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Implication of calpain in caspase activation during B cell clonal deletion

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In the absence of costimulating signals, B cell receptor (BCR) crosslinking on immature B cells triggers the apoptotic cell death program. In the WEHI-231 B cell lymphoma model, anti-IgM crosslinking triggers activation of caspase-7 independently of caspase-8, followed by apoptosis. Two main mechanisms for caspase-7 activation have been proposed: (i) caspase-8 recruitment to death receptors (Fas or tumour necrosis factor); and (ii) changes in mitochondrial membrane permeability and cytochrome *c* release, which activate caspase-9. Here we report that caspase-7 activation induced by BCR crosslinking is independent of caspase-8 and cytochrome *c* translocation from mitochondria to the cytosol, as well as of mitochondrial depolarization. In addition, in a cell-free system, the S-100 fraction of anti-IgM-treated WEHI-231 cells induces a caspase activation pattern different from that activated by cytochrome *c* and dATP. We demonstrate that calpain specifically triggers activation and processing of caspase-7 both *in vitro* and *in vivo*, and that both processes are inhibited by calpain inhibitors. Furthermore, calpain activation is associated with decreased expression levels of calpastatin, which is upregulated by CD40 ligation. These data confirm a role for calpain during BCR crosslinking, which may be critical for cell deletion by apoptosis during B cell development and activation.

Keywords: apoptosis/B cells/caspases/cell-free system/cytochrome *c*

Introduction

Programmed cell death is a biological process critical for the development, homeostasis and regulation of the immune system (Evan and Littlewood, 1998; Green and Reed; 1998; Raff, 1998; Izquierdo *et al.*, 1999). Most programmed cell death processes, independently of the origin of the death stimulus, are executed through a stereotyped pattern of cellular and biochemical events known as apoptosis (Nagata, 1997). The emerging view of apoptosis is that this highly conserved execution mechanism is activated through complex regulatory pathways whose essential components are the caspases (cysteinyl aspartate-specific proteinases) (Alnemri *et al.*, 1996). In mammalian cells >10 caspases have been cloned that share homology with the *Caenorhabditis elegans* ced-3. The caspases are present as inactive precursors (procaspases) composed of three domains, a prodomain and two subunits, the larger of which contains the active pentapeptide site QACXG (X = R, Q, G). During apoptosis, the procaspases are activated by cleavage between the large and small subunits at Asp-X sites. Most caspases execute the apoptotic program through a sequential activation cascade of initiator and executioner procaspases (Salvesen and Dixit, 1997). Mammalian caspases also have a variety of roles in apoptosis and inflammation (Nicholson and Thornberry, 1997).

The activation of initiator caspases requires specific cofactors, such as triggering of caspase-8 (FLICE), which is recruited to death receptors via an adapter, FADD (Fas-associated protein with death domain) (Muzio *et al.*, 1996). Alternatively, under stress conditions, mitochondrial cytochrome *c* is released into the cytosol (Liu *et al.*, 1996; Reed, 1997), where it activates the cofactor Apaf-1 (apoptotic protease activating factor-1) with subsequent proteolytic processing of caspase-9 (another initiator caspase) (Li *et al.*, 1997; Zou *et al.*, 1997). Mitochondrial depolarization ($\Delta\Psi_m$) takes place upon activation of cell death, and has been proposed as the cause of cytochrome *c* translocation (Kroemer *et al.*, 1997; Bossy-Wetzel *et al.*, 1998). Nevertheless, it has been reported that a specific channel formed by the Bax and ANT (adenine nucleotide translocator) proteins can participate in cytochrome *c* release (Marzo *et al.*, 1998). In this regard, Bcl-2 blocks all mitochondrial activity during apoptosis (Kroemer *et al.*, 1997), including cytochrome *c* release, preventing caspase activation (Kluck *et al.*, 1997; Yang *et al.*, 1997).

The immature B cell lymphoma WEHI-231 has been used as a model of self-induced B cell tolerance (Boyd and Schader, 1981). These cells express surface IgM (Ralph, 1979; Boyd and Schader, 1981), and anti-IgM treatment leads to cell cycle arrest and apoptosis (Benhamov *et al.*, 1990; Mahesmaran *et al.*, 1993; Brás *et al.*, 1999); this latter event also occurs following antigenic recognition in spleen B cells (Boscá *et al.*, 1995; Genaro *et al.*, 1995). We recently showed that anti-IgM treatment of WEHI-231 cells and spleen B cells triggers caspase-7 activation independently of caspase-8 (Brás *et al.*, 1999). Furthermore, the initial processing of procaspase-7 did not appear to involve a classical caspase, as it was not inhibited by z-VAD-fmk (a broad caspase inhibitor); nonetheless, subsequent processing was prevented by z-VAD-fmk, indicating later caspase dependence. Here we demonstrate that caspase activity after anti-IgM crosslinking is independent of cytochrome *c* release and mitochondrial depolarization. We provide compelling evidence that initial caspase-7 processing by BCR crosslinking in B cells is calpain mediated, promoting later activation of caspase-7.

Results

Bcl-2, caspase inhibitors, or CD40 ligation prevented mitochondrial membrane depolarization induced by IgM crosslinking

Previously we showed that BCR crosslinking induces selective activation of the CPP32 subfamily member caspase-7 (Brás *et al.*, 1999). Caspase-7 can thus be cleaved directly by caspase-8 or caspase-9, the two most important initiator caspases, and also by granzyme B (Fernandes-Alnemri *et al.*, 1996; Muzio *et al.*, 1997; Srinivasula *et al.*, 1998). The molecular mechanism of caspase-7 activation in BCR crosslinking is unknown since: (i) BCR crosslinking does not induce caspase-8 activation; (ii) the initial processing of procaspase-7 is not inhibited by z-VAD-fmk (Brás *et al.*, 1999); and (iii) WEHI-231 cells do not express granzyme B (data not shown). We therefore tested whether activation of caspase-9 is the mechanism implicated in caspase-7 processing.

To assess the role of caspase-9 in direct cleavage of procaspase-7, we first analyzed the ability of IgM crosslinking to trigger caspase-9 activation through changes in $\Delta\Psi_m$ required for cytochrome *c* release and caspase-9 activation (Susin *et al.*, 1999b). Changes in plasma membrane potential including $\Delta\Psi_m$ and increase in ROS (reactive oxygen species) were detected 48 h after anti-IgM crosslinking in WEHI-231 cells, as determined by $DiOC_6$ (Salvioli *et al.*, 1997) and hydroethidine (HE) staining, respectively (Figure 1A). After BCR crosslinking, caspase-7 activity kinetics are more rapid than those of $\Delta\Psi_m$, as activity is detected at 8 h and peaks at 24 h (Figure 1D), a time at which changes in $\Delta\Psi_m$ and ROS are not yet detectable. To ensure that we were indeed measuring mitochondrial depolarization, we also performed experiments using JC-1, which showed similar results (not shown). This argues that the latter events are downstream of caspase-7 activation. In addition, Bcl-2 overexpression, CD40L or z-VAD-fmk treatment, all previously shown to inhibit caspase-7 activity (Brás *et al.*, 1999), prevent $\Delta\Psi_m$ and ROS increase (Figure 1B and C). In summary, the results show that a reduction in $\Delta\Psi_m$ is not required for caspase activation in anti-IgM-induced apoptosis but, in contrast, that caspase activation is required for the changes in plasma membrane potential, including $\Delta\Psi_m$.

