

# CD40 and adenosine A2 receptor agonist–cyclic adenosine monophosphate rescue B-cell antigen receptor–induced apoptosis through independent pathways and converge to prevent caspase activation

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**Background:** Antigen receptor ligation induces apoptosis of B lymphocytes, but the molecular mechanisms underlying induction of apoptosis remain unclear, although the growing family of IL-1 $\beta$ -converting enzyme cysteine proteases (caspases) are recognized to be major effectors of cellular death.

**Objective:** We sought to delineate and compare the rescue of B-cell apoptosis through CD40 ligand-CD40 interaction and cyclic adenosine monophosphate (cAMP)–dependent protein kinase A in human B cells.

**Methods:** By using tonsillar B cells and the B-lymphoblastoid cell line Ramos, rescue from B-cell apoptosis was compared, as were signaling pathways after activation of cells through CD40 and the adenosine A2 receptor.

**Results:** Both CD40 ligand-CD40 interaction and activation of intracellular cAMP rescue B cells from apoptosis after antigen receptor ligation. Although these pathways do not overlap, they converge by preventing the anti-IgM–induced activation of CPP32 (caspase 3), a member of the IL-1 $\beta$ -converting enzyme protease family.

**Conclusion:** These data indicate that the cAMP-protein kinase A–dependent and CD40-signaling pathways regulate B-cell survival and converge at a common point, the inhibition of antigen receptor–induced activation of caspases. (*J Allergy Clin Immunol* 2000;105:522-31.)

**Key words:** B cells, apoptosis, cyclic adenosine monophosphate, CD40

Apoptosis has been observed in many types of cells after addition of diverse stimuli and plays an important role in the establishment and maintenance of the human immune system. Deletion of autoreactive immature T cells by apoptosis in the thymus is believed to underlie the mechanism of thymic negative selection. Failure of apop-

## Abbreviations used

|         |                                                                                |
|---------|--------------------------------------------------------------------------------|
| ATP:    | Adenosine triphosphate                                                         |
| cAMP:   | Cyclic adenosine monophosphate                                                 |
| dbcAMP: | N <sup>6</sup> ,O <sup>2</sup> -dibutyryl-adenosine 3',5'-cyclic monophosphate |
| DMSO:   | Dimethyl sulfoxide                                                             |
| ERK:    | Extracellular signal-regulated kinase                                          |
| JNK:    | c-Jun N-terminal kinase                                                        |
| MBP:    | Major basic protein                                                            |
| MEK:    | Mitogen-activated protein kinase kinase                                        |
| PKA:    | Protein kinase A                                                               |

toxis leads to lymphoproliferative disorders. B-lymphoblastoid cell lines, such as Ramos or WEHI-231, have been used as in vitro models for studying clonal selection of immature B cells.<sup>1-3</sup> These cells undergo apoptosis after antigen receptor ligation, and the cells are rescued by CD40 ligation. However, the molecular mechanisms of induction or rescue remain poorly defined.

Antigen receptor ligation of B cells leads to the rapid activation of nonreceptor-type protein tyrosine kinases, including p53/56 Lyn, p55 Blk, p59 Fyn, and p72 Syk. These kinases phosphorylate the tyrosine residues of a number of intracellular substrates, including phosphatidylinositol-3 kinase,<sup>4</sup> phospholipase C- $\gamma$ ,<sup>5</sup> and Ras GTPase-activating protein.<sup>6</sup> These phosphorylated substrates then serve to transmit additional signals. Among these, extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase is activated.<sup>7,8</sup> This activation is mediated through Ras/Raf-1/mitogen-activated protein kinase kinase (MEK) activation.<sup>8</sup> Recently, we and others found that signaling through CD40 activates yet another mitogen-activated protein kinase family member, c-Jun N-terminal kinase (JNK)/stress-activated protein kinase in human B cells.<sup>9,10</sup> These data suggested that opposing influences on apoptosis by antigen receptor and CD40 may be regulated by the ERK- and JNK-signaling pathways. A protective effect of JNK activation has recently been proposed during T-cell development.<sup>11</sup>

Cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA) inhibits B-cell responses not only by blocking cell cycle progression in

