

# Ligation of CD40 rescues Ramos-Burkitt lymphoma B cells from calcium ionophore- and antigen receptor-triggered apoptosis by inhibiting activation of the cysteine protease CPP32/Yama and cleavage of its substrate PARP

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**Abstract** The new and growing family of interleukin-1 $\beta$ -converting enzyme (ICE) cysteine proteases are now recognised to be major effectors of cellular death by apoptosis. Like other members of this family, the CPP32/Yama proform is activated by processing to its active heterodimeric enzyme or apopain when it likely contributes to the process of apoptosis by cleaving poly(ADP-ribose) polymerase (PARP) and thereby inhibiting much of its DNA repair activity. Apoptosis plays a fundamental role in the regulation of the immune system where it is involved in the selection of both T and B lymphocytes bearing antigen receptor (AgR) for non-self. Cells of the Ramos Epstein-Barr virus (EBV)-genome-negative Burkitt lymphoma (BL) B cell line (Ramos-BL) can be triggered into growth arrest and apoptosis by treating with the calcium ionophore ionomycin or by cross-linking their surface AgR with antibodies directed against immunoglobulin (Ig)M (anti-IgM). Ionomycin- and AgR-triggered growth arrest and apoptosis are arrested by signals transduced through the surface CD40 of Ramos-BL B cells. Both ionomycin and anti-IgM trigger activation of CPP32 and cleavage of PARP prior to the onset of apoptosis; this process is abrogated by treatment with anti-CD40 and is independent of Bcl-2 expression. A tripeptide inhibitor of ICE family cysteine proteases, Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) inhibits ionomycin- and AgR-triggered CPP32 activation, PARP cleavage and apoptosis, but not growth arrest, in Ramos-BL B cells. Thus, in this report we demonstrate that in a physiological system, activation of endogenous members of the ICE family, including CPP32, and cleavage of the death substrate PARP act as major effectors of apoptotic death.

**Key words:** Antigen receptor; Apoptosis; B cell; CD40; CPP32/Yama; ICE; PARP

## 1. Introduction

Apoptosis is a highly conserved form of cell death which plays a fundamental role in development, homeostasis and in the regulation of the immune system [1,2]. The concept that the mechanism of apoptosis is conserved derives from observations consistently demonstrating that all mammalian cells undergoing this form of death exhibit common morphological

characteristics [3,4]. These features include cellular shrinkage, membrane blebbing, chromatin condensation and degradation and culminate with the rapid phagocytosis of the apoptotic cells or their fragments by neighbouring cells or professional phagocytes [1–4]. Conservation of the molecular machinery of apoptosis also finds strong support from studies of programmed cell death in the nematode *Caenorhabditis elegans*. Here, the protein products of *ced-3* and *ced-4* are required for all somatic deaths that occur during the development of this nematode, while the *ced-9* gene product affords protection from *ced-3*- and *ced-4*-induced death. The Ced-9 protein is homologous to mammalian Bcl-2, a proto-oncogene product that affords protection against apoptosis, such that the function of mutationally inactivated *ced-9* can be partially restored by expression of the human *bcl-2* gene [5]. Although no mammalian homologues of Ced-4 have been discovered to date, Ced-3 exhibits homology to the new and growing family of interleukin-1 $\beta$ -converting enzyme (ICE) cysteine proteases [6–9].

The ICE family is now known to comprise at least five members including CPP32/Yama/apopain [10–12], Nedd-2/ICH-1 [13,14], TX/ICH-2/ICE<sub>rel</sub>II [15–17], ICE<sub>rel</sub>III [17] and Mch-2 [18]; Fernandes-Alnemri et al. [19] and Duan et al. [20] have recently described novel additions to the family, Mch-3 and ICE-LAP3 respectively, which are closely related to CPP32/Yama. All family members to date are synthesised as proenzymes that are proteolytically processed to form active heterodimeric proteases that contain the pentameric peptide, QACRG, which surrounds the putative catalytic residue cysteine [10–20]. Ectopic expression of ICE and its relatives results in the apoptosis of a variety of host cells which can be arrested by the cowpox serpin Crm A or by tetrapeptide (YVAD) inhibitors of ICE-like proteases [14,21,22].

The article now presented queries how ICE and its family members contribute to the process of apoptosis: although yet to be elucidated, it is thought that the activation of a cascade of proteases and the subsequent proteolytic cleavage of their specific substrates likely leads to the structural changes or to the activation of other effectors critical for death [4]. It is known that ICE-like proteases degrade lamins during apoptosis and may thereby trigger collapse of the chromatin [23,24]. A partially characterised 24-kDa protease has been shown to induce DNA degradation characteristic of apoptosis when added to purified nuclei possibly by activating an endonuclease as it does not itself exhibit any nuclease activity [25]. Activated CPP32/Yama or apopain can cleave poly-(ADP-ribose) polymerase (PARP) and thereby inhibit most of its DNA repair activity [11,12,18,19,26].