Caspase-7 activation by anti-IgM crosslinking is independent of cytochrome c

$\Delta\Psi_m$ is not required for caspase-7 activation in anti-IgM-induced apoptosis. Cytochrome *c* can be released from mitochondria independently of mitochondrial depolarization (Bossy-Wetzel *et al.*, 1998), triggering caspase activation by binding to Apaf-1. It is thus possible that apoptosis induced by BCR crosslinking uses the cytochrome *c* pathway.

In Western blotting, no significant cytochrome *c* release was observed in WEHI-231 cells following IgM treatment in kinetic studies from 4 to 48 h post-treatment, even with overexposure of the film; at this latter time, 35% of the cells show an apoptotic phenotype (Figure 2A). This was not due to lack of assay sensitivity, since in actinomycin D-induced apoptosis, cytochrome *c* release was detected 4 h after treatment (Figure 2A). Furthermore, the cytochrome *c* pathway is active in WEHI-231 cells, as the caspase activation profile by cytochrome *c* in S-100 extracts of these cells is comparable to that induced by the same stimuli in HEK-293 cells (Figure 2B). To confirm that cytochrome *c* release is not involved in anti-IgM-induced apoptosis, we also carried out immunocytochemical studies of cytochrome *c* distribution using confocal microscopy, in untreated as well as anti-IgM-treated cells. WEHI-231 cells showed cytochrome *c* expression in a punctate pattern. Co-localization of cytochrome *c* with a mitochondria-specific antibody showed that the punctate pattern corresponded to mitochondria (Figure 2C). Upon apoptosis induction with anti-IgM, marked mitochondrial aggregation but no cytochrome *c* release was found at 24 h post-treatment, at which time a peak of caspase-7 activity appears. In contrast, cytochrome *c* release was found in WEHI-231 cells treated with actinomycin D; this suggests that cytochrome *c* release depends on the apoptosis stimuli. These results indicate that anti-IgM-induced apoptosis does not use this pathway for early caspase activation events, in which cytochrome *c* triggers Apaf-1 and caspase-9 through their respective caspase recruitment domains (CARD).

In addition, anti-IgM treatment induced marked caspase-3-like and very low caspase-9 activity levels, but no caspase-1 or caspase-6 activity (data not shown). In contrast, activation of the cytochrome *c* pathway by addition of dATP and cytochrome *c* strongly induced caspase-3-like, caspase-9 and caspase-6 activities; neither treatment induced caspase-1-like activity (data not shown). Moreover, addition of cytochrome *c* and dATP to WEHI-231 S-100 induced caspase-3 and caspase-9 processing, whereas anti-IgM treatment of WEHI-231 cells for different time periods caused neither caspase-3 nor caspase-9 processing in S-100 from these cells (Figure 3). These data therefore suggest that cytochrome *c* and anti-IgM treatment activate different caspase activity patterns. In addition, in anti-IgM-induced apoptosis, we detect caspase-9 activity independent of processing, as shown above. These results support those published recently, showing that caspase-9 activation is also independent of processing, although the mechanism of caspase-9 activation is unknown (Stennicke *et al.*, 1999).

Finally, to explore the differences between anti-IgM- and cytochrome *c*-induced apoptosis, we used procaspase-7 to study the proteolytic processing of this caspase. Incubation of procaspase-7 with S-100 from WEHI-231 cells treated with anti-IgM for 24 h [S100(IgM24)] resulted in its processing to the p32 form. In contrast, full caspase-7 processing was observed when procaspase-7 was incubated with untreated WEHI-231 S-100 to which dATP and cytochrome *c* had previously been added, with generation of the p32 subunit which was further cleaved into p20 and p12 subunits (Figure 4).

The sum of these results, i.e. the lack of cytochrome *c* release, the distinct caspase pattern and the different procaspase-7 processing profile, clearly indicates that the cytochrome *c* pathway is not involved in BCR crosslinking-induced caspase-7 activation in WEHI-231 cells.

Caspase-7 activates an autocatalytic process in a cell-free system

The initiator caspases-8 and -9 can process procaspase-7 (p35) by cleavage at D198, giving rise to the p23 and p12