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activated cells but also by regulating apoptosis in resting cells.<sup>12,13</sup> Intracellular cAMP levels also appear to be involved in the regulation of apoptosis in germinal center B cells.<sup>14</sup> In the present study we investigated the interactions between cell-permeable cAMP analogs and signals activated by antigen receptor ligation with the apoptotic response of the human Burkitt's lymphoma-derived cell line Ramos and tonsillar B cells. Ramos cells retain a vestige of the normal selection process. Not only do these cells express a phenotype characteristic of germinal center B cells but they also easily undergo apoptosis. Increases in cytosolic levels of cAMP effectively rescued these cells and tonsillar B cells from apoptosis induced by antigen receptor ligation.

## METHODS

### Cells

The human Burkitt's lymphoma cell line Ramos was obtained from the American Type Tissue Culture Collection (Rockville, Md), and cells were maintained in RPMI-1640 medium supplemented with 50 U/mL penicillin-streptomycin, 2 mmol/L glutamine, 50 mmol/L 2-mercaptoethanol, and 10% FCS. Exponentially growing cells were used in all experiments. Tonsillar B cells were purified as described previously.<sup>9</sup>

### Reagents

Anti-IgM (F[ab']<sub>2</sub> goat anti-human IgM antibody) was purchased from Cappel (Durham, NC). The mouse monoclonal anti-human CD40 antibody (G28-5) was generously provided by Dr A. Aruffo (Bristol-Myer Squibb Pharmaceutical Research Institute, Seattle, Wash). The cAMP analogs, proteinase K, and RNase were obtained from Sigma (St Louis, Mo). H89 was obtained from Calbiochem (La Jolla, Calif). The adenosine agonist (CGS 21680) was obtained from RBI (Natick, Mass). Polyclonal goat anti-ERK2 and monoclonal mouse anti-Bcl-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), and myelin major basic protein (MBP) was obtained from Upstate Biotechnology (Lake Placid, NY). PD 98059 was generously provided by Dr A. Saltiel (Parke Davis, Mich).

### Cell viability assay

Cell viability was evaluated by trypan blue dye exclusion. Cells ( $5 \times 10^5$ /mL) were cultured in the presence of various agents for up to 48 hours. Cells were mixed with an equal volume of 0.85% saline containing 0.4% trypan blue (Gibco BRL), and numbers of stained and unstained cells were counted under the microscope. The percentage of viable cells was calculated by dividing the number of cells excluding trypan blue by the total number of cells.

### Propidium iodide staining and flow cytometry analysis

Hypodiploid DNA was analyzed by using flow cytometry. Cells were treated for 24 hours as indicated;  $10^6$  cells were washed once in PBS and fixed in 70% ethanol at  $-20^\circ\text{C}$  overnight, washed once in cold PBS, incubated with 0.25 mg/mL RNase in PBS at  $37^\circ\text{C}$  for 30 minutes, and then stained with 25  $\mu\text{g}/\text{mL}$  propidium iodide at room temperature for 30 minutes. Apoptotic cell nuclei containing hypodiploid DNA were analyzed by flow cytometry on an EPICS Profile (Coulter, Hialeah, Fla). For analysis, all cells in the population were analyzed, and the DNA content was recorded on a linear scale. Apoptotic cell nuclei containing hypodiploid DNA were enumerated as a percentage of the total population.

### Measurement of cAMP

Intracellular cAMP concentrations were determined by using a cAMP enzyme immunoassay system (RPN 225; Amersham, Arlington Heights, Ill). Briefly, after cell stimulation,  $10^6$  cells were washed once in PBS and suspended in PBS. Extraction of cAMP was conducted by the addition of ice-cold ethanol to result in a 65% ethanol concentration. Insoluble material was removed by centrifugation. The supernatant was dried in a vacuum centrifuge and then dissolved in 200  $\mu\text{L}$  of assay buffer. The cAMP concentration was determined by using a nonacetylation method.