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**Abbreviations:** Ag, antigen; AgR, antigen receptor; BL, Burkitt lymphoma; EBV, Epstein-Barr virus; ICE, interleukin-1 $\beta$ -converting enzyme; Ig, immunoglobulin; PARP, poly(ADP-ribose) polymerase; sIg, surface immunoglobulin; zVAD-fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(O-methyl)-fluoromethylketone

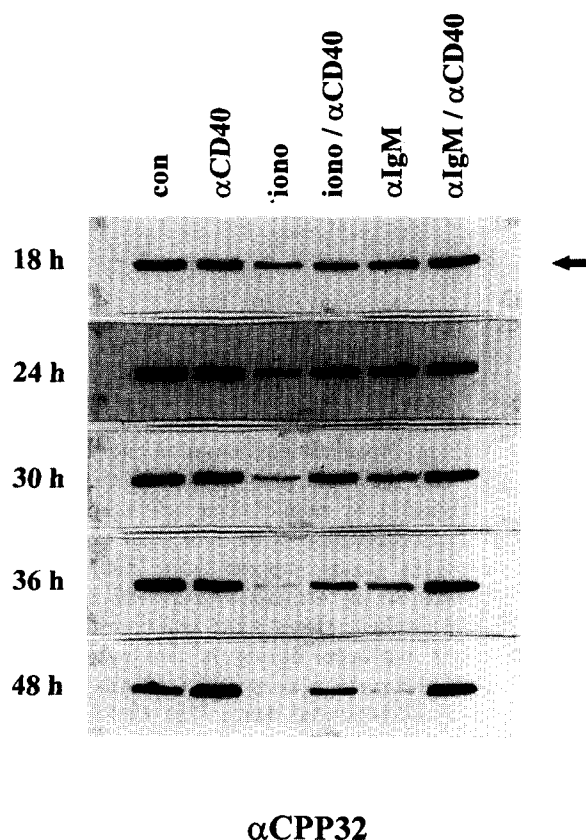


Fig. 1. Anti-CD40 inhibits ionomycin- and anti-IgM-triggered CPP32 processing and apoptosis in Ramos-BL B cells. Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured for 18, 24, 30, 36 and 48 h alone or with ionomycin (1  $\mu$ g/ml) or with anti-IgM (20  $\mu$ g/ml) in the absence or presence of anti-CD40 (1  $\mu$ g/ml) and expression of the CPP32 proform determined by immunoblotting. The results of one experiment, representative of two, are shown and the CPP32 proform is denoted by an arrow.

In this study, we investigate CPP32/Yama/apopain-mediated cleavage of PARP as a putative effector of apoptosis in a cell line model of B lymphocyte selection during maturation of the humoral immune response. Briefly, in the germinal centres (GC) of secondary lymphoid tissues, B cells undergo somatic hypermutation on their immunoglobulin (Ig) variable genes and are selected for expression of high-affinity antigen receptor (AgR), which comprises Ig, for non-self by competing for limited antigen: non-selected GC B cells are deleted by apoptosis and removed by tingible body macrophages [27–29].

Table 1  
Anti-CD40 rescues Ramos-BL B cells from ionomycin- and anti-IgM-triggered growth arrest

Time (h)	$[^3\text{H}]$ thymidine incorporation (cpm)					
	Con	$\alpha$ CD40	Iono	Iono + $\alpha$ CD40	$\alpha$ IgM	$\alpha$ IgM + $\alpha$ CD40
18	11 926	11 624	7 529	8 266	4 719	4 741
24	37 754	18 993	8 373	13 626	12 109	13 592
30	38 944	30 806	9 823	19 647	23 323	30 544
36	39 801	32 967	17 643	26 162	21 642	19 740
48	71 831	36 722	14 400	29 401	22 801	31 809

Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured alone, or with ionomycin (Iono, 1  $\mu$ g/ml) or with anti-IgM ( $\alpha$ IgM, 20  $\mu$ g/ml) in the absence or presence of anti-CD40 ( $\alpha$ CD40, 1  $\mu$ g/ml) for the appropriate time and  $[^3\text{H}]$ thymidine incorporation was assessed during the last 4 h of culture. The results of one experiment, representative of two, are presented as mean counts per minute (cpm) for triplicate cultures with standard deviation typically less than 15%.

A vestige of this normal selection process appears to be retained in Burkitt lymphoma (BL) B cells which are Epstein-Barr virus (EBV)-genome-negative such as Ramos (Ramos-BL). Not only do these cells express a phenotype characteristic of GC B cells but they also readily undergo apoptosis in response to a number of stimuli including calcium ionophore. However, in marked contrast to GC B cells antibody directed against Ig actually drives apoptosis in these cells [30]. Ligation of the type I surface glycoprotein CD40 delivers a survival signal in both normal and neoplastic B cell types [29,30].

We now present evidence that signals transduced through CD40 [30] or treatment with the zVAD-fmk tripeptide inhibitors of the ICE-family cysteine proteases [31,32] prevent calcium ionophore- and AgR-triggered processing of CPP32, cleavage of PARP and apoptosis in Ramos-BL B cells.

## 2. Materials and methods

### 2.1. Reagents, antibodies and their sources

**2.1.1. Reagents.** Benzylloxycarbonyl-valinyl-alaninyl-aspartyl(*O*-methyl)-fluoromethylketone (zVAD-fmk), a cell-permeable, irreversible tripeptide inhibitor of the ICE family of cysteine proteases, was supplied by Enzyme Systems Products, Inc. (Dublin, CA, USA). Ionomycin was obtained from Calbiochem (Nottingham, UK) and dimethyl sulphoxide (DMSO) from Sigma (Poole, UK). Stock solutions of zVAD-fmk (50 mM in DMSO) and ionomycin (1 mg/ml in ethanol) were kept at  $-20^\circ\text{C}$  and final dilutions made immediately prior to use.  $[^3\text{H}]$ Thymidine (185 GBq/mmol) and the enhanced chemiluminescence (ECL) Western blotting system were purchased from Amersham Life Science (Aylesbury, UK).