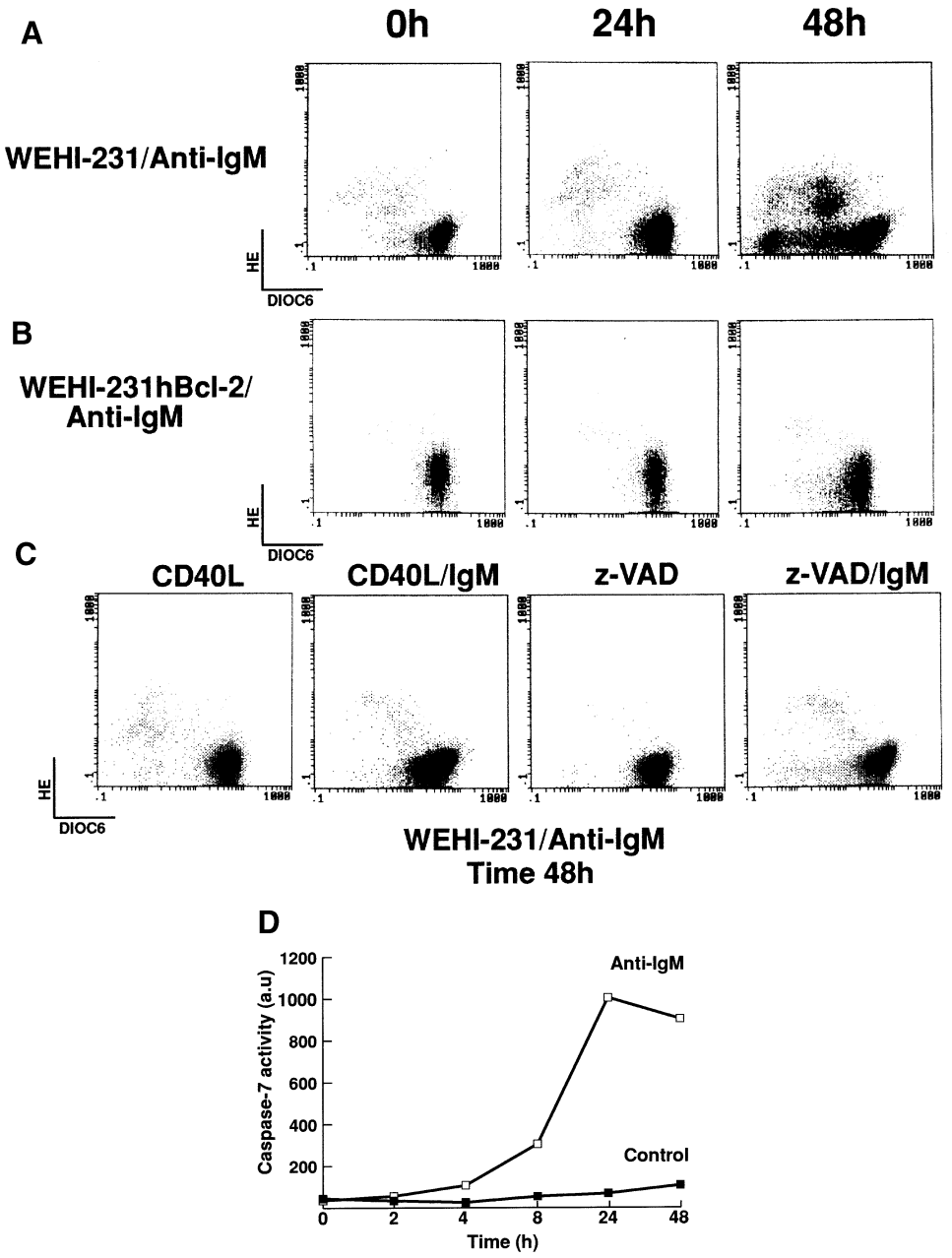

Fig. 1. Anti-IgM crosslinking induces mitochondrial depolarization and an increase in ROS. (**A**) WEHI-231 cells (5×10^5 cells/ml) were cultured in the presence of 10 μg/ml of anti-IgM for the times indicated. Samples were collected, washed with PBS and prepared as described (Materials and methods). (**B**) A human Bcl-2-transfected subclone of WEHI-231 cells (WEHI-hBcl-2) was treated with anti-IgM for the times indicated and prepared as described (Materials and methods). (**C**) WEHI-231 cells were incubated alone or with z-VAD-fmk (100 μM) for 1 h before addition of anti-IgM (10 μg/ml). The cells were treated in the presence of anti-IgM, CD40L (40% supernatant containing the fusion protein mCD40L–mCD8), or both. In all cases, mitochondrial depolarization and ROS were measured. (**D**) Cells (0.25×10^6 cells/ml) were cultured in the presence of anti-IgM (10 μg/ml) for the times indicated. Caspase-7 activity was determined in cytosolic extracts as the fluorescence emission of the cleaved substrates.

subunits (Muzio *et al.*, 1997; Srinivasula *et al.*, 1998), whereas caspase-3 processes procaspase-7 at D23, generating a p32 subunit (Orth *et al.*, 1996). The caspase-7 processing observed in S100(IgM24) created a p32 subunit. To analyze the origin of the p32 subunit, we prepared S100(IgM24) with or without the caspase inhibitors z-VAD-fmk (a broad caspase inhibitor) or DEVD-CHO (a specific inhibitor of caspase-3-like activity), which was then incubated with procaspase-7. Anti-IgM treatment induced procaspase-7 processing from p35 to p32, which is blocked by the caspase inhibitors (Figure 5A). Since anti-IgM treatment activates caspase-7 (Brás *et al.*, 1999) and very low caspase-9 activity, the processing from p35 to p32 is caused by caspase-7 activity in these extracts, and subsequent processing is impaired by the low caspase-9 activity. Diminished processing was observed in S-100 from anti-IgM plus CD40L-treated cells (Figure 5B), which also showed low caspase-7 activity. Moreover, S-100 from WEHI-231 overexpressing Bcl-2 and treated with anti-IgM for 24 h showed no caspase-7 activity; these S-100 were unable to induce processing from p35 to p32 (Figure 5C). These data further suggest that

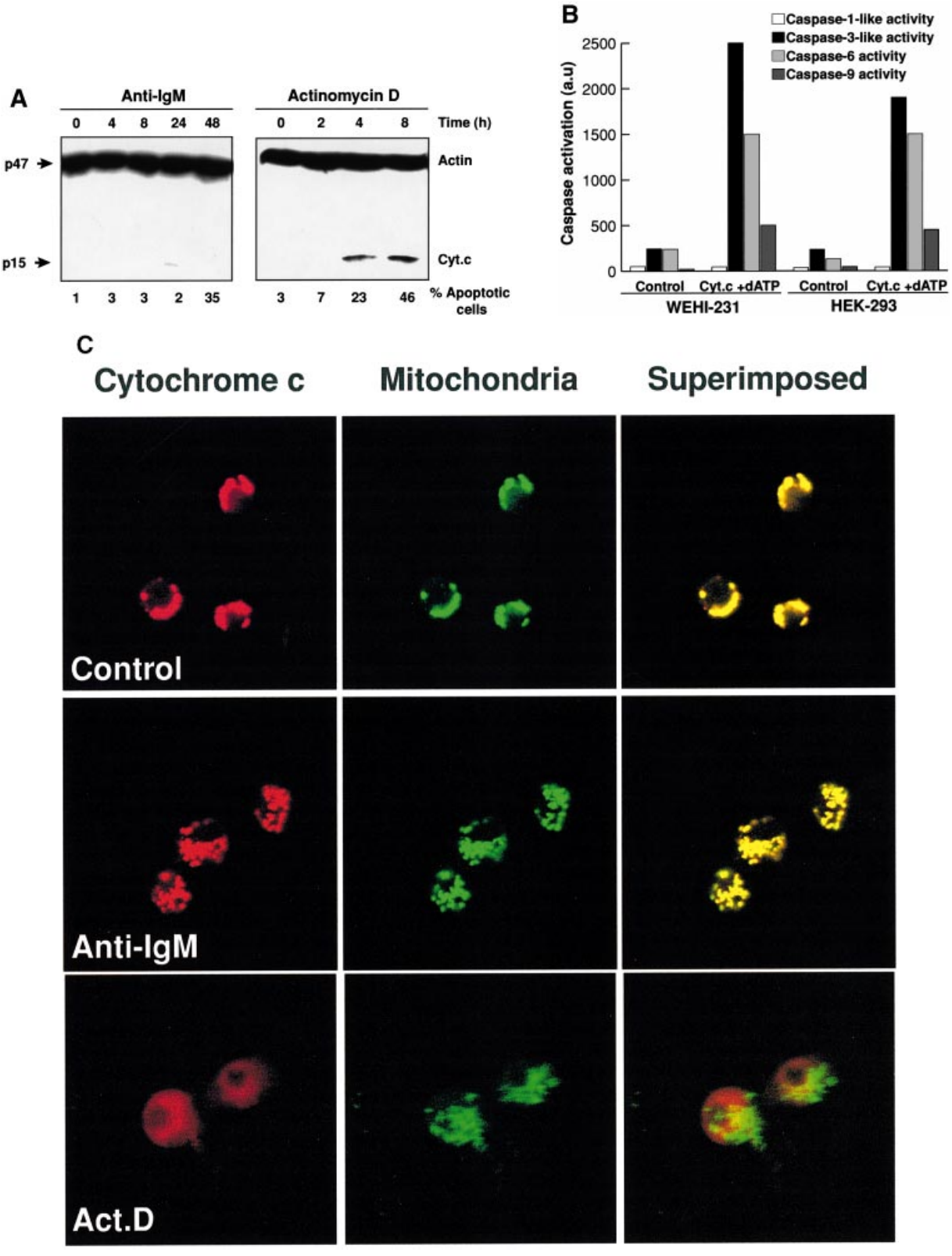

Fig. 2. Mitochondrial cytochrome *c* is not released to the cytoplasm after anti-IgM stimulation. (**A**) Cytosolic extracts were prepared as described (Materials and methods) at the times indicated after anti-IgM (10 μg/ml) and actinomycin D (0.1 μg/ml) treatment; cytochrome *c* and actin were analyzed by Western blot analysis. Actinomycin D was used as a positive control for cytochrome *c* release, and actin as a protein loading control. At the same time the samples were collected, cell pellets were permeabilized and stained with PI. Apoptosis corresponds to the amount of fragmented DNA in the hypoploid subG_0/G_1 peak of the cell cycle. Values are expressed as percentages. (**B**) An aliquot (50 μl) of HEK-293 or of WEHI-231 cell S-100 (50 μg) was incubated with dATP (1 mM) plus cytochrome *c* (1 μM) at 37°C for 2 h; samples were collected and caspase-1-like, caspase-3-like, caspase-6 and caspase-9 activity determined as the fluorescence emission of the cleaved substrates. The values shown are representative of three independent experiments. (**C**) The pictures show immunofluorescent detection of cytochrome *c* (red) and mitochondria (green).