### Measurement of PKA activity

After cell stimulation,  $2 \times 10^6$  cells were lysed in 100  $\mu\text{L}$  of extraction buffer (5 mmol/L EDTA, 50 mmol/L TRIS-HCl [pH 7.5], and 0.5% Triton X-100). Insoluble material was removed by centrifugation at  $4^\circ\text{C}$ . Ten microliters of supernatant was subjected to the following kinase assay. The kinase assay was performed in 40  $\mu\text{L}$  of kinase buffer containing 50 mmol/L TRIS-HCl (pH 7.5), 10 mmol/L  $\text{MgCl}_2$ , and 100  $\mu\text{mol}/\text{L}$  adenosine triphosphate (ATP) containing  $\gamma$ -phosphorous 32 [ $^{32}\text{P}$ ]-ATP ( $1-2 \times 10^5$  dpm/nmol; 4 nmol/assay tube), 0.25 mg/mL BSA, and 50 mmol/L Kemptide as a substrate for 5 minutes at  $30^\circ\text{C}$ . The kinase reaction was terminated by the addition of 20  $\mu\text{L}$  of stop buffer (100 mmol/L ATP and 100 mmol/L EDTA). Thirty microliters was spotted onto a phosphocellulose paper (Whatman, Clifton, NJ). The paper was washed several times with 75 mmol/L phosphoric acid, and then radioactivity was determined by means of liquid scintillation counting. A unit (picomoles per minute) is defined as the amount of kinase that catalyzes the transfer of 1 pmol of  $^{32}\text{P}$  from ATP to Kemptide substrate in 1 minute.

### Measurement of ERK2 kinase activity

ERK2 kinase assay was performed as previously described.<sup>9</sup> A unit (picomoles per minute) is defined as the amount of kinase, which catalyzes the transfer of 1 pmol of  $^{32}\text{P}$  from ATP to MBP substrate in 1 minute.

### Measurement of CPP32 (or CPP32-like) activity

Cells ( $10^6$ /mL) were treated with medium;  $\text{N}^6, \text{O}^2$ -dibutyryl-adenosine 3',5'-cyclic monophosphate (dbcAMP); or anti-CD40 (1  $\mu\text{g}/\text{mL}$ ) for 30 minutes before the addition of 10  $\mu\text{g}/\text{mL}$  anti-IgM antibody. After 24 hours, cells were lysed in 50 mmol/L TRIS (pH 7.4), 1 mmol/L EDTA, and 10 mmol/L ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid for 10 minutes at  $37^\circ\text{C}$ . Lysate protein (60  $\mu\text{g}$ ) was incubated with 5  $\mu\text{mol}/\text{L}$  acetylasp-glu-val-asp-amino-4-methylcoumarin (Bachem Biosciences, King of Prussia, Pa), a fluorogenic substrate selective for CPP32,<sup>15</sup> in 1 mL of 50 mmol/L TRIS (pH 7.4), 1 mmol/L EDTA, and 10 mmol/L ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid for 20 minutes at  $37^\circ\text{C}$ . Fluorescence was monitored with an excitation wavelength of 380 nm and emission wavelength of 460 nm. Fluorescence of the substrate alone was subtracted in each case.

### Immunoblot analysis

Twenty-four hours after incubation with reagents, cells were lysed in 100  $\mu\text{L}$  of lysis buffer (25 mmol/L TRIS-HCl [pH 7.6], 50 mmol/L NaCl, 0.5% Na deoxycholate, 2% NP-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 50  $\mu\text{g}/\text{mL}$  aprotinin, and 50  $\mu\text{mol}/\text{L}$  leupeptin). Equal amounts of lysates (20  $\mu\text{g}$ ) were electrophoresed through a 15% SDS-PAGE gel, and proteins were transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (25 mmol/L TRIS-HCl [pH 8.0], 125 mmol/L NaCl, 0.1% Tween-20, 2% BSA, and 0.1%  $\text{NaN}_3$ ) at  $4^\circ\text{C}$  overnight,

monoclonal mouse anti-Bcl-2 antibody (1:250) was added to the blocking buffer, and blots were incubated for an additional 2 hours at room temperature. The blots were washed 3 times in TBST (25 mmol/L TRIS-HCl [pH 8.0], 125 mmol/L NaCl, and 0.025% Tween-20) and incubated with anti-mouse Ig and horseradish peroxidase-linked whole antibody (Amersham, Arlington Heights, Ill) 1:500 in TBST for 1 hour at room temperature. The blots were washed 3 times in TBST, and proteins were then detected by using the ECL system (Dupont NEN, Boston, Mass).

### Statistical analysis

Values were compared by using the Student *t* test.

## RESULTS

### cAMP analogs or adenosine analogs rescue B cells from apoptosis induced by anti-IgM antibody

Ramos cells express cell-surface IgM on the cell membrane and undergo apoptosis after addition of anti-IgM antibody. Ramos cells ( $5 \times 10^5$  cells/mL) were treated with 10  $\mu$ g/mL anti-IgM antibody, and cell viability was evaluated by trypan blue dye exclusion (Fig 1, A). Cell viability decreased to  $65\% \pm 4\%$  (mean  $\pm$  SD) after 48 hours. In the presence of various concentrations of dbcAMP, cell viability was recovered in a dose-dependent fashion. When cell yields were examined under the various experimental conditions, it was observed that addition of anti-IgM antibody not only decreased cell viability but also inhibited the proliferation of Ramos cells (doubling time, approximately 48 hours). At 24 hours, there was little increase in the number of (untreated) cells, and the effect of anti-IgM antibody was in reducing cell viability, which was restored in the presence of dbcAMP. At 48 hours, dbcAMP restored viability but had only a partial effect (approximately 50%) in restoring cell numbers. The Ramos cells were also treated with anti-IgM antibody alone or in the presence of various concentrations of dbcAMP for 24 hours and stained with propidium iodide (Fig 1, B). The percentage of cells containing hypodiploid DNA detected by flow cytometry was increased from  $2.4\% \pm 1.5\%$  (mean  $\pm$  SD) in the control (untreated) sample to  $21.3\% \pm 4.9\%$  in the presence of anti-IgM antibody. In the presence of increasing concentrations of dbcAMP, the percentage of apoptotic nuclei decreased in a dose-dependent manner. These results were confirmed by using DNA fragmentation to monitor apoptosis; in the presence of either anti-CD40 or 2 mmol/L dbcAMP, DNA fragmentation induced by anti-IgM was blocked in Ramos cells (data not shown).

Virtually identical effects were observed by using other cAMP analogs, such as 8-chlorophenylthio-cAMP and benzylamine cAMP (data not shown). Treatment of the cells with dbcAMP alone, even at high concentrations (2 mmol/L), did not affect cell proliferation and DNA synthesis, cell viability, or cell cycle distribution (data not shown). These findings indicate that cell-permeable cAMP analogs prevented apoptosis induced by antigen receptor ligation of Ramos cells but did not alter the growth properties of the cells.

The studies with cAMP analogs indicated that the cAMP-PKA pathway was associated with the rescue of cells from apoptosis. Activation of the adenosine A2 receptor increases intracellular cAMP levels, and adenosine plays a role in protecting organs from induced stress.<sup>16</sup> We therefore investigated whether the increases in intracellular cAMP levels by adenosine-adenosine A2 receptor interaction could rescue Ramos cells from apoptosis induced by anti-IgM antibody. Ramos cells were treated with the adenosine analog (CGS 21680), and intracellular cAMP levels were measured. CGS 21680 increased intracellular cAMP levels in a dose-dependent fashion (Fig 1, C). In parallel, cell viability after treatment with anti-IgM antibody was also recovered in the presence of CGS 21680 in a dose-dependent fashion (Fig 1, D). Thus the adenosine A2 receptor is a physiologically relevant regulator of the apoptotic response in antigen receptor-stimulated Ramos cells.

We also tested whether the effect of cAMP on apoptosis is demonstrable in isolated tonsillar B cells. Cells were incubated in medium containing anti-IgM antibody (10  $\mu$ g/mL) or anti-IgM antibody and dbcAMP (0.5 or 2 mmol/L) for 24 hours, and then cell viability was measured by means of trypan blue dye exclusion. Addition of anti-IgM antibody decreased cell viability, whereas samples pretreated with dbcAMP maintained cell viability similar to that of control samples (Fig 1, E). At 24 hours, cell yields were virtually identical under all experimental conditions.

These findings indicate that cAMP-PKA activation pathways, including activation through the adenosine A2 receptor, are associated with the delivery of a rescue signal against antigen receptor-induced apoptosis in freshly isolated tonsillar B cells and Ramos cells.

### PKA activation is associated with the rescue signal provided by cAMP but not the rescue signal after CD40 ligation

We investigated whether a PKA inhibitor could alter the reduction in apoptosis induced by cAMP. Ramos cells were treated with H89, a specific inhibitor of PKA (Ki value,  $0.048 \pm 0.008$   $\mu$ mol/L)<sup>17</sup> for 30 minutes before the addition of dbcAMP. The cells were then treated with 10  $\mu$ g/mL anti-IgM antibody for 24 hours, and the percentage of apoptotic nuclei was evaluated by means of flow cytometry (Fig 2, A). In the presence of 10  $\mu$ mol/L H89, the protective effects of 0.5 and 2.0 mmol/L dbcAMP on anti-IgM-induced apoptosis were eliminated. H89 alone did not affect cell viability. These findings suggest that dbcAMP rescues B cells from apoptosis through the activation of PKA.

CD40 ligation also rescues B cells from apoptosis induced by anti-IgM antibody.<sup>9</sup> As previously reported,<sup>9</sup> addition of anti-CD40 restored cell viability to  $94\% \pm 2\%$  of control values. We investigated whether the mechanism of rescue from apoptosis by CD40 ligation was similarly associated with the cAMP pathway. Intracellu-

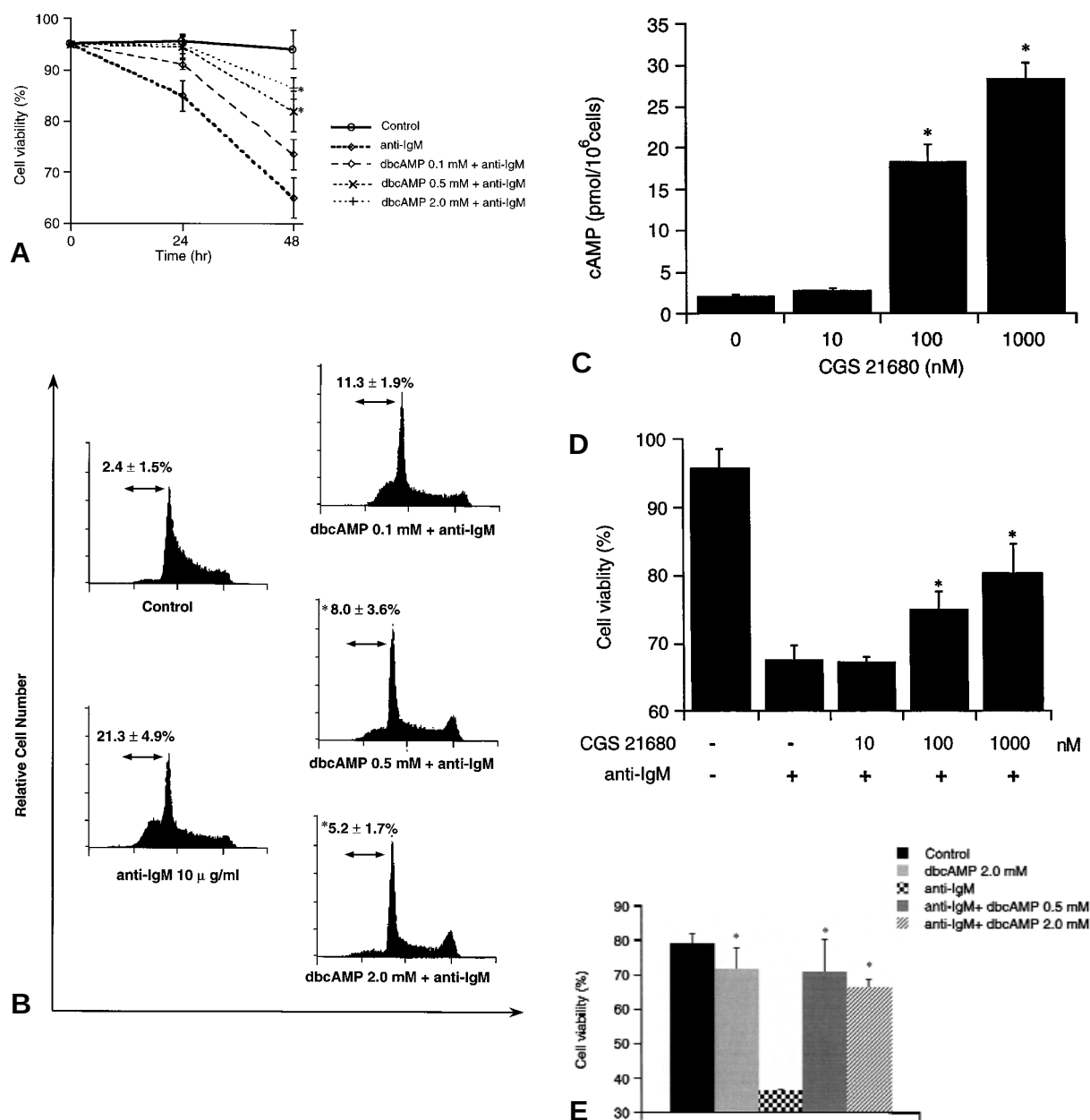


**FIG 1. A**, Dibutyryl cAMP rescues Ramos cells from apoptosis induced by anti-IgM antibody. Ramos cells (at a concentration of  $5 \times 10^5$  cells/mL) were treated with 10 µg/mL anti-IgM antibody in the presence of 0.1, 0.5, or 2.0 mmol/L dbcAMP, and cell viability was evaluated by trypan blue dye exclusion 24 and 48 hours later. Results represent the mean percentage of cell viability  $\pm$  SD from 3 independent experiments. **B**, Ramos cells (at a concentration of  $5 \times 10^5$  cells/mL) were treated with 10 µg/mL anti-IgM antibody in the presence of 0.1, 0.5, or 2.0 mmol/L dbcAMP for 24 hours. The percentage of hypodiploid nuclei was analyzed as described in the "Methods" section. Results represent the mean percentage  $\pm$  SD from 5 independent experiments. Statistically significant differences are identified by an asterisk ( $P < .05$ ). **C**, Adenosine analogs increase intracellular cAMP levels and rescue Ramos cells from apoptosis induced by anti-IgM. Ramos cells were treated with various concentrations of adenosine analog (CGS 21680) for 10 minutes. cAMP was extracted by cold ethanol, and the concentration was determined as described in the "Methods" section. Results show the average cAMP concentration per  $10^6$  cells. **D**, Ramos cells were treated with various concentration of adenosine analog (CGS 21680) for 10 minutes before the addition of 10 µg/mL anti-IgM antibody. Cell viability was evaluated by trypan blue dye exclusion 48 hours later. Results show the average percentage of cell viability  $\pm$  SD from 3 independent experiments. Statistically significant differences are identified by an asterisk ( $P < .05$ ). **E**, Dibutyryl cAMP rescues tonsillar B cells from apoptosis. Isolated tonsillar B cells were incubated in medium containing 10 µg/mL anti-IgM antibody or dbcAMP (0.5 or 2 mmol/L) plus anti-IgM antibody for 24 hours. Cell viability was measured by trypan blue dye exclusion. Results represent the mean percentage of cell viability  $\pm$  SD from 3 separate experiments. Statistically significant differences are identified by an asterisk ( $P < .05$ ).

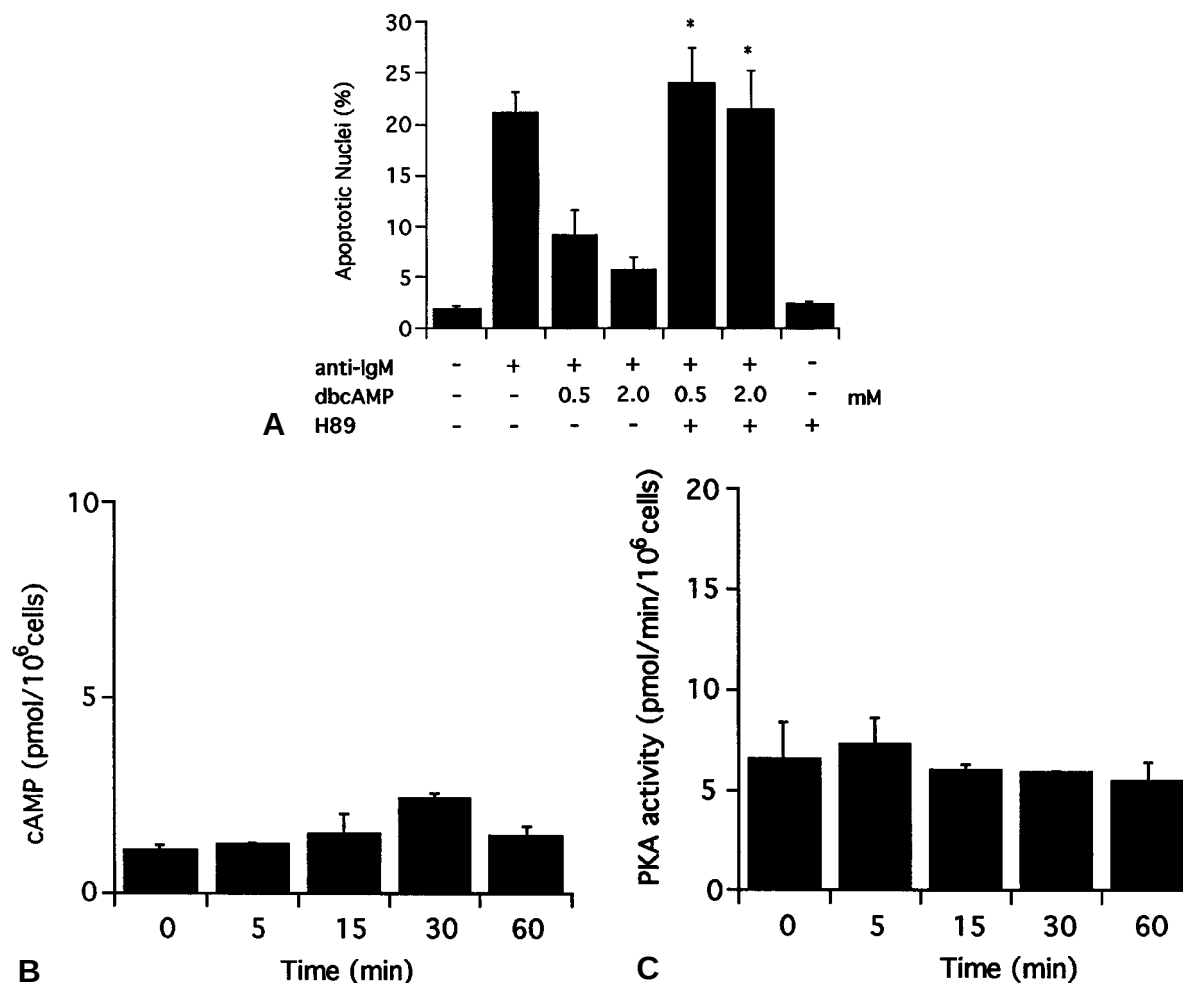


**FIG 2.** PKA activation is associated with the mechanism of rescue from apoptosis by dbcAMP but not anti-CD40 antibody. **A**, Ramos cells (at a concentration of  $5 \times 10^5/\text{mL}$ ) were treated with  $10 \mu\text{g/mL}$  anti-IgM antibody alone, treated with 0.5 or 2.0 mmol/L dbcAMP for 15 minutes before the addition of  $10 \mu\text{g/mL}$  anti-IgM antibody, or preincubated with  $10 \mu\text{mol/L}$  H89 for 30 minutes and treated with 0.5 or 2.0 mmol/L dbcAMP for 15 minutes before the addition of anti-IgM antibody. After 24 hours, the percentage of hypodiploid nuclei was evaluated as described in the "Methods" section. Results show the average percentage of apoptotic nuclei  $\pm$  SD from 3 independent experiments. Statistically significant differences are identified by an asterisk ( $P < .05$ ). **B**, Ramos cells were treated with  $2 \mu\text{g/mL}$  anti-CD40 antibody (G28-5) for various times. cAMP was extracted by cold ethanol and the concentration was determined as described in the "Methods" section. Results show the average cAMP concentration per  $10^6$  cells  $\pm$  SD from 3 independent experiments. **C**, Ramos cells were treated with  $2 \mu\text{g/mL}$  anti-CD40 antibody for various times. PKA activity was measured as described in the "Methods" section. Results show the average level of PKA activation (pmol/min per  $10^6$  cells  $\pm$  SD) from 3 independent experiments.

lar cAMP levels were measured after treatment of Ramos cells with  $2 \mu\text{g/mL}$  anti-CD40 antibody, a concentration of antibody fully effective in rescuing Ramos cells from apoptosis,<sup>9</sup> and the basal levels of cAMP were virtually unchanged 30 minutes after treatment (Fig 2, B). PKA activation was similarly not detected after treatment with anti-CD40 antibody (Fig 2, C). In addition, anti-CD40 antibody treatment still rescued the cells from apoptosis in the presence of  $10 \mu\text{mol/L}$  H89 (data not shown). These findings indicate that the cAMP pathway is parallel and largely independent of CD40-mediated rescue of B cells from apoptosis.

#### cAMP analogs do not modify $\text{Ca}^{2+}$ mobilization, ERK, or Ras activation by anti-IgM antibody, and ERK2 activation by anti-IgM is not necessary for apoptosis

Antigen receptor ligation stimulates a rapid increase in intracellular  $\text{Ca}^{2+}$  concentrations, as well as the activation of Ras and ERK2.<sup>8,9</sup> In data not shown, anti-IgM antibody treatment ( $10 \mu\text{g/mL}$ ) rapidly induced increases in intracellular  $\text{Ca}^{2+}$  and the activation of Ras and ERK2. In the presence of 2 mmol/L dbcAMP, these responses were unchanged, which is consistent with the

effect of PKA being largely independent of Ras and ERK regulation.

### **ERK2 activation by anti-IgM antibody is not necessary for inducing apoptosis**

To determine whether ERK activation is associated with the induction of apoptosis after anti-IgM cross-linking, we measured ERK activation in the presence of dbcAMP and also receptor-induced apoptosis in the presence of compound PD 98059, a specific MEK inhibitor, which is known to inhibit ERK activation.<sup>18</sup> ERK is rapidly activated after treatment with anti-IgM antibody, reaching peak levels at 20 minutes and then decreasing to basal levels within 60 minutes in the Ramos cells<sup>9</sup>; the major form of ERK in Ramos cells is ERK2. When Ramos cells were treated with various concentrations of dbcAMP for 15 minutes before the addition of 10  $\mu$ g/mL anti-IgM antibody and then lysed and assayed for ERK2 activity, it was observed that ERK2 activation was not affected by the presence of dbcAMP (Fig 3, A).

In Ramos cells pretreatment with 50  $\mu$ mol/L of PD 98059 for 1 hour significantly inhibited ERK2 activation by anti-IgM antibody (Fig 3, B). The percentage inhibition of ERK2 activation by anti-IgM in the presence of 4, 10, or 50  $\mu$ mol/L of the inhibitor was 55%, 75%, and 78%, respectively. The basal kinase activity of ERK2 slightly increased in the presence of 0.1% dimethyl sulfoxide (DMSO) as a vehicle; however, ERK2 activation by anti-IgM antibody was not affected in the presence of 0.1% DMSO (data not shown). In the presence or absence of the MEK inhibitor, apoptotic nuclei 48 hours after treatment with anti-IgM antibody was observed. Differences were not significant in 6 independent experiments (Fig 3, C). These data indicate that surface IgM-mediated apoptosis likely does not require ERK2 activation; moreover, the protective effect of dbcAMP PKA activation also appeared independent of ERK2 activation.

### **cAMP does not affect the JNK activation pathway**

We previously reported that treatment of B cells with anti-CD40 antibody and soluble gp39-activated JNK in Ramos cells.<sup>9</sup> JNK activation has been proposed as providