg protein were loaded per lane and data presented are representative of 3 independent repeat experiments.

DISCUSSION

The current aim of our research is to identify early steps in the pathway initiating apoptosis. To achieve this, we have employed a simple, readily manipulated cell model system to study the regulation of apoptosis. In view of several reports implicating a reduction in intracellular pH as a proapoptotic signal (Barry et al., 1993; Li and Eastman, 1995; Rajotte et al., 1992), we have investigated systematically early changes in acid/base homeostasis in cells stimulated to undergo apoptosis or in cells prevented from doing so (without concomitant proliferation) by activation of v-Abl PTK.

Activation of v-Abl PTK elicits multiple measurable cellular changes including the activation of protein kinase C (Evans et al., 1995). v-Abl PTK associates with the signalling adaptor protein SHC (Owen-Lynch et al., 1995). Pertinent to this work is the observation that v-Abl PTK activation caused a cellular alkalinization which could be partially attenuated by chronic exposure to the phorbol ester TPA (Owen et al., 1993). Here we have confirmed that in the absence of both v-Abl PTK activity and IL-3 there is a reproducible decrease in pH_i as early as 2 hours after cytokine removal, we have investigated potential mechanisms of acidification and we have determined whether changes in pH_i are functionally important with respect to the engagement of apoptosis.

Acidification in apoptosis is due to increased acid loading and decreased Na^+/H^+ exchange

Intracellular pH is determined by an equilibrium between all cellular acidification and alkalinizing processes. We have sought to analyse the changes to this equilibrium which occur during the early stages of apoptosis. When Na^+/H^+ exchange is blocked by addition of 10 μM NMIA to viable IC.DP cells (i.e. either in the presence of IL-3 or with v-Abl PTK activated) they acidify, indicating that the steady-state pH_i of these cells is the result of the Na^+/H^+ exchange activity causing the efflux of H^+ and other acid-loading processes which allow entry or accumulation of H^+. The identity of the acid-loading process described (Fig. 3) remains to be fully determined. It is unlikely to be Cl^-/HCO_3^- exchange as the acid-loading process could occur in the nominal absence of CO_2/HCO_3^- (Fig. 4A) and could not be blocked by 0.5 mM DIDS (data not shown), which is known to inhibit Cl^-/HCO_3^- exchange in lymphocytes (Garcia-Soto and Grinstein, 1990). More likely candidates for the acid-loading process are metabolic acid production, a proton leak in the plasma membrane and/or efflux of protons from acidic micro vesicles into the cytoplasm (Bronk and Gores, 1991).

Although we remain uncertain of the precise identity of this acid-loading process, measurement of its rate, taking into account cellular capacity for H^+, indicates that its activity is substantially increased 18 hours after IL-3 withdrawal. In contrast, quantification of Na^+/H^+ exchange at the same time point showed it to be only slightly reduced. These changes in transport activity are prevented by activation of v-Abl PTK mirroring its prevention of cellular acidification following IL-3 withdrawal. Previous studies have suggested that a reduction in the 'set point' of Na^+/H^+ exchange causes acidification in IL-2 dependent apoptosis (Li and Eastman, 1995). The changes in Na^+/H^+ exchange kinetics (Fig. 4C) we observed are consistent with a reduction in the 'set point' though it should be noted that it was not possible to acidify the cells sufficiently to allow accurate measurement of the V_{max} or Hill coefficient. Thus the fall in pH_i observed during the precommitment phase of apoptosis is the result of an increase in the rate of intracellular H^+ production and/or entry (Fig. 4A) and is exacerbated by a reduction of Na^+/H^+ exchange activity. Efflux of H^+ from the cytosol via vacuolar H^+-ATPase has been implicated in the delay of G-CSF to delay apoptosis in neutrophils by granulocyte colony-stimulating factor (Gottlieb et al., 1995). However, in our model bafilomycin A, which blocks vacuolar H^+-ATPase had no effect on cellular pH (data not shown).

Role of acidification in the precommitment phase of apoptosis

Our findings that cells undergoing cytokine withdrawal-induced apoptosis acidify suggests that an early decrease in pH_i may herald apoptosis and also that a sustained fall in pH_i may

facilitate the completion of this death pathway. The observation that complete inhibition of Na^+/H^+ exchange by 10 μM NMIA induced apoptosis in cells with active v-Abl PTK suggests that abrogation of cellular acidification may contribute to v-Abl PTK mediated cell survival (Fig. 5A). This is unlikely to be a non-specific effect of NMIA as an equimolar concentration of amiloride which does not prevent the acidification did not block the protective effect of v-abl PTK. Moreover NMIA is not intrinsically toxic as it does not kill IC.DP in the presence of IL-3 (Fig. 5B). This result also implies that IL-3 prevents apoptosis via a different pathway from v-abl PTK. There are other examples of apoptosis in which intracellular acidification appears to be important. For example, Na^+/H^+ activity is required for the suppression of apoptosis in MOE-7 cells by granulocyte macrophage colony stimulating factor (Rajotte et al., 1992). Acidification is also found in Jurkat cells which have been induced to undergo apoptosis by either anti-Fas IgM, UV light or cycloheximide (Gottlieb et al., 1996). Surprisingly, both acidification and apoptosis can be prevented by incubating the cells with the weak base imidazole and chloroquine (Gottlieb et al., 1996). These data support the idea that acidification is essential for apoptosis. However, it should not be assumed from these studies that acidification is essential for all types of apoptosis or for all types of lethal stimuli as exceptions have been described (Li and Eastman, 1995), particularly as the role of cellular pH has not been studied extensively in apoptosis induced by DNA damage.

In conclusion, these data imply there is at least one step in the pathway to cytokine deprivation induced apoptosis (at least in some haemopoietic cell types) which is either enhanced or dependent upon an acidic intracellular environment. The acidification process is the result of an increase in an acid loading process(es) and a decrease in Na^+/H^+ exchange activity. Interestingly, the three-dimensional structure of the apoptosis suppressor protein Bcl-x_L is reminiscent of a pH-dependent membrane pore (Muchmore et al., 1996). It is also possible that decreased intracellular pH facilitates the activity of apoptotic proteases or the generation of ceramide by acidic sphingomyelinase (Cifone et al., 1994) but these remain to be determined. Further studies are required to elucidate the exact nature of the acidification process(es), to identify the molecules involved and to map the pathway leading from acidification to the irreversible engagement of apoptosis.

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