**2.1.2. Antibodies.** The sheep polyclonal antibodies to human IgM (anti- $\mu$ -chain-specific) was obtained from The Binding Site Ltd (Birmingham, UK). The murine monoclonal antibody to human CD40 (G28.5) was purified from the supernatant of hybridoma HB9110 (American Type Culture Collection, Rockville, MD, USA). The sheep anti-mouse Ig horseradish peroxidase (HRP)-linked whole antibody was purchased from Amersham. The mouse monoclonal antibody to human CPP32 was supplied by Transduction Laboratories (Lexington, KY, USA). The mouse monoclonal antibody to mammalian PARP (anti-PARP) was kindly donated by Professor W.C. Earnshaw of The Institute of Molecular and Cellular Biology, The University of Edinburgh (UK). The mouse monoclonal antibody to human Bcl-2 (anti-Bcl-2) was a kind gift from Dr. D.Y. Mason of The Department of Cellular Science, John Radcliffe Hospital, Oxford (UK).

### 2.2. BL cell lines

The BL B cell line used in this study, Ramos-BL, is EBV-genome-negative and was derived from a 3-year-old Caucasian male with histologic diagnosis of American BL [33–35]. The Ramos-BL B cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). These cells were maintained in exponential culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (hiFCS) from pre-screened batches.

### 2.3. Cell culture

Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were established in RPMI 1640 medium enriched with 10% hiFCS (CM) in 24-well (for samples for Western blotting) and 96-well (for measurement of [ $^3$ H]thymidine incorporation and apoptosis) flat-bottomed microculture plates in duplicate and triplicate respectively at 37°C, 5% CO<sub>2</sub> for the appropriate time.

### 2.4. Assessment of DNA synthesis

DNA synthesis was determined for the final 4 h of incubation for Ramos-BL B cell culture by pulsing each well with 0.5  $\mu$ Ci (18.5 kBq) of [ $^3$ H]thymidine contained in 50  $\mu$ l of CM.

### 2.5. Detection of apoptosis

Ramos-BL B cell apoptosis quantified routinely by flow cytometry (Becton Dickinson FACScan analyzer, Oxford, UK) as previously described [30,36]. The results presented are expressed as percent apoptotic events which is calculated from the proportion of cellular events classified as apoptotic for each culture.

### 2.6. Detection of CPP32, PARP and Bcl-2 by Western blotting

CPP32, PARP and Bcl-2 were detected by immunoblotting with the appropriate murine monoclonal antibodies. Briefly,  $6 \times 10^5$  Ramos-BL B cells per sample were pelleted by microfuge, washed twice with PBS and resuspended in 50  $\mu$ l SDS-sample buffer. Samples for detection of PARP were run on 7.5% SDS-polyacrylamide gels and samples for detection of CPP32 and Bcl-2 on 14% SDS-polyacrylamide gels. Following transfer to nitrocellulose membrane, the immunoblots were blocked by incubating with 5% milk in Tris-HCl pH 7.5 with 0.1% Tween 20 and probed for 2 h at room temperature or overnight at 4°C with murine anti-CPP32 (0.25  $\mu$ g/ml) or with murine anti-PARP (1 in 2000) or with murine anti-Bcl-2 (1 in 20) diluted to the appropriate concentration in Tris-HCl pH 7.5 with 0.1% Tween 20 and 0.001% sodium azide. The immunoblots were then probed with HRP-conjugated anti-mouse Ig (1 in 7500 in 5% milk in Tris-HCl pH 7.5) and developed using the ECL system.

## 3. Results

### 3.1. Anti-CD40 inhibits ionomycin- and anti-IgM-triggered

#### CPP32 processing and apoptosis in Ramos-BL B cells

We have previously shown that cells of the EBV-genome-negative Ramos-BL B cell line can be rescued from calcium ionophore- and surface IgM (sIgM)-triggered growth arrest and apoptosis by signals transduced through the type I surface glycoprotein CD40 [30]. To determine whether the cysteine protease CPP32, which is highly expressed in cell lines of lymphocytic origin [10] including Ramos-BL (Fig. 1), is a component of the ionomycin- and sIgM-triggered pathway(s) leading to apoptosis, we assessed expression of the endogenous CPP32 proform for Ramos-BL B cells cultured alone or

Table 2

Anti-CD40 rescues Ramos-BL B cells from ionomycin- and anti-IgM-triggered apoptosis

Time (h)	Percent apoptotic events					
	Con	$\alpha$ CD40	Iono	Iono + $\alpha$ CD40	$\alpha$ IgM	$\alpha$ IgM + $\alpha$ CD40
18	7.2	9.8	30	16	12	16
24	6.3	9.6	35	19	14	19
30	7.3	11	46	20	17	16
36	4.2	9.0	54	23	20	15
48	2.6	4.9	67	25	27	11

Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured as described for Table 1 and apoptosis was quantified by flow cytometry following the appropriate incubation period. The results of one experiment, representative of two, are presented as percent apoptotic events for pooled triplicate cultures.