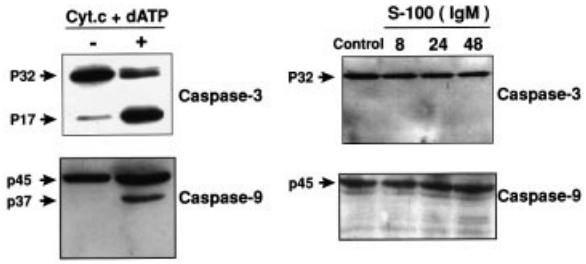

Fig. 3. Anti-IgM and cytochrome *c* induce distinct caspase activity patterns. Analysis of caspase-3 and caspase-9 by Western blotting. WEHI-231 cell S-100 were incubated with dATP (1 mM) and cytochrome *c* (1 μM) for 2 h at 37°C, and S-100 of WEHI-231 cells stimulated with anti-IgM for the times indicated.

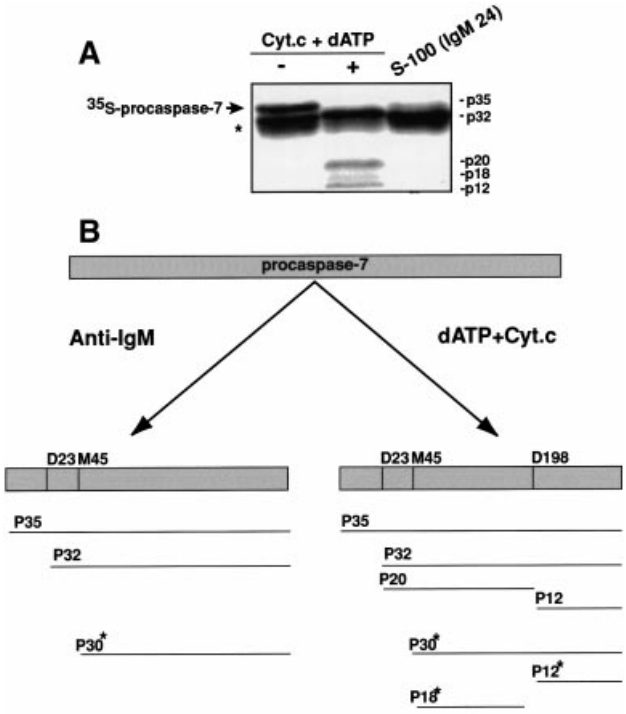

Fig. 4. Anti-IgM induces differential procaspase-7 processing. (**A**) *In vitro*-translated ^{35}S-labeled procaspase-7 was added to cytosolic extracts of anti-IgM-treated WEHI-231 cells, which were then incubated (2 h, 37°C). Anti-IgM-induced processing of procaspase-7 was monitored by SDS–PAGE and autoradiography. The positions of the processed caspase-7 subunits are indicated by p32. The asterisk indicates the 30 kDa internal translation product starting at Met45. ^{35}S-labeled procaspase-7 was incubated with WEHI-231 cell S-100 which was incubated (37°C, 2 h) with or without cytochrome *c* (1 μM) and dATP (1 mM). The positions of the processed subunits are indicated by p32, p20, p18 and p12. (**B**) Procaspase-7 has two possible cleavage sites, at D23 and D198; the cleavage of procaspase-7 by cytochrome *c* plus dATP generates a 32 kDa band that is further processed to the 20 and 12 kDa bands corresponding to full processing, whereas anti-IgM treatment produces a single cleavage event at D23 to form the p32 subunit. The asterisks indicate the 30 kDa internal translation product starting with M45 and the subsequent processed subunits (p18 and p12), which can only be produced by cytochrome *c* plus dATP.

caspase-7 processing from p35 to the p32 form is due to the caspase-7 autoproteolytic activity.

Caspase-7 belongs to the group II caspases (as do caspase-3 and -2), all of which recognize the DEVD sequence. When procaspase-7 is incubated with recombinant caspase-3, a p32 subunit is produced by cleavage at this site (data not shown). Since the only caspase activated

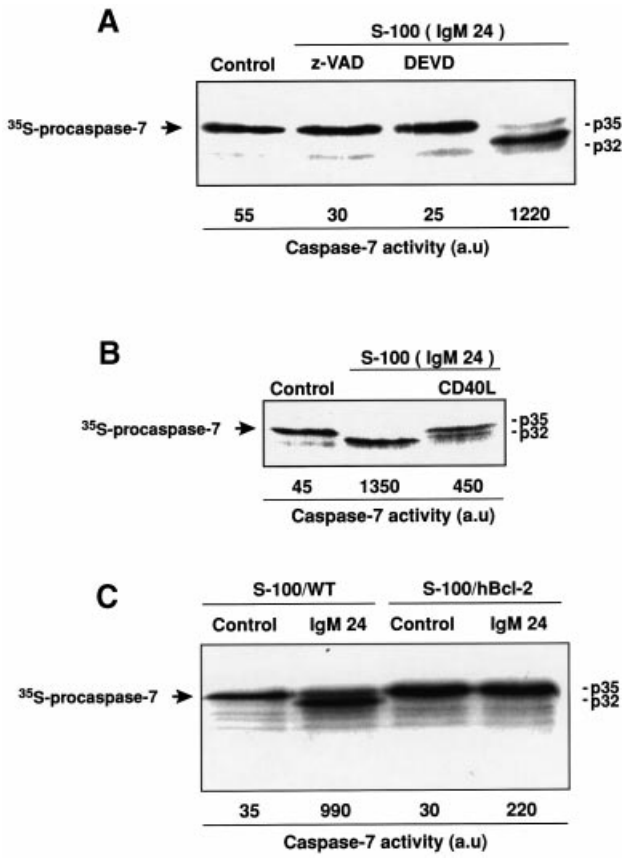

Fig. 5. CD40L, Bcl-2 overexpression and caspase inhibitors block anti-IgM-induced caspase-7 processing. (**A**) ^{35}S-labeled procaspase-7 was incubated (37°C, 2 h) with S-100 from WEHI-231 cells stimulated with anti-IgM plus caspase inhibitors (z-VAD-fmk and DEVD-CHO). (**B**) ^{35}S-labeled procaspase-7 was incubated with S-100 from WEHI-231 cells stimulated with anti-IgM, CD40L or both. (**C**) ^{35}S-labeled procaspase-7 was incubated (37°C, 2 h) with S-100 of untreated or anti-IgM-treated WEHI-231 cells or WEHI-231 cells transfected with Bcl-2. All samples were collected and S-100 prepared as described (Materials and methods). Caspase-7 activity was determined as the fluorescence emission of the cleaved substrates. Samples were separated by SDS–PAGE, dried and exposed to X-ray film. The positions of the processed caspase-7 subunits are indicated as p32.

in BCR crosslinking is caspase-7, and the caspase-7 processing observed in S100(IgM24) gives rise to a p32 subunit, the subunit obtained may thus be a consequence of the caspase-7 activity in S100(IgM24). This would indicate the ability of caspase-7 to process pro-caspase-7 into the active caspase.

