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Caspase Activity Is Required for Stimulated B Lymphocytes to Enter the Cell Cycle

N. Eric Olson,2* Jonathan D. Graves,2† Geraldine L. Shu,2* Elizabeth J. Ryan,* and Edward A. Clark3*,†

Following activation with proliferative stimuli, including ligation of CD40, dense human tonsillar B cells (>98% cells in G0) have increased cleavage and activation of caspase-8 and -6 accompanied by decreased caspase-3 activation and apoptosis. Proliferation was blocked by either a broad specificity caspase inhibitor or inhibitors selective for caspase-6 or caspase-8. In contrast, an inhibitor selective for caspase-3 was without effect. Furthermore, induction of cyclin D and cyclin-dependent kinase 4 mRNA and protein was blocked upon inhibition of caspase-6, but not caspase-3. Thus, caspase-6-like activity is required for quiescent B cells to increase the expression of genes required for entry into G1. In support of this model, the transcriptional suppressor special AT-rich sequence-binding protein 1, a preferred caspase-6 substrate, was cleaved upon B cell stimulation. Caspase activity was not required for all signaling events, as caspase inhibitors did not affect the phosphorylation of p42/44 mitogen-activated protein kinase, the expression of the survival factor cellular inhibitor of apoptosis 2, or the production of IL-6 by stimulated G0 B cells. These findings suggest a mechanism by which caspase-6 may selectively allow entry of quiescent B cells into the cell cycle. The Journal of Immunology, 2003, 170: 6065–6072.

The mechanisms that regulate the survival and homeostatic proliferation of mature B lymphocytes are only partially understood. The number of mature B cells is not influenced as significantly by daily rates of B cell production in the bone marrow as by active mechanisms in the periphery (1). The B cell Ag receptor complex is required for mature B cells to survive (2), and as yet poorly understood B cell Ag receptor-dependent selective mechanisms, such as the regulation of transitional B cells in the spleen (3), appear to regulate B cell pool size (4).

Quiescent B cells express a distinct set of genes from either anergic or activated B cells (5), suggesting that the decision to remain quiescent is an actively regulated process. A number of proteins with known or predicted inhibitory functions have been implicated in this process (5, 6). For example, lung Kruppel-like factor and special AT-rich sequence-binding protein 1 (SATB1)4 function as transcriptional repressors that maintain T cells in a quiescent state (7, 8). It remains unclear whether lung Kruppel-like factor or SATB1 plays a similar role in B cell homeostasis, which is regulated separately from T cell homeostasis (4).

Another mechanism known to contribute to the homeostatic regulation of B cell populations is programmed cell death or apoptosis. The main effectors of cell death are a family of cysteine proteases called caspases (reviewed in Refs. 9 and 10). The best-characterized pathway for caspase activation is via the CD95/Fas receptor. Activated CD95/Fas trimers recruit the pro form of caspase-8 via the adapter protein Fas-associated death domain protein (FADD) (9, 10). The resulting oligomerization of pro-caspase-8 results in autoprocessing and activation. Caspase-8 then cleaves and activates downstream caspases such as caspase-3, -6, and -7. Once activated, caspases target cleavage of a highly specific and restricted group of cellular proteins that regulate cell morphology, signal transduction, and survival (9). Among the known targets of caspases are a number of proteins that function to regulate the cell cycle. These include the retinoblastoma protein (Rb), a critical regulator of cell cycle progression (11), and the cyclin-dependent kinase inhibitors, p21Cip1/Waf1 and p27Kip1 (12). Cleavage of these proteins contributes to cell cycle arrest and serves a permissive role in the induction of apoptosis.

Several reports suggest that caspases can function to regulate processes besides apoptosis, including proliferation and differentiation (13). One of the first hints that caspase-dependent pathways may regulate proliferation came from analyses of FADD-deficient mice and mice expressing dominant-negative FADD in the T cell compartment. Using both these approaches, it was shown that FADD is required for T cell activation and IL-2-dependent proliferation (14–16). Subsequent studies suggested a positive role for caspases during proliferation of CD3-stimulated normal human T cells (17, 18). These studies found that caspase-8 activity is associated with T cell proliferation, and that either a broad specificity caspase inhibitor or an inhibitor selective for caspase-8 could block proliferation. More recently, Chun et al. (19) identified a caspase-8 mutation in patients with autoimmune lymphoproliferative syndrome-related symptoms. T, B, and NK cells from

*Correspondence and reprint requests to Dr. Edward A. Clark, Department of Microbiology, Box 357242, University of Washington, Seattle WA 98195. E-mail address: ecclark@u.washington.edu