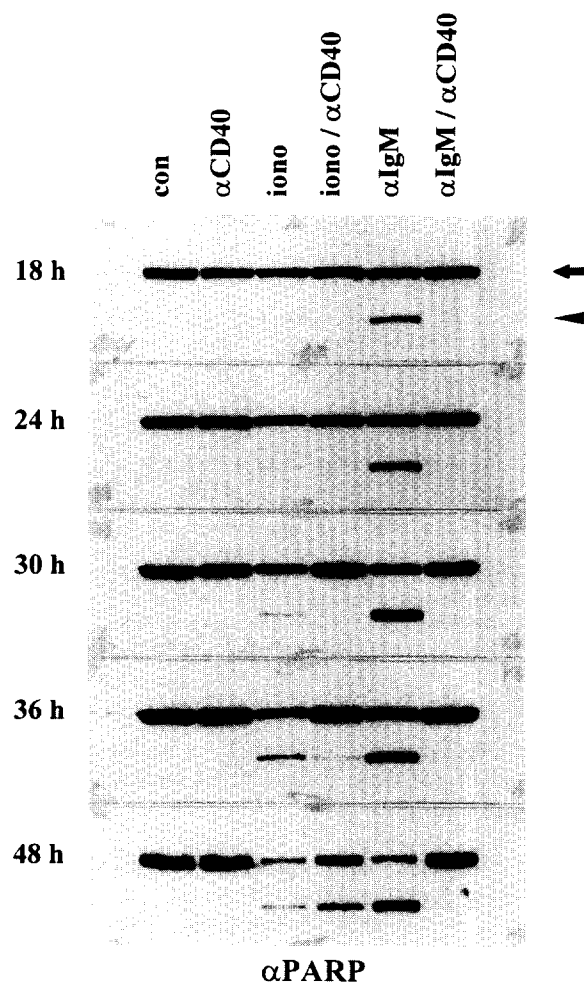


Fig. 2. Anti-CD40 inhibits ionomycin- and anti-IgM-triggered cleavage of PARP in Ramos-BL B cells. Ramos-BL B cells were cultured as described for Fig. 1 and expression of PARP was determined by immunoblotting. The results of one experiment, representative of two, are shown. The 130-kDa PARP is denoted by an arrow and its 89-kDa fragment by an arrowhead.

with ionomycin or anti-IgM in the absence or presence of anti-CD40 over a 48-h culture period.

Ramos-BL B cells treated with ionomycin exhibit a significant decrease from 18- to 48-h culture of detectable levels of CPP32 proenzyme, presumably reflecting activation by processing to its 17-kDa and 12-kDa subunits which comprise apopain [12]; these data are consistent with our findings that ionomycin induces significant growth arrest (Table 1) and apoptosis (Table 2) by 18-h culture with maximal effect achieved by 48 h. Anti-IgM also triggers a steady decrease in detectable levels of the CPP32 proform from 24 h post stimulation, which is consistent with the ability of anti-IgM to induce significant apoptosis of Ramos-BL B cells from 30-h culture; the late onset of apoptosis likely reflects our findings that anti-IgM-induced apoptosis is linked to the cell cycle [30]. Since anti-IgM induces growth arrest as early as 18 h, it is likely that this AgR-triggered pathway is distinct from that to CPP32 activation and apoptosis.

Co-culture with anti-CD40 significantly reduces ionomycin-triggered processing of CPP32 and abrogates anti-IgM-triggered CPP32 cleavage over the 48-h culture period. This is consistent with our data that treatment of Ramos-BL B cells

Table 3  
zVAD-fmk does not inhibit ionomycin- and anti-IgM-triggered growth arrest in Ramos-BL B cells

	zVAD-fmk ( $\mu$ M)	[ $^3$ H]Thymidine incorporation (cpm)		
		Exp. 1	Exp. 2	Exp. 3
Con	0	58 632	63 435	45 196
	10	67 252	62 423	39 788
	50	61 809	68 934	54 086
	100	75 378	70 842	54 115
Iono	0	10 265	9 756	12 900
	10	11 271	12 193	16 561
	50	8 025	12 785	9 897
	100	12 083	7 895	12 138
$\alpha$ IgM	0	29 364	21 186	27 995
	10	24 001	22 051	28 477
	50	14 903	12 247	13 718
	100	22 016	17 666	22 184

Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured alone, or with ionomycin (Iono, 1  $\mu$ g/ml) or with anti-IgM ( $\alpha$ IgM, 20  $\mu$ g/ml) in the absence or presence of zVAD-fmk (10–100  $\mu$ M) and [ $^3$ H]thymidine incorporation was assessed during the last 4 h of a 48-h culture. The results of three experiments are presented as mean counts per minute (cpm) for triplicate cultures with standard deviation typically less than 15%.

with anti-CD40 significantly enhances the proliferative capacity and viability of those cells cultured with calcium ionophore or with anti-IgM.

### 3.2. Anti-CD40 inhibits ionomycin- and anti-IgM-triggered cleavage of PARP in Ramos-BL B cells

In order to confirm that ionomycin and anti-IgM trigger activation of CPP32 and that the observed decrease in detectable levels of CPP32 can be attributed to processing of the proenzyme to its active apopain form [12], we investigated expression of PARP, which is cleaved from a 113–116-kDa form to an 85–89-kDa fragment by CPP32 [11,12,26,37].