Calpain involvement in activation and processing of caspase-7

The intracellular thiol proteases μ-calpain and m-calpain exist as inactive proenzymes, composed of two non-identical subunits of 30 and 80 kDa. The small subunits of μ- and m-calpain are identical, and both proteins are distinguished by their *in vitro* calcium requirement (Murachi, 1989). Calpain translocates from the cytosol to the membrane in a calcium-dependent manner; following translocation, it undergoes two N-terminal autoproteolytic events to form a 78 and a 76 kDa subunit (Croall and DeMartino, 1991). Like caspases, calpains are cysteinyl proteases, but lack the constraints of a defined sequence-

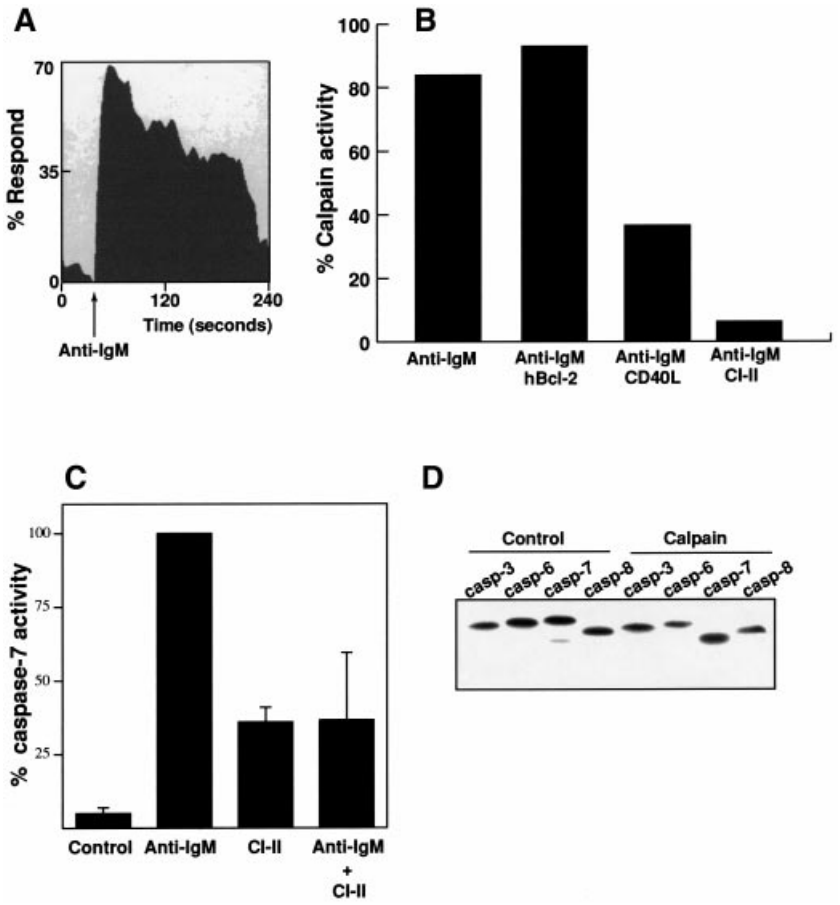

Fig. 6. Calpain is upstream of caspase-7 activation. (**A**) To measure calcium influx, cells were treated as described (Materials and methods). (**B**) WEHI-231 cells and WEHI-231 cells overexpressing Bcl-2 were serum-starved for 1 h before anti-IgM addition (10 μg/ml). WEHI-231 cells were stimulated with either anti-IgM plus calpain inhibitor (CI-II, 1 h before) or anti-IgM plus CD40L. Samples were collected and calpain activity determined as the fluorescence emission of the cleaved substrates at the peak of activity (30 min after stimulation). (**C**) WEHI-231 cells were incubated alone or with CI-II for 1 h before anti-IgM addition (10 μg/ml), samples were collected and calpain activity determined as the fluorescence emission of the cleaved substrates. WEHI-231 cells were stimulated with anti-IgM and treated with or without CI-II for 1 h before stimulation with anti-IgM. The samples were collected and caspase-7 activity determined as the fluorescence emission of the cleaved substrates. Each point represents averages calculated using triplicate experiments; error bars are mean ± 1 SD. (**D**) ^{35}S-labeled caspases were incubated with μ-calpain (100 μM, 30°C, 2 h). Calpain buffer was used for incubation and activation of μ-calpain. After incubation, samples were separated by SDS–PAGE, dried and exposed to X-ray film.

specific cleavage site within their target substrates (Sasaki *et al.*, 1984). Calpains are implicated in the proteolysis of proteins involved in maintaining and regulating the cytoskeletal structure of the cell; they are also implicated in the degradation of transcription factors, growth factor receptors and signal transducing proteins (Wang *et al.*, 1989). Calpain inhibitors have been shown to delay cell death in T cell receptor crosslinking-induced apoptosis (Sarin *et al.*, 1993), although a direct role in caspase activation has not been demonstrated.

To test whether IgM crosslinking indeed activates calpain, we first showed that BCR crosslinking triggers a sustained Ca^{2+} influx, which is required for calpain activation (Figure 6A). To determine whether BCR crosslinking induces calpain activity, we assayed proteolytic activity for the calpain substrate Suc-Leu-Tyr-AMC in WEHI-231 cells as well as in WEHI-231 cells overexpressing Bcl-2. Calpain activation is detected between 5 and 45 min after IgM crosslinking in both cell types, before any caspase activity is detectable. In addition, CD-40L treatment in wild-type cells prevented this activation, although marked prevention is observed in cells treated with the specific calpain inhibitor CI-II. These results thus indicate that BCR crosslinking triggers calpain activity. Calpain is insensitive to z-VAD-fmk *in vivo* (McGinnis *et al.*, 1998), and is thus a potential candidate to induce caspase-7; we therefore used the inhibitor CI-II to analyze whether calpain has a role in the caspase-7 activity detected following anti-IgM treatment. WEHI-231 cells were treated with several CI-II concentrations; after selection of a concentration at which the toxic effect is minimal (1 μM) and no interference with the proteosome is detected (Palombella *et al.*, 1994), we studied its effects at 12 h post-stimulation. At this concentration, CI-II abolished the caspase-7 activity induced by anti-IgM treatment; the remaining caspase-7 activity was comparable to that observed in cells treated with CI-II alone (Figure 6C). To rule out the proteosome involvement in this process, we used the specific proteosome inhibitor lactacystin (Dick *et al.*, 1996); lactacystin treatment had no effect on the caspase-7 activity detected following BCR crosslinking (data not shown). We employed a cell-free system to analyze the capacity of calpain to process to caspase-7 directly. Thus, incubation of procaspases-3, -6 and -7, as