†Address correspondence and reprint requests to Dr. Edward A. Clark, Department of Microbiology, Box 357242, University of Washington, Seattle WA 98195. E-mail address: ecclark@u.washington.edu

4 Abbreviations used in this paper: SATB1, special AT-rich sequence-binding protein 1; Ac-DEVD-AMC, acetyl Asp-Glu-Val-Asp 7-amido-4-methyl coumarin; Ac-IETD-AMC, acetyl Ile-Glu-Thr-Asp 7-amino-4-methylcoumarin; Ac-VEID-AMC, acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin; Ac-IETD-fmk, benzyloxycarbonyl (Cbz)-N-acetyl-Ile-Glu-Thr-Asp-al; ZVAD-fmk, benzyloxycarbonyl (Cbz)-Val-Ala-Asp(Ome)-fluoromethylketone; ZVAD-CHO, N-acetyl-Val-Ala-Asp(Ome)-fluoromethylketone; ZVAD-fmk, benzyloxy carbonyl (Cbz)-Val-Ala-Asp(Ome)-fluoromethylketone; iDAP, cellular iDAP.
patients exhibited defects in activation consistent with a role for caspase-8 in lymphocyte activation. However, which caspases function downstream of caspase-8 and how caspases might act as positive regulators of the cell cycle has not been determined.

In this study we investigated the role of caspases in the activation of normal quiescent human B cells. We found that both caspase-8 and -6 activities increased following B cell activation by a variety of proliferative stimuli, and that inhibitors selective for these caspases blocked B cell proliferation. Inhibition of caspase-6 antagonized both mRNA and protein expression of cyclin-dependent kinase 4 (cdk4) and cyclin D, suggesting that resting B cells may require caspase-6 activity to enter into the G1 phase of the cell cycle. Neither CD40-mediated IL-6 production nor cellular inhibitor of apoptosis 2 (cIAP2) expression was affected by caspase inhibition, suggesting that caspase activity is required for cell cycle entry, but not cytokine or survival programs. We propose a model that caspases may regulate cell cycle entry by targeting proteins that actively maintain the quiescent state, thereby activating transcription of early cell cycle genes.

Materials and Methods

Cells and Abs

Dense tonsillar B cells were prepared as described previously (20, 21); briefly, E rosette-negative (Er–) cells were layered over a Percoll step gradient, and, after centrifugation, cells were isolated that were >60% Percoll density (98% CD20+, >90% in G0 based on acridine orange staining). Mouse dense B cells were purified and stimulated as previously described (22). The G28-5 mAb to human CD40 (23) and the G28-8 mAb to Bgp95, recently designated CD180/RP105 (20, 24), were used alone or together to activate resting B cells. The IC10 mAb to mouse CD40 was used to stimulate mouse B cells. In some experiments F(ab′)2 of goat anti-mouse μ chains or goat anti-human μ sera (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to stimulate B cells.

Reagents

The peptide caspase inhibitors, benzoylloxycarbonyl (Cbz)-Val-Ala-Asp(Fmoc)-fluoromethylketone (ZVAD-fmk), N-acetyl-Val-Ala-Asp(Fmoc)-fluoromethylketone (IETD-fmk), benzoylloxycarbonyl (Cbz)-Asp-Glu-Val-al(ZVAD-CHO), benzyloxycarbonyl (Cbz)-Val-Glu-Ile-Asp(Ome)-fluoromethylketone (DEVD-fmk), and an emission wavelength of 510 nm with the use of a CytoFluor II signal was detected.

Caspase activity assays

Following stimulation, cells were washed once in PBS and resuspended at 2 × 10^6/ml in hypotonic lysis buffer (50 mM NaCl, 40 mM β-glycerophosphate, 10 mM HEPES (pH 7.0), 5 mM EGTA, and 2 mM MgCl2). The lysate was then subjected to four freeze-thaw cycles before centrifugation at 10,000 × g for 10 min (100 mM HEPES (pH 7.5), 10% sucrose, 0.1% 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate, 10 mM DTT, and 0.1 mg/ml OVA). Protein concentrations were determined, and 25 μg of cell extract was incubated for 4 h at 37°C with 50 μM Ac-DEVD-AMC, Ac-VEID-AMC, or Ac-IETD-AMC. Assays were performed in the presence or the absence of 1 μM ZVAD-CHO or DEVD-CHO inhibitor as a control for broad specificity caspase activity and caspase-3 activity, respectively. Protease activity was determined by monitoring the release of 7-amino-4-trifluoromethyl coumarin at an excitation wavelength of 400 nm and an emission wavelength of 510 nm with the use of a CytoFluo II 96-well plate spectrofluorometer (PerSeptive Biosystems, Framingham, MA). Relative caspase activity was determined by dividing the activity observed at each time point by the values detected at time zero.