As shown in Fig. 2 PARP is expressed as a single polypeptide protein of 130 kDa, a slightly higher molecular weight than that reported by others [11,12,26,37], in untreated and anti-CD40-stimulated Ramos-BL B cells over a 48-h culture period. By contrast, anti-IgM triggers cleavage of PARP, as witnessed by increasing levels of the 89-kDa cleavage fragment, and this is abrogated by co-culture with anti-CD40. While our observations that anti-IgM-triggered cleavage of PARP, apparent by 18-h culture, precedes activation of CPP32, which is not apparent until 30 h, may reflect the different sensitivities of the antibodies used for immunoblotting or differences in concentrations of protein expressed, it may also indicate that signals transduced through sIgM activate another member of the ICE family that is able to cleave PARP, prior to activation of CPP32. We favour the latter of these alternatives since ionomycin, which clearly activates CPP32 activation by 18 h, does not induce cleavage of PARP until 30 h and thus precludes the possibility that differences in the sensitivities of our assays for CPP32 and PARP cleavage account for our observations. The data obtained from ionomycin-treated cultures raise the possibility that CPP32 activated by an ionomycin-triggered pathway acts on a substrate other than PARP. As for anti-IgM, anti-CD40 is able to at least partially inhibit ionomycin-triggered cleavage of PARP.

Table 4  
zVAD-fmk inhibits ionomycin- and anti-IgM-triggered apoptosis in Ramos-BL B cells

	zVAD-fmk ( $\mu$ M)	Percent apoptotic events		
		Exp. 1	Exp. 2	Exp. 3
Con	0	11	7.5	4.1
	10	10	6.5	3.5
	50	8.3	5.4	3.0
	100	9.0	5.3	3.2
Iono	0	52	73	67
	10	48	73	60
	50	47	47	34
	100	33	25	21
$\alpha$ IgM	0	47	51	26
	10	41	42	21
	50	35	36	20
	100	31	32	16

Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured as described for Table 3 and apoptosis was quantified by flow cytometry following 48-h culture. The results of three experiments are presented as percent apoptotic events for pooled triplicate cultures.

### 3.3. Anti-CD40-mediated inhibition of ionomycin- and anti-IgM-triggered CPP32 processing and PARP cleavage occurs independently of Bcl-2 expression

Although it is now well established that expression of the 26-kDa protein product of the *bcl-2* proto-oncogene can afford protection against apoptosis [5], it is as yet unclear at which specific step in the apoptotic pathway Bcl-2 may act: Kumar [38] has postulated that Bcl-2 may inhibit activation of ICE family members by subverting upstream signal transduction events or by blocking proteolytic cleavage of these cysteine proteases to their active forms or by sequestering their downstream targets. We decided to address this issue by mon-

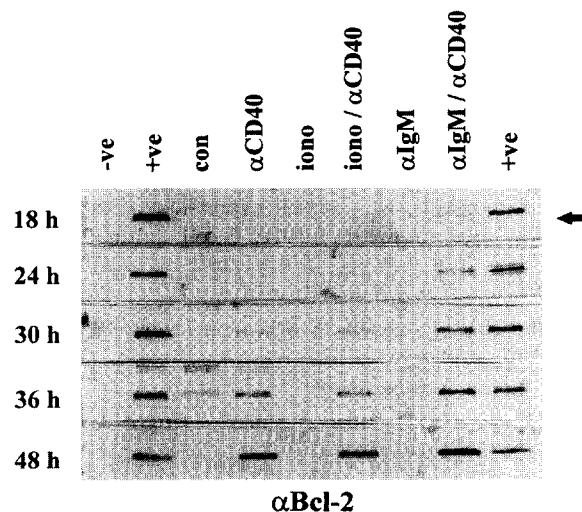


Fig. 3. Anti-CD40-mediated inhibition of ionomycin- and anti-IgM-triggered CPP32 processing and PARP cleavage occurs independently of Bcl-2 expression. Ramos-BL B cells were cultured as described for Fig. 1 and expression of Bcl-2 was determined by immunoblotting. The results of one experiment, representative of two, are shown and Bcl-2 is denoted by an arrow; negative (–ve) controls comprise time zero samples of Ramos-BL B cells and positive (+ve) controls comprise Ramos-BL B cells stimulated for 48 h with anti-CD40 (1  $\mu$ g/ml).

itoring expression of Bcl-2 in Ramos-BL B cells cultured on their own or with ionomycin or anti-IgM in the absence or presence of anti-CD40 (Fig. 3).

We were unable to detect Bcl-2 by immunoblotting in control untreated as well as in ionomycin- and anti-IgM-treated Ramos-BL B cells cultured over a 48-h period. However, expression of Bcl-2 became apparent from 36 h on culture of Ramos-BL B cells with anti-CD40 alone or with ionomycin and anti-CD40. Since treatment with anti-CD40 prevents processing of CPP32 as early as 18 h post stimulation and cleav-

age of PARP at 30-h culture, which is prior to expression of Bcl-2, the ability of anti-CD40 to subvert ionomycin-triggered activation of CPP32 and subsequent cleavage of PARP occurs independently of Bcl-2 expression.