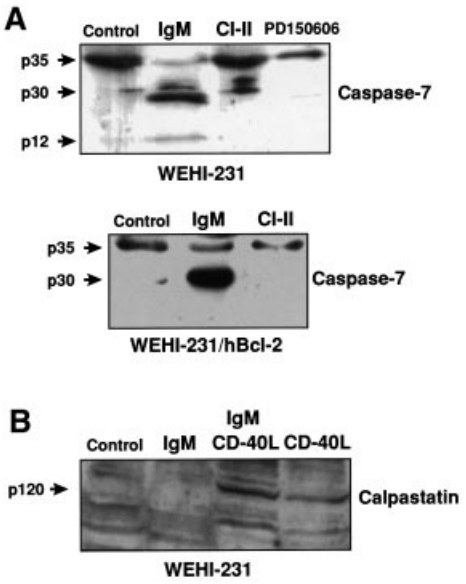

Fig. 7. Caspase-7 is activated *in vivo* by calpain. (**A**) Western blot analysis of caspase-7 processing in S-100 from WEHI-231 cells and WEHI-231 cells overexpressing hBcl-2. Wild-type cells were preincubated for 2 h with calpain inhibitors (1 μM CI-II or 10 μM PD150606), then treated with anti-IgM for 18 h. (**B**) CD40L induces calpastatin expression. Western blot of S-100 from anti-IgM- and CD40L-treated WEHI-231 cells (18 h).

well as a clone of caspase-8 (Orth *et al.*, 1996; Muzio *et al.*, 1997) with purified calpain, resulted in specific cleavage only of caspase-7, creating a p30 subunit (Figure 6D).

To demonstrate the role of calpain in *in vivo* caspase-7 processing, we analyzed S-100 from WEHI-231 cells treated with anti-IgM alone, with anti-IgM plus CI-II, or with anti-IgM plus a calcium binding site calpain inhibitor (PD150606), as well as from anti-IgM-treated WEHI-231 overexpressing hBcl-2. In Western blot analysis using a specific anti-caspase-7 antibody that recognizes the C-terminal domain, we observed that BCR crosslinking induced caspase-7 processing from p35 into a p30 subunit. This processing was inhibited only by the calpain inhibitors CI-II and PD150606, but not by Bcl-2 overexpression (Figure 7A).

Since CD40L treatment prevents calpain activity as well as caspase-7 activity, as shown above, we analyzed the expression of the endogenous calpain inhibitor, calpastatin (Murakami *et al.*, 1981), by Western blotting in anti-IgM- and CD40L-treated WEHI-231 cells. The CD40L treatment resulted in calpastatin expression (Figure 7B), suggesting the mechanism by which CD40L impedes calpain activity, and consequently caspase-7 activity.

In conclusion, these results clearly demonstrate that calpain triggers specific caspase-7 processing and is required for caspase-7 activity. These results show that BCR crosslinking triggers calpain activity, and also indicate that calpain plays a role in the induction and processing of caspase-7.

Discussion

During B cell development, DNA rearrangement of immunoglobulin genes results in the generation of lymphocytes bearing a complete set of antigen receptor specificities. One consequence of random V(D)J recombination is the generation of B cells that recognize endogenous self-antigens. In B cell lineages, clonal deletion is thus an important mechanism for the elimination of self-reactive cells (Nossal, 1994; Rajewsky, 1996). The clonal deletion process occurs by apoptosis; the identification of the mechanisms involved in B cell deletion is thus of interest for the understanding of B cell biology. To gain insight into the molecular mechanisms implicated in the triggering of apoptosis following antigen recognition by B cells, we studied tolerance in the WEHI-231 B cell lymphoma model, as these cells undergo cell death following BCR crosslinking.

We have characterized the mechanism implicated in activation of the apoptotic pathway triggered by BCR crosslinking. Here we demonstrate that caspase-7 activity induced by anti-IgM crosslinking in WEHI-231 cells is upstream of the mitochondrial depolarization decrease, since the loss in $\Delta\Psi_m$ was not observed until 48 h post-treatment, whereas caspase-7 activity appears at 8 h. Moreover, $\Delta\Psi_m$ is a consequence of caspase activation, since treatments that prevent caspase-7 activation and apoptosis, such as z-VAD-fmk, CD40 stimulation or Bcl-2 overexpression, inhibit depolarization. This concurs with reports in other systems in which the $\Delta\Psi_m$ reduction shown to occur in Fas-induced apoptosis is blocked by z-VAD-fmk (Boise and Thompson, 1997; Susin *et al.*, 1997), confirming that caspase activity is upstream of the changes in plasma membrane potential, including $\Delta\Psi_m$. Caspase-induced mitochondrial dysfunction may therefore work as a positive feedback system in which different apoptotic factors such as AIF (apoptosis-inducing factor) or caspase-9 are released from mitochondria (Marchetti *et al.*, 1996; Susin *et al.*, 1996, 1999a,b), increasing the apoptotic phenotype. In addition, we demonstrate that BCR crosslinking does not induce cytochrome *c* release. The caspase-7 activity observed therefore reflects the activation of a pathway that is independent of cytochrome *c*. These findings are supported by the results showing that the caspase pattern activated by cytochrome *c in vitro* is different from that induced by anti-IgM crosslinking. Bcl-2 inhibits caspase-7 activation and BCR crosslinking-induced apoptosis, although the mechanisms by which it prevents these effects in WEHI-231 cells are unknown. Bcl-2 and Bcl-x_L are reported to act by inhibiting cytochrome *c* release from mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997). This is not the case in this model, since this pathway is not activated by BCR crosslinking.

WEHI-231 cells express caspases-1, -2, -3, -6, -7, -8 and -9, of which caspase-7 is the only one to be processed by anti-IgM crosslinking (Brás *et al.*, 1999). In a cell-free system, the initiator caspases-8 and -9 can process procaspase-7 by cleavage at D198 (Muzio *et al.*, 1997; Srinivasula *et al.*, 1998), whereas caspase-3 processes procaspase-7 at D23 (Orth *et al.*, 1996). The caspase-7 processing observed in S100(IgM24) is thus due to caspase-7 activity in these extracts, which would cleave the molecule at the unique N-terminal DEVD site of caspase-7 (D23). This is supported by the observation that full processing is not detected and that DEVD-CHO, zVAD-fmk, CD40L treatment and Bcl-2 overexpression