Cytokine assays

B cells were stimulated as described above. Then supernatants were removed at 24 h, and the concentrations of IL-6, IL-10, and IL-1 were determined by immunoassays. Matched pairs of Abs were as follows: IL-10, JES3-19F1 (capture) and JES3-6B11 (detection); IL-6, MQ2-13A5 (capture) and MQ2-9C3 (detection); and IL-1β, 508A7G8 and 508AA42 (capture) and 508A3H12 (detection). Results were expressed as mean concentrations extrapolated from a standard curve prepared with recombinant cytokines (BD Biosciences), for each stimulation condition performed in triplicate. The limit of sensitivity of each assay was 15 pg/ml.

Caspases and B cell proliferation

Human tonsillar B cells were pretreated where indicated with caspase inhibitors for 3 h before stimulation. At the indicated time, 1.5 × 10^6 cells were rinsed with cold PBS and lysed in a buffer containing 1% SDS and 50 mM NaCl (pH 7.6). Samples were passed through a 25-gauge needle 10 times, and then insoluble matter was removed by centrifugation. A small sample of the resulting supernatant was reserved for protein determination; the rest was diluted 1/1 in sample buffer containing 5% β-ME and boiled for 5 min. The protein concentration of each sample was determined using a bichinonic acid assay (Pierce, Rockford, IL) with BSA as a standard. Equal amounts of total protein were loaded onto polyacrylamide gels, electrophoresed, and transferred to nitrocellulose. The blot was then immunostained using Abs specific for cyclin D3 (Upstate Biotechnology, Lake Placid, NY), caspase-3, caspase-8 (BD PharMingen, San Diego, CA), caspase-6 (MLB International, Watertown, MA), cyclin A, SATB1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Rb, phospho-extracellular signal-regulated kinase (phospho-ERK), Akt/protein kinase B (Cell Signaling Technology, Beverly, MA), or p27kip1 (BD Biosciences). Briefly, the blot was first incubated in 5% nonfat dried milk in TBS-T (10 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) for 1 h. The blot was then incubated with primary Ab diluted in TBS-T buffer for 1 h. After rinsing, the blot was exposed to a HRP-conjugated secondary Abs diluted in TBS-T. After rinsing, blots were incubated in ECL (Amersham Pharmacia Biotech) reagent for 1 min, blotted dry, and then exposed to film until a signal was detected.

Western blot analysis

Human tonsillar B cells were pretreated with caspase inhibitors for 3 h before stimulation. At the indicated time, 1.5 × 10^6 cells were rinsed with cold PBS and lysed in a buffer containing 1% SDS and 50 mM NaCl (pH 7.6). Samples were passed through a 25-gauge needle 10 times, and then insoluble matter was removed by centrifugation. A small sample of the resulting supernatant was reserved for protein determination; the rest was diluted 1/1 in sample buffer containing 5% β-ME and boiled for 5 min. The protein concentration of each sample was determined using a bichinonic acid assay (Pierce, Rockford, IL) with BSA as a standard. Equal amounts of total protein were loaded onto polyacrylamide gels, electrophoresed, and transferred to nitrocellulose. The blot was then immunostained using Abs specific for cyclin D3 (Upstate Biotechnology, Lake Placid, NY), caspase-3, caspase-8 (BD PharMingen, San Diego, CA), caspase-6 (MLB International, Watertown, MA), cyclin A, SATB1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Rb, phospho-extracellular signal-regulated kinase (phospho-ERK), Akt/protein kinase B (Cell Signaling Technology, Beverly, MA), or p27kip1 (BD Biosciences). Briefly, the blot was first incubated in 5% nonfat dried milk in TBS-T (10 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) for 1 h. The blot was then incubated with primary Ab diluted in TBS-T buffer for 1 h. After rinsing, the blot was exposed to a HRP-conjugated secondary Abs diluted in TBS-T. After rinsing, blots were incubated in ECL (Amersham Pharmacia Biotech) reagent for 1 min, blotted dry, and then exposed to film until a signal was detected.
Results

Stimulation of human G0 B cells increases proliferation and caspase activity and decreases apoptosis

In initial experiments we compared various signals for their ability to stimulate human dense G0 tonsillar B cells to enter the cell cycle and proliferate (Fig. 1A). The B cells used, as previously reported (20, 21), were >98% CD20+ and >98–99% in G0. These G0 B cells, when left unstimulated for 3 days in culture, exhibited little or no proliferation. After 3 days in culture the cells were 12–15% annexin V positive, reflecting a basal level of apoptosis in the unstimulated normal cells (Fig. 1B). Stimulation with Abs to surface IgM, CD40, or CD180 (RP105/Bgp95) or with rIL-4 increased B cell proliferation at 72 h 4- to 5-fold as determined by thymidine incorporation (Fig. 1A). Anti-CD40 with other signals (anti-CD180, anti-IgM, or IL-4) led to a significant increase in proliferation relative to that induced by single stimuli (Fig. 1A).