By contrast, co-culture of Ramos-BL B cells with anti-IgM and anti-CD40 induces detectable expression of Bcl-2 by 24 h post stimulation, suggesting that anti-IgM-triggered signal transduction events impinge upon and amplify the CD40-triggered biochemical events that lead to expression of Bcl-2. Since this induction of Bcl-2 expression precedes sIgM-trig-

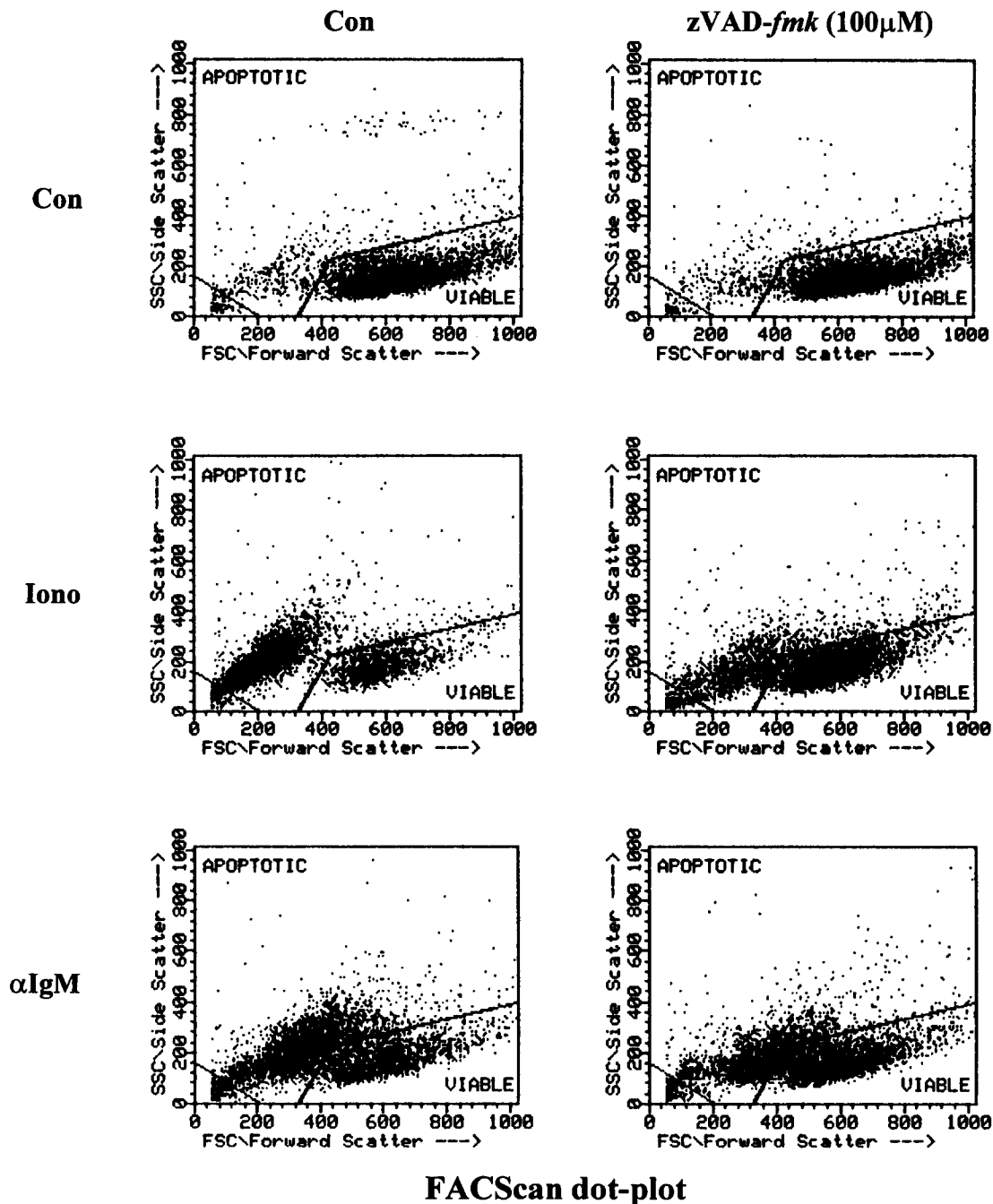


Fig. 4. FACS dot-blot light scatter profiles: zVAD-fmk inhibits ionomycin- and anti-IgM-triggered apoptosis in Ramos-BL B cells. Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured for 48 h alone or with ionomycin (1  $\mu$ g/ml) or with anti-IgM (20  $\mu$ g/ml) in the absence or presence of zVAD-fmk (100  $\mu$ M) and apoptosis was assessed by flow cytometry. The viable population contains cells with relatively high forward-scatter and low side-scatter properties while cells undergoing apoptosis appear in the low-forward-scatter/high-side-scatter zone. The results of one experiment, representative of three, are shown.

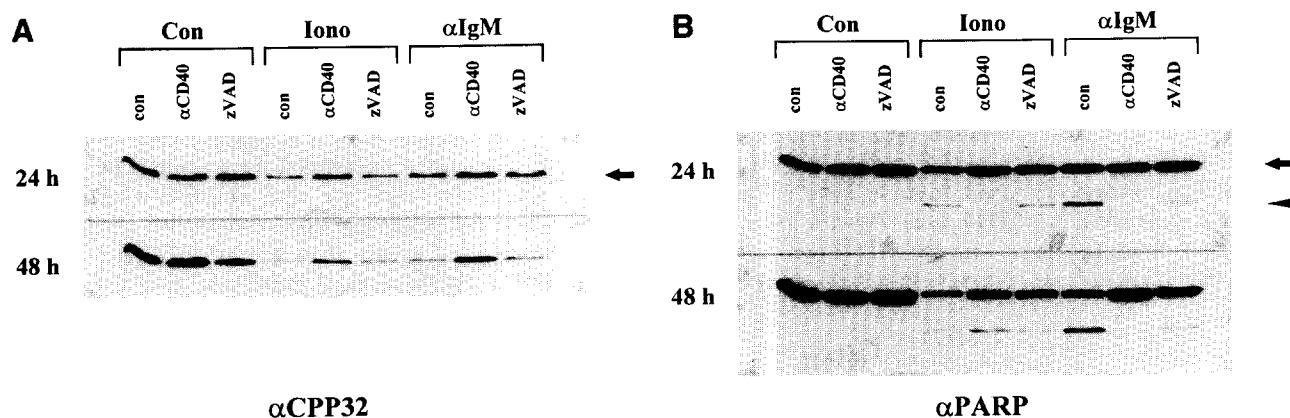


Fig. 5. zVAD-fmk inhibits ionomycin- and anti-IgM-triggered CPP32 processing and PARP cleavage in Ramos-BL B cells. Ramos-BL B cells ( $3 \times 10^5$  cells/ml) were cultured for 24 h or 48 h alone or with ionomycin (1  $\mu$ g/ml) or with anti-IgM (20  $\mu$ g/ml) in the absence or presence of anti-CD40 (1  $\mu$ g/ml) or zVAD-fmk (100  $\mu$ M) and expression of the CPP32 proform and of PARP was determined by immunoblotting. The results of one experiment, representative of three, are shown.

gered processing of CPP32, which does not become apparent until 30 h, it is possible that Bcl-2 may prevent CPP32 processing and activation in this scenario. However, the anti-CD40-mediated inhibition of sIgM-triggered PARP cleavage is likely to be Bcl-2-independent since anti-IgM triggers cleavage of PARP at 18 h, possibly in a CPP32-independent manner, prior to anti-CD40-triggered expression of Bcl-2.

#### 3.4. zVAD-fmk inhibits ionomycin- and anti-IgM-triggered CPP32 processing, PARP cleavage and apoptosis, but not growth arrest, in Ramos-BL B cells

The data collected thus far demonstrate that both the calcium ionophore ionomycin and cross-linking sIgM trigger activation of CPP32 and cleavage of PARP and may thus lead to the apoptosis of Ramos-BL B cells. In order to test this hypothesis further, we examined the effect of zVAD-fmk, an irreversible, cell-permeable tripeptide inhibitor of ICE family cysteine proteases [31,32], on ionomycin- and anti-IgM-triggered processing of CPP32, PARP cleavage and on growth arrest and apoptosis in Ramos-BL B cells.

The results presented in Table 3 demonstrate that following 48-h culture, zVAD-fmk (10–100  $\mu$ M) slightly increases basal levels of [ $^3$ H]thymidine incorporation but has little or no effect on ionomycin- and anti-IgM-triggered growth arrest in Ramos-BL B cells. By marked contrast zVAD-fmk, which fails to affect basal levels of apoptosis, significantly reduces both ionomycin- and anti-IgM-triggered levels of apoptosis in a dose-dependent fashion (Table 4; Fig. 4). However, the maximal dose of 100  $\mu$ M zVAD-fmk used here does not quite reduce ionomycin- and anti-IgM-triggered levels of apoptosis to those of the control, which suggests that another factor, independent of the ICE family of enzymes, contributes to the execution of apoptosis in Ramos-BL B cells. We confirmed that CPP32 is a critical component of ionomycin- and anti-IgM-triggered apoptosis in Ramos-BL B cells by demonstrating that zVAD-fmk, used at a concentration of 100  $\mu$ M, can at least partially prevent both ionomycin- and anti-IgM-triggered processing of CPP32 and cleavage of PARP at 24 h and at 48 h post stimulation; however, zVAD-fmk is by no means as effective as treatment with anti-CD40 in the prevention of these processes (Fig. 5).

Taken together, these data suggest that both ionomycin- and anti-IgM-triggered apoptosis, but not growth arrest, is

at least partially dependent on the activity of members of the ICE family of cysteine proteases.

#### 4. Discussion

The process of cell death by apoptosis is of fundamental importance in the regulation of the immune system where it is involved in the selection of both immature T and B lymphocytes bearing functional AgR to non-self and in the selection of mature B cells bearing high-affinity AgR for non-self during maturation of the humoral immune response [39]. The latter of these scenarios, in which mature B cells undergo selection by apoptosis at the GC site, is represented here by the EBV-genome-negative Ramos-BL cell line [30]. In such cellular populations subject to regulation through apoptosis, dysregulation of the apoptotic mechanism can have devastating consequences by contributing to the pathogenesis of malignancy. Indeed two tumours arising at the GC site, BL and follicular centre cell lymphoma, show aberrations in their ability to undergo apoptosis [39].

In this report we demonstrate that in a physiological system of stimulus-triggered apoptosis, endogenous members of the new and growing family of ICE cysteine proteases [4,38] act as critical effectors of apoptotic death. Our evidence for this conclusion is two-fold. First, zVAD-fmk, an inhibitor of ICE family protease activity [31,32], rescues Ramos-BL B cells from both calcium ionophore- and AgR-triggered apoptosis (Table 4; Fig. 4). However, zVAD-fmk fails to completely reduce both ionomycin- and anti-IgM-driven death to control levels of apoptosis and thus suggests the existence of a mediator(s)/effector(s) of Ramos-BL B cell apoptosis which does not belong to, nor is regulated by, the ICE family of cysteine proteases. Li et al. [40] and Kuida et al. [41] recently demonstrated that mice deficient in ICE develop normally and that their thymocytes and macrophages undergo apoptosis in response to dexamethasone and ionising radiation but not to anti-Fas antibody. While these data can be interpreted to indicate the redundancy of multiple members of the ICE family in their apoptotic function [38,40,41], they do not preclude the possibility of a route to apoptotic death independent of the ICE cysteine proteases. In toto, our data suggest that both calcium ionophore and anti-IgM trigger Ramos-BL B cell apoptosis primarily through an ICE-family-dependent pathway.

Our second line of evidence derives from the direct study of CPP32/Yama (Fig. 1). Engaging the AgR of Ramos-BL B cells triggers evident CPP32 processing approximately 6 h prior to the onset of apoptosis at 30-h culture. Thus, the activation of CPP32/Yama to apopain and subsequent cleavage of its substrates, such as PARP [11,12,18,19,26], may deliver the final fatal blow in an amplifying cascade of protease activity. AgR-triggered degradation of PARP is apparent at 18 h and thus precedes the activation of CPP32/Yama at 24 h; the cleavage of PARP then appears to be greatly accelerated following activation of CPP32/Yama (Fig. 2). It follows that signals transduced through the AgR of Ramos-BL B cells must result in the activation of a member(s) of the ICE family, prior to the activation of CPP32/Yama, able to cleave PARP such as TX [15,42], Mch-2 [18] or Mch-3 [19]. This process of AgR-triggered, CPP32/Yama-independent degradation of PARP is in itself insufficient to trigger apoptosis and likely indicates the ability of cellular mechanisms to repair initial proteolytic damage; a critical point then appears to be attained following the activation of CPP32/Yama when proteolysis likely outpaces repair and cellular collapse into apoptotic death ensues [4]. Although it is known that ICE can process CPP32/Yama to its active apopain form [9–12], the precise molecular mechanisms by which the activity of CPP32/Yama, and indeed the ICE family, is regulated remains to be elucidated.

In the Ramos-BL B cell model, 24 h elapses between AgR ligation and CPP32 activation suggesting that, as for cellular entry into apoptosis [43], the regulation of cysteine protease activity may be related to progression through the cell cycle. The complexity of the regulatory mechanisms likely to be operating here is further highlighted by our observations that ionomycin-triggered activation of CPP32/Yama at 18 h precedes cleavage of PARP at 30 h; these data, when considered in conjunction with the AgR-driven time course of CPP32/Yama activation and PARP degradation, suggest that the initial biochemical events triggered by an apoptotic stimulus may influence the ultimate substrate preference of CPP32/Yama. As well as acting on PARP, CPP32/Yama has recently been shown to cleave sterol regulatory element binding protein-1 (SREBP-1) and SREBP-2 in vitro, although the significance of this observation to the apoptotic mechanism is not known [44].

Calcium ionophore- and AgR-triggered activation of CPP32/Yama and degradation of PARP is arrested by signals transduced through the surface CD40 of Ramos-BL B cells; this CD40-mediated inhibition of cleavage appears to be of a greater efficacy than that observed for zVAD-fmk (Fig. 5). Although ligation of CD40 is known to trigger activation of the protein tyrosine kinase Lyn and of phosphatidylinositol 3-kinase, stress-activated protein kinases and NF- $\kappa$ B [45–47], the molecular mechanism(s) by which anti-CD40 elicits such effective inhibition of the apoptotic pathway is unknown. It is clear that while both anti-CD40 and zVAD-fmk rescue Ramos-BL B cells from ionomycin- and AgR-driven apoptosis, only anti-CD40 is able to alleviate the growth arrest observed following such treatments (Tables 1–4). These data indicate that pathways for growth arrest and apoptosis are distinct and this is supported by data demonstrating that AgR-triggered growth arrest precedes both CPP32/Yama processing and apoptosis.

It has been postulated that Bcl-2 may afford protection

against apoptosis by inhibiting the activity of ICE family members [38]. In Ramos-BL B cells, anti-CD40-mediated inhibition of ionomycin-triggered CPP32/Yama activation and PARP cleavage together with AgR-triggered, CPP32/Yama-independent, degradation of PARP precedes Bcl-2 expression (Fig. 3). Thus, CD40-mediated survival signals must subvert the ionomycin- and AgR-triggered apoptotic pathways upstream from activation of ICE family members by proteolysis. However, co-culture of Ramos-BL B cells with anti-IgM and anti-CD40 triggers Bcl-2 expression prior to AgR-triggered processing of CPP32/Yama; in this scenario, inhibition of the apoptotic pathway mediated by CPP32/Yama may be coincident with or alternatively dependent upon expression of Bcl-2.

In summary, we have demonstrated in a physiological system of stimulus-triggered apoptosis that activation of endogenous members of the ICE family, including CPP32/Yama, and cleavage of the death substrate PARP act as critical effectors of apoptosis.

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