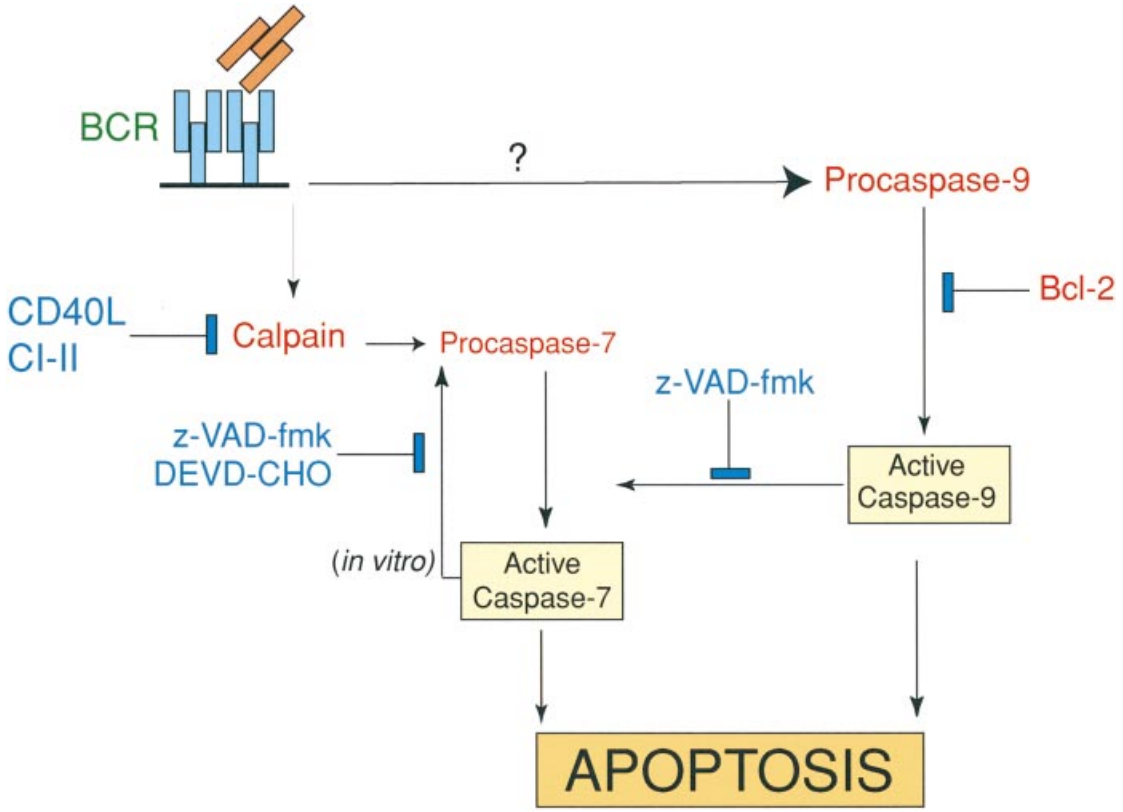

Fig. 8. Schematic representation of the B cell clonal deletion model. BCR crosslinking activates the apoptotic pathway by proteolyzing calpain, which in turn activates caspase-7. In the model, both calpain/caspase-7 and caspase-9/Bcl-2 systems are critical for caspase activation in B cell clonal deletion.

abolish this cleavage, as well as the caspase-7 activity, indicating a case of caspase activation by autoproteolysis.

Finally, BCR crosslinking induces calpain activity, and this activation is blocked by the calpain inhibitor CI-II and partially by CD40L, but not by Bcl-2 overexpression. These data, the fact that CI-II and PD150606 block caspase-7 processing and that calpain processes procaspase-7, all indicate that calpain has a role in BCR crosslinking-mediated caspase activation. The initial processing of procaspase-7 did not appear to involve a classical caspase, as it was not inhibited by z-VAD-fmk, whereas the subsequent processing is caspase dependent (McGinnis *et al.*, 1998; Brás *et al.*, 1999); these data allow us to discuss a tentative model in which BCR crosslinking activates the apoptotic pathway required for clonal deletion (Figure 8). In Figure 8, we indicate that procaspase-7 undergoes two processing events, one mediated by calpain and the other probably triggered by unprocessed caspase-9. As discussed above, it is intriguing that we detect caspase-9 activity at 24 h after IgM crosslinking, a time at which $\Delta\Psi_m$ reduction has not appeared and processing is not detected, suggesting alternative mechanisms for caspase-9 activation. As described, Bcl-2 forms a ternary complex with Apaf-1 and caspase-9, blocking caspase-9 activation; we detect caspase-9 activity that is inhibited by Bcl-2 (data not shown). In addition, as Apaf-1 interacts only with caspases with a large prodomain, and caspase-7 has a small prodomain (Pan *et al.*, 1998), we postulate that Bcl-2 controls caspase-9 and not procaspase-7. In our model, the calpain/caspase-7 and caspase-9/Bcl-2 systems are critical for caspase activity in BCR-induced apoptosis.

Calpain involvement in apoptosis has been studied in several models of apoptosis (Squier *et al.*, 1997; Waterhouse *et al.*, 1998; Debiasi *et al.*, 1999). Here we demonstrate that calpain is required to activate caspase-7, in contrast to other models in which calpain is downstream of caspase activity (Wood and Newcomb, 1999). In these systems, calpain activity is proposed to be caused by caspase degradation of the endogenous calpain inhibitor calpastatin (Murakami *et al.*, 1981; Porn-Ares *et al.*, 1998; Squier *et al.*, 1999). In BCR crosslinking-induced apoptosis calpain is upstream of caspase-7 however, since it triggers caspase-7 cleavage. The specific cleavage site in caspase-7 is nonetheless difficult to analyze, since calpains lack the constraints of a defined sequence-specific cleavage site within their target substrates (Sasaki *et al.*, 1984). We detect the p30 subunit *in vivo* using an antibody against the C-terminal domain, suggesting that calpain cleaves caspase-7 at a hypothetical N-terminal cleavage site. In addition, experiments using fluorogenic peptides show that calpain tends to cleave Met, Tyr, Arg or Lys at P1 when preceded by a hydrophobic amino acid residue at P2 (Sasaki *et al.*, 1984). Comparison of the human and murine caspase-7 protein sequences shows a hypothetical cleavage site that does not appear in other caspases; experiments are currently under way to confirm the caspase-7 cleavage site. All together, these results offer a new mechanism by which recognition through clonally distributed receptors activates a biochemical pathway leading to clonal deletion.

Materials and methods

Cell culture

The WEHI-231 cell line was cultured in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 10 mM HEPES and 50 μM 2-mercaptoethanol (Sigma, St Louis, MO). HEK-293 human cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin; both were maintained at 37°C in a humidified atmosphere with 5% CO_2. Transfected J558 cells secreting the mCD40L–mCD8 fusion protein (a gift of Dr P.Lane, Basel, Switzerland) were maintained in supplemented RPMI-1640 containing 2 mg/ml geneticin (Calbiochem, San Diego, CA). For production of CD40L-containing supernatants, cells were cultured at 10^6/ml for 24 h with no selection agent, the cell-free supernatants harvested, sterile filtered and stored at –70°C until use.

Assessment of apoptotic cell death

Apoptosis was evaluated by staining cellular DNA content with the DNA intercalator propidium iodide (PI) in a semi-automatic procedure (DNA-Prep Reagents; Coulter, Miami, FL), followed by analysis on an EPICS XL flow cytometer (Coulter). Briefly, cells (10^5–10^6) were recovered by centrifugation, resuspended in 100 μl of phosphate-buffered saline (PBS), permeabilized and stained by addition of 100 μl of detergent reagent followed by 1 ml of PI solution. After vortexing, samples were incubated at 37°C for 30 min and analyzed by flow cytometry. Apoptosis was determined as the percentage of DNA located in the hypoploid subG_0/G_1 peak of the cell cycle.

Mitochondrial potential and intracellular ROS production

Cells (5×10^5) were incubated (15 min, 37°C) with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$, 40 nM) to measure mitochondrial potential ($\Delta\Psi_m$), and with 2 μM HE to measure superoxide anion generation causing HE oxidation to ethidium; cells were then analyzed on an EPICS XL flow cytometer. Control experiments were performed using the specific inhibitor of mitochondrial depolarization, bongkrekic acid (Calbiochem) and JC-1 (Molecular Probes, Eugene, OR) to measure $\Delta\Psi_m$.

Antibodies and reagents

Polyclonal rabbit anti-mouse caspase-3 was the kind gift of Drs T.Mak and R.Hakem (Ontario Cancer Institute, Toronto, Canada), anti-caspase-9 antibodies were generated by immunizing rabbits with purified recombinant caspase-9, monoclonal anti-cytochrome *c* antibodies were purchased from Pharmingen (San Diego, CA), polyclonal anti-caspase-7 antibody was from Stressgen (Victoria, BC, Canada) and anti-calpastatin was from Santa Cruz (Santa Cruz, CA). Goat anti-mouse IgM, μ chain-specific (10 μg/ml, Jackson ImmunoResearch, West Grove, PA) was used for induction of cell death. Anti-mitochondrial antibodies were obtained from the serum of a patient with primary biliary cirrhosis, shown to recognize the E2 polypeptide of the mammalian mitochondrial pyruvate dehydrogenase complex (Clavería *et al.*, 1998).

For *in vivo* assays, 1 μM of the calpain inhibitor *N*-Ac-Leu-Leu-methioninal (CI-II; Calbiochem), 10 μM of calcium binding site calpain inhibitor (PD150606; Calbiochem), 100 μM of the tetrapeptide protease inhibitors acetyl-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO) or the tripeptide z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk) (Bachem, Bubendorf, Switzerland) were added to cultures 1 h before anti-μ treatment. Actinomycin D, bovine heart cytochrome *c* and nucleotide triphosphate (dATP) were purchased from Sigma; [^{35}S]methionine was from Amersham (Aylesbury, UK). Lactacystin and μ-calpain were purchased from Calbiochem.

Cloning of caspase-7 and in vitro transcription/translation

Caspase-7 cDNA was cloned from Jurkat cell RNA by RT–PCR, using superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and the expand long template PCR system (Boehringer Mannheim, Mannheim, Germany). The PCR product obtained with primers LAP3.5′*Bam*HI (GGATCCACCATGGCAGATGATCAGGGCTG) and LAP3.3′*Eco*RI (GAATTCCTATTGACTGAAGTAGAGTTCCTTG) was cloned in a pCR2.1 vector (Invitrogen, San Diego, CA). The positive clones were subcloned in pCDNA3 (Invitrogen) under the T7 promoter, and their sequences confirmed by sequence analysis. Plasmid templates were used in coupled *in vitro* transcription–translation reactions to generate [^{35}S]methionine-labeled proteins (Promega, Madison, WI).

Preparation of S-100 cytosolic fraction and caspase-7 processing assay

WEHI-231 cells were harvested, washed in ice-cold PBS and pellets resuspended in 5 vol of ice-cold buffer A [20 mM HEPES–KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin]. After incubation on ice for 15 min, cells were lysed by three freeze–thaw cycles, centrifuged (10^5 *g*, 1 h) and the resulting S-100 used for the *in vitro* assay. Briefly, S-100 was incubated in the presence of the translated [^{35}S]methionine-labeled caspase-7 (2 h, 37°C), after which sodium dodecylsulfate (SDS) loading buffer was added to each reaction, boiled and separated in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. For the calpain-induced caspase processing assay, samples were incubated (2 h, 30°C) in calpain buffer (60 mM imidazole, 5 mM L-cysteine pH 7.3, 2.5 mM 2-mercaptoethanol and 5 mM $CaCl_2$).

Western blot analysis

The protein content of the lysate was quantified using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Following SDS–PAGE, proteins were transferred to nitrocellulose membranes (Bio-Rad) and membranes blocked overnight with 5% non-fat dry milk in TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl). Subsequent antibody incubations and membrane washes were performed in TBS-T buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20) containing 5% non-fat dry milk. After 2 h antibody incubation and 2 h washing, the blot was developed with peroxidase (PO)-conjugated anti-rabbit or -mouse antibodies using enhanced chemiluminescence (ECL) reagents (Amersham).

Enzyme assay for caspase and calpain activity

The caspase activity assay was performed on S-100 fractions. Twenty micrograms of cytosolic protein, estimated by the bicinchoninic acid method (Smith *et al.*, 1985), were diluted 5-fold in caspase buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM DTT and 0.1 mg/ml ovalbumin) and incubated with 10 μM of the fluorescent substrates Ac-YVAD-AMC (acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin), Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin), Ac-VEID-AMC (acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin) or Ac-LEHD-AMC (acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin) to measure caspase-1-like, caspase-3-like, caspase-6 or caspase-9 activity, respectively. After incubation (1 h, 37°C), the reaction was terminated by addition of HPLC buffer [water/acetonitrile (75/25), 0.1% trifluoroacetic acid (TFA)]. Cleaved substrate fluorescence was determined by C_{18} reverse phase HPLC using fluorescence detection (338 nm excitation, 455 nm emission). Control experiments confirmed linearity with time and protein concentration of substrate release.

For the calpain activity assay, cells were collected, washed in ice-cold PBS and resuspended in extraction buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 1% NP-40, 0.4 mM Na_3VO_4). After 30 min incubation on ice, the cell lysate was centrifuged (20 000 *g*, 30 min) and the supernatant used as cytosolic extract. Twenty micrograms of cytosolic proteins were diluted 5-fold in caspase buffer and incubated with 10 μM of the fluorescent substrate Suc-Leu-Tyr-AMC (Calbiochem). After incubation (1 h, 37°C), the reaction was terminated by addition of HPLC buffer [water/acetonitrile (75/25), 0.1% TFA]. Cleaved substrate fluorescence was determined by C_{18} reverse phase HPLC using fluorescence detection (338 nm/455 nm). Control experiments were performed using the specific calpain inhibitor *N*-Ac-Leu-Leu-methioninal.

Subcellular fractionation

WEHI-231 cells were washed once in PBS, resuspended in isotonic buffer A containing 250 mM sucrose, and homogenized in a Teflon homogenizer. Nuclei and unbroken cells were separated (120 *g*, 5 min), the supernatant centrifuged (10 000 *g*) to collect the mitochondrial pellet, and the supernatant analyzed for cytochrome *c*. The mitochondrial pellet was analyzed as a positive control of the subcellular fraction (not shown).

Immunofluorescence

For immunofluorescence, WEHI-231 cells were washed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, pre-incubated in 5% BSA, and incubated for 1 h with anti-cytochrome *c* (1:1000), together with anti-mitochondrial antibodies (1:1000) in PBS containing 0.5% BSA and 0.1% Triton X-100. After incubation, cells were washed three times in the same buffer and incubated for 1 h with Cy2-conjugated anti-human and Cy3-conjugated anti-rabbit secondary antibodies (Jackson Immuno-

Research). Samples were pipetted onto slides and optical sections obtained using an Ar-Kr laser and a TCS-NT Leica confocal imaging system.

Calcium determination

Changes in intracellular calcium concentration were monitored using the fluorescent probe Fluo-3AM (Calbiochem). Cells (2.5×10^6/ml) were resuspended in RPMI containing 10% FCS and 10 mM HEPES, then incubated with Fluo-3AM (300 μM in dimethylsulfoxide, 10 μl/10^6 cells) for 15 min at 37°C. After incubation, cells were washed and resuspended in complete medium containing 2 mM $CaCl_2$ and maintained at 4°C until just before anti-IgM addition to minimize membrane trafficking and to eliminate spontaneous Ca^{2+} entry. Calcium mobilization in response to 20 μM of anti-IgM was determined at 37°C in an EPICS XL flow cytometer at 525 nm, and includes background level stabilization and determination of the level of probe loaded for each sample. Only samples with similar load, as assessed by determination of ionophore (5 μg/ml ionomycin, Sigma)-induced Ca^{2+} mobilization, are considered acceptable.

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