Previous studies suggested that caspase-8 was cleaved and activated in stimulated normal human T cells (17, 18). To determine whether caspase-8 was cleaved upon stimulation of normal B cells, Western blot analyses for caspase-8 were performed 48 h after stimulation using an Ab that recognizes the pro form as well as the cleaved activated form of caspase-8. Cleaved caspase-8 was not detectable in the unstimulated cells and was only detected in cells stimulated via CD40 alone or via CD40 in combination with CD180, surface IgM, or IL-4 signals (Fig. 1B). In subsequent experiments we stimulated G0 B cells with anti-CD40 plus anti-CD180, since this combination consistently induced the most proliferation. Stimulation of B cells with CD40 ligand, rather than anti-CD40, in combination with anti-CD180 gave similar levels of proliferation and caspase cleavage (data not shown). These results show that the anti-CD180 and anti-IgM combinations, which resulted in the highest level of proliferation and the lowest level of apoptosis, led to caspase-8 cleavage.

To determine whether caspase activity was also increased by proliferative stimuli, we performed caspase activity assays on lysates prepared from B cells that were either unstimulated or stimulated via CD40/CD180 for 12–48 h. For these experiments we used the peptide reporter substrates DEVD-AMC, VEID-AMC, and IETD-AMC, which are preferred substrates for caspase-3, -6, and -8, respectively. These substrates have been widely used to assess caspase activity. All peptide cleavage activity was completely inhibited by the broad specificity caspase inhibitor ZVAD-CHO and therefore attributable to caspases. In unstimulated B cells, DEVDase (caspase-3-like) activity was detectable at all times measured and increased over the time course of the assay (Fig. 2A). This activity was completely blocked by 1 μM DEVD-CHO, supporting the idea that caspase-3 was probably responsible (data not shown). To exclude contaminating caspase-3 activity, assays for caspase-6 and -8 were performed in the presence of 1 μM DEVD-CHO. In contrast to DEVDase activity, little or no VEIDase (caspase-6-like) activity or IETDase (caspase-8-like) activity was detectable in unstimulated cells at any time point (Fig. 2, B and C). Stimulation via CD40/CD180 led to decreased DEVDase activity by 24 and 48 h, but increased VEIDase and IETDase activity (Fig. 2). Cleavage of VEID-AMC was detected as early as 12 h after stimulation and peaked at 24 h, while IETD-AMC cleavage was detected first at 24 h and peaked 48 h after stimulation (Fig. 2, B and C). These data are consistent with an increase in caspase-8 and -6, but a decrease in caspase-3, activity following the proliferative stimulation of normal human B cells.

We next performed Western blotting for caspase-3 and -6 in cells stimulated for 24 h with CD40/CD180. Cleavage of caspase-3, indicative of activation, was observed in unstimulated B cells, but not in B cells stimulated with anti-CD40/anti-CD180 (Fig. 3A, upper panel). In contrast, significant caspase-6 cleavage was only detected in stimulated B cells (Fig. 3A, lower panel). To gain further insight into the activation state of caspase-3 and -6, we examined the cleavage state of endogenous substrates for either caspase-3 or caspase-6 in unstimulated and stimulated B cells. Accumulated evidence suggests that poly-ADP ribose polymerase (PARP) is a preferred substrate for caspase-3, while the transcriptional repressor SATB1 is primarily a substrate for caspase-6 (26, 27). While cleavage of PARP was observed in unstimulated cells, little or no PARP cleavage was seen in B cells stimulated with anti-CD40/anti-CD180 (Fig. 3B, upper panel). In contrast, cleavage of SATB1 was only seen in stimulated B cells (Fig. 3B, lower panel).

We then tested the effect of cell-permeable tetrapeptide inhibitors, selective for either caspase-3 (DEVD-fmk) or caspase-6 (VEID-fmk), on PARP and SATB1 cleavage in unstimulated and
stimulated B cells. Pretreatment with DEVD-fmk blocked cleavage of PARP in unstimulated B cells, but not of SATB1 in stimulated cells (Fig. 3B). Conversely, VEID-fmk inhibited SATB1 cleavage by 70%, but only blocked PARP cleavage by 20% (Fig. 3B). These data suggest that caspase-6 activity is most likely responsible for SATB1 cleavage in stimulated B cells. Collectively, our Western blotting data are consistent with the data obtained by the peptide activity assay in suggesting that caspase-8 and -6 activities are elevated in proliferating normal B cells, while caspase-3 activity is reduced.

Caspase activity is required for G0 B cells to proliferate

We next determined whether the caspase activity induced after stimulating resting B cells is required for B cell proliferation. Dense human B cells were pretreated for 3 h with the broad spectrum caspase inhibitor ZVAD-fmk and then stimulated with anti-CD40 in combination with anti-CD180, anti-IgM, or IL-4. Pretreatment with ZVAD-fmk inhibited B cell proliferation in response to all stimuli used (data not shown). Thus, caspase activity is required for B cells to be induced to proliferate via these stimuli. We compared the dose-response of caspase-selective inhibitors for their ability to inhibit human B cell proliferation. Dense human B cells were pretreated for 3 h with the indicated concentration of either ZVAD-fmk (a broad specificity inhibitor), IETD-fmk (selective for caspase-8), VEID-fmk (selective for caspase-6), or DEVD-fmk (selective for caspase-3). The cells were then stimulated with anti-CD40 in combination with anti-CD180, and thymidine incorporation was determined after 48 h of stimulation (Fig. 4A). VEID-fmk inhibited CD40/CD180-mediated B cell proliferation over a dose range of 20–80 μM, with complete inhibition seen at 80 μM (Fig. 4A). ZVAD-fmk was effective over a similar concentration range as VEID-fmk, with 50% inhibition occurring between 40 and 50 μM. In contrast, DEVD-fmk had no effect on CD40/CD180-mediated proliferation at any concentration examined. The caspase-8 inhibitor IETD-fmk achieved ~50% inhibition at 80 μM and was consistently less effective than either ZVAD-fmk or VEID-fmk.

We next sought to determine whether the ability of caspase inhibitors to block proliferation was restricted by stimulus, cell type, or species. The proliferation of primary B cells in response to other combinations of stimuli, including anti-CD40 with IL-4 and anti-CD40 in addition to anti-IgM, was also blocked by caspase inhibitors, indicating that the inhibitory effects of caspase inhibitors were not stimulus specific (data not shown). In contrast, the proliferation of human T cells stimulated with anti-CD3 plus anti-CD28 was not inhibited by either DEVD-fmk or VEID-fmk under experimental conditions where B cell proliferation was significantly inhibited (Fig. 4B). We next compared the effects of ZVAD-fmk, VEID-fmk, IETD-fmk, and DEVD-fmk on anti-CD40- or LPS-mediated proliferation in

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**FIGURE 2.** Caspase activity following CD40/CD180 stimulation. Human B cells were either unstimulated (○, △, and ■) or stimulated with anti-CD40/CD180 (●, ■, and □) for the indicated time. Cell extracts were prepared and caspase activity measured using DEVD-AMC (A), VEID-AMC (B), or IETD-AMC (C) as substrate. DEVD-AMC activity was completely antagonized by the inclusion of 1 μM DEVD-CHO (data not shown), while data presented for VEIDase and IETDase activity were measured in the presence 1 μM DEVD-CHO. DEVDase (caspase-3-like) activity was decreased by anti-CD40/CD180 stimulation, while VEIDase (caspase-6-like) and IETDase (caspase-8-like) activities were increased. The results presented are representative of three independent experiments.

**FIGURE 3.** Cleavage and activation of caspase-6 but not caspase-3 in proliferating B cells. Human B cells were either untreated or pretreated with 80 μM DEVD-fmk or VEID-fmk for 3 h before stimulation with anti-CD40 and anti-CD180 for 48 h. Western blot analysis was then performed on cell extracts using Abs specific for caspase-3 or caspase-6 (A), or PARP or SATB1 (B). Caspase-3 and PARP cleavage were observed in unstimulated cells, but not in proliferating cells. In contrast, caspase-6 and SATB1 cleavage was only detected in proliferating cells. The results presented are representative of three independent experiments.
FIGURE 4. Effects of caspase inhibitors on human and mouse lymphocyte cell proliferation. A, Human B cells were either untreated (○) or pretreated with the indicated concentration of IETD-fmk (□), DEVD-fmk (△), ZVAD-fmk (■), or VEID-fmk (●) before stimulation with anti-CD40 in combination with anti-CD180. B, Human tonsilar B cells or E$^+$ rosette-forming T cells were either untreated (○) or pretreated with DEVD-fmk (△) or VEID-fmk (■) before stimulation with anti-CD40 and anti-CD180 (B cells) or anti-CD3 plus anti-CD28 (T cells). C, Murine B cells were either untreated (○) or pretreated with 80 μM IETD-fmk (□), DEVD-fmk (△), ZVAD-fmk (■), or VEID-fmk (●) before stimulation with either LPS or anti-CD40. D, Human B cells were either untreated (○) or pretreated with DEVD-fmk (△) or VEID-fmk (■) before stimulation with anti-CD40 in combination with anti-CD180. After 72 h, proliferation was measured by incorporation of $[^{3}H]$thymidine, and RNA synthesis was measured by incorporation of $[^{3}H]$uridine. The results presented are representative of three independent experiments.

Caspase activity is required for entry into the cell cycle

Since the B cell population used in this study is highly quiescent, we examined the effects of caspase inhibitors on various parameters of cell cycle entry. Two early changes that accompany cell cycle entry include induction of RNA synthesis and an increase in cell size. To test whether caspase inhibitors blocked the induction of RNA synthesis we labeled cells with $[^{3}H]$uridine following 72 h of stimulation in the presence or the absence of caspase inhibitors (Fig. 4D). While DEVD-fmk had little effect, pretreatment with VEID-fmk almost completely abolished the incorporation of $[^{3}H]$uridine induced by anti-CD40 and anti-CD180 (Fig. 4D, right panel). Similar results were obtained at 48 h (data not shown). In addition, analysis of forward vs side scatter FACS profiles indicated that the early increase in cell size was also blocked by caspase inhibitors (data not shown).

To more precisely determine the mechanism by which caspase inhibitors blocked cell cycle entry of stimulated B cells, we measured the induction of critical cell cycle regulators. Since the levels of cell cycle regulators can be influenced by both transcriptional and post-translational mechanisms, we measured both mRNA and protein levels. Stimulation of quiescent B cells with anti-CD40/anti-CD180 for 24 h significantly increased the levels of mRNAs for cyclins D1 and D2 and the cyclin-dependent kinase cdk4 (Fig. 5, A and B). Pretreatment with VEID-fmk blocked induction of $\alpha$-type cyclin and cdk4 mRNA by anti-CD40/CD180 almost to basal levels (Fig. 5, A and B). However, DEVD-fmk did not inhibit the induction of any of these mRNAs. In contrast to its effect on $\alpha$-type cyclins, VEID-fmk did not influence message levels for the cyclin-dependent kinase inhibitors p21 or p27 (Fig. 5, A and B).

These findings implicate caspase-6, but not caspase-3, in induction of $\alpha$-type cyclin and cdk4 mRNA by anti-CD40/CD180.

Western blot analysis using Abs specific for cell cycle proteins was then employed to determine the expression level of these proteins (Fig. 5C). Stimulation of quiescent B cells with anti-CD40/CD180 for 48 h led to an increase in cyclin D2, cyclin D3, and cdk4 protein levels. Phosphorylation of the Rb and expression of cyclin A were also significantly increased by stimulation, while levels of the cdk inhibitor p27Kip1 were decreased. The stimulated accumulation of cyclin D2, cyclin D3, and cdk4 was blocked by preincubation with VEID-fmk, but not DEVD-fmk (Fig. 5C).

VEID-fmk also reduced Rb phosphorylation and blocked the decrease in p27Kip1 levels observed following stimulation. The fact that caspase-6 inhibitors block the induction of both cdk4 and $\alpha$-type cyclins suggests that caspase-6 activity is required for resting B cells to enter the G1 phase of the cell cycle.

Caspase activity is not required for all CD40-dependent activation events

We next tested whether the caspase inhibitors selectively blocked other signaling events associated with the stimulation of quiescent B cells. CD40 ligation has been previously shown to up-regulate the expression of cIAP2 (28), a protein that inhibits apoptosis by selectively binding and inhibiting caspase-3 and caspase-7, but not caspases-6 and -8 (29). Western blot analysis of lysates from CD40/CD180-stimulated B cells showed that CD40/CD180 stimulation increased cIAP2 expression. Pretreatment with ZVAD-fmk had no effect on cIAP2 protein levels (Fig. 6A). We also examined...
the rapid phosphorylation of the ERK/MAPK and protein kinase B/Akt kinases following treatment with the caspase inhibitor ZVAD-fmk. An increase in ERK phosphorylation was observed as early as 15 min after combined stimulation via CD40/CD180 (Fig. 6B), and ERK remained highly phosphorylated for at least 6 h following stimulation. Treatment with ZVAD-fmk did not affect ERK phosphorylation. ZVAD-fmk also had no effect on the phosphorylation of Akt induced by CD40/CD180 stimulation (data not shown).

CD40 or CD180 stimulation up-regulates IL-6 expression in dense human B cells (30), and IL-10 expression is regulated by CD40 in normal human B cells (31). Using an ELISA to measure IL-6 and IL-10 production in supernatants, we found that CD40/CD180 ligation stimulated B cells to produce both IL-10 and IL-6 (Fig. 6C). Treatment with ZVAD-fmk or VEID-fmk dramatically reduced the level of IL-10 in the supernatant of stimulated cells (Fig. 6C and data not shown). DEVD-fmk was again without effect. IL-6 levels, however, were not affected by any of the caspase inhibitors tested. Thus, caspase activity is selectively required for the induction of cell cycle genes (Figs. 4 and 5) and the B cell growth factor IL-10 (31).

Discussion
Proliferative stimuli activate a pattern of caspase activity that is distinct from apoptotic stimuli

Our results demonstrate that treatment of dense human B cells with proliferative stimuli, such as ligation of CD40 and CD180, leads to the selective activation of caspase-8 and caspase-6, while caspase-3 activity and apoptosis are reduced. These conclusions are based upon collective data including the use of peptide substrates that are selective for caspases-3, -6, and -8. Proliferative...
stimuli increased VEIDase and IETDase activities, while DEV-Dase activity was reduced. Although these substrates are modeled after preferred substrate sequences for the respective caspases, their specificity is not absolute. However, in addition to this evidence, Western blotting using Abs specific for these caspases as well as for preferred substrates was also performed. Cleavage of caspase-8, caspase-6, and SATB1, but not caspase-3 or PARP, was observed after B cells were activated. Together these data provide strong evidence to suggest that a pattern of caspase activation distinct from that involved in apoptosis, involving increased caspase-8 and caspase-6 activity, is induced by proliferative stimuli.

How might B cells activate caspase-8 and caspase-6 while preventing caspase-3 activation and apoptosis? The decrease in apoptosis may result from both the up-regulation of prosurvival factors and the selective inactivation of effector caspases. In this respect, CD40 ligation of human B cells not only increases the expression of antiapoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-X, and A1 (32–34), but also up-regulates the expression of IAPs such as cIAP1 and cIAP2 (28). IAPs selectively bind and inhibit caspase-3 and -7, but not caspase-6 and -8 (29). In addition, cIAP2 has been shown to ubiquitinate caspase-3 and stimulate its degradation by the proteasome (35). Thus, IAPs may selectively inactivate caspase-3 without affecting the caspases required for B cells to enter the cell cycle. Interestingly, antisera specific for caspase-3 consistently recognized several species that migrated below the pro form in proliferating cells (Fig. 3A). The fact that these bands are also observed upon treatment of B cells with proteasome inhibitors (J. D. Graves, unpublished observations) supports the idea that caspase-3 might be inactivated by ubiquitination in proliferating cells. The precise mechanism by which the specificity of caspase activation is maintained in response to proliferative stimuli is currently under investigation.

Caspase activity is required for B cell proliferation

Our data based upon the use of cell-permeable caspase inhibitors demonstrate that caspase activity is required for dense B cells to proliferate. The broad specificity caspase inhibitor ZVAD-fmk, which is an efficient inhibitor of apoptosis in many systems, blocked B cell proliferation in response to ligation of CD40 and CD180. VEID-fmk, an inhibitor selective for caspase-6, was an especially effective inhibitor of proliferation, while DEVD-fmk, which is selective for caspase-3, was ineffective (Fig. 3B). While it is important to recognize that these peptide caspase inhibitors are not absolutely specific, several lines of evidence suggest that they exhibit a high degree of selectivity in this system. VEID-fmk completely blocked cleavage of the caspase-6 substrate SATB1, but only partially blocked cleavage of the caspase-3 substrate PARP, while DEVD-fmk had no effect on SATB1 cleavage, but abolished that of PARP (Fig. 3B). These data also argue against differences in permeability making a significant contribution to the differential effectiveness of VEID-fmk and DEVD-fmk. The fact that caspase-6 and not caspase-3 activity is elevated in these cells provides further evidence suggesting that VEID-fmk is likely to be exerting its effects via inhibition of caspase-6.

Based upon its apical position in the apoptotic caspase cascade induced by CD95/Fas, it is tempting to conclude that caspase-8 functions upstream of caspase-6 in regulating B cell proliferation. In this respect, Kennedy et al. (18) showed that caspase-8 is activated in CD3-stimulated normal T cells and that a caspase-8 inhibitor efficiently blocked CD3-mediated proliferation. Further evidence for an important role for caspase-8 can be derived from a recent study that identified a functional deficit in caspase-8 in patients with a lymphoproliferative disorder (19). However, while VEIDase activity was maximal 12 h after CD40/CD180 stimulation of quiescent B cells, caspase-8 activity did not peak until 24 h (Fig. 2, B and C). Furthermore, IETD-fmk was a weak and inconsistent inhibitor of B cell proliferation (Fig. 4B and data not shown). An alternative possibility is suggested by a recent study that indicates caspase-6 functions upstream of caspase-8 in the apoptotic pathway initiated by cytochrome c (36). Thus, it is possible that caspase-6 functions upstream of caspase-8 in response to proliferative stimuli in B cells. In support of this hypothesis, IETD-fmk did not block processing and activation of caspase-6 in response to anti-CD40/CD180 (data not shown).

Although ZVAD-fmk is a potent antagonist of B cell proliferation, caspase activity is not required for all activation events. For example, caspase inhibitors had no effect on ERK/MAPK phosphorylation induced via CD40/CD180 (Fig. 6B). Since the ERK pathway is required for B cell proliferation (37), this argues against the possibility that caspase inhibitors might exert a nonspecific or toxic effect blocking the activation of signaling pathways. Also, no significant decrease in cell viability was observed following incubation with VEID-fmk for periods up to 72 h (data not shown). Further evidence for the specificity of caspase involvement comes from data showing that VEID-fmk antagonized anti-CD40/CD180-induced IL-10 secretion, but not secretion of IL-6 (Fig. 6C). These data support the idea that caspases function downstream of, or parallel to, early signaling events that regulate the proliferation, survival, and function of quiescent B cells.

While the identity of the caspase(s) required for B cell proliferation cannot be definitively deduced from our present data, our results are consistent with the hypothesis that caspase-6 and/or another caspase, sensitive to VEID-fmk yet relatively insensitive to DEVD-fmk, is required for human B cell proliferation. Interestingly, a recent study involving the generation of a caspase-6-deficient chicken cell line has provided evidence that caspase-6 may be important for B cell proliferation and/or survival (38). Experiments in caspase-6-deficient mice assessing the role of caspase-6 as a critical effector of B cell proliferation and maturation are underway.

Caspase-6 activity is required for entry into the cell cycle

Pretreatment of quiescent B cells with VEID-fmk blocked induction of RNA synthesis, increases in cell size, and induction of cyclin D2, cyclin D3, and cdk4 proteins while increasing levels of the cdk inhibitor p27Kip1 in response to anti-CD40/CD180 (Figs. 4D and 5). This suggests that caspase-6 activity is required at the G_{0}/G_{1} restriction point of cell cycle entry. Although p27Kip1 is cleaved during apoptosis, several reports also suggest that caspase cleavage of p27 may serve a regulatory function during B cell proliferation (39). Thus, one mechanism by which caspase-6 might regulate primary B cell proliferation is via cleavage of p27Kip1. Another potential mechanism is suggested by the fact that VEID-fmk blocks the induction of mRNA encoding cdk4 and d-type cyclins (Fig. 5, A and B). Further evidence to suggest that caspase inhibitors block early events can be inferred from the fact that VEID-fmk or ZVAD-fmk must be added within the first 4 h following stimulation to block proliferation (data not shown). Accordingly, caspase-6 might target transcriptional activators or repressors that regulate early cell cycle gene expression. Of the several defined substrates for caspase-6, SATB1 was of particular interest. SATB1 is a matrix attachment region DNA-binding protein (40), is expressed in B cells (5) (Fig. 3B), is known to down-regulate gene transcription (8, 41), and after caspase cleavage loses its ability to bind to chromatin (19). Activation of human B cells leads to increased cleavage of SATB1 that is antagonized by VEID-fmk. This raises the possibility that caspase-6 functions to...
down-regulate transcriptional repressors such as SATB1 during proliferation.

The homeostatic regulation of B cell populations is tightly regulated by a balance among cell death, survival, and proliferation. Our data indicate that caspases, already established as critical effectors of cell death, also play an important role in B cell proliferation. While further analysis is required to understand the mechanism by which caspases contribute to these contrasting cell fates, these findings raise the possibility that caspase-6 might constitute an attractive therapeutic target for lymphoproliferative or autoimmune diseases.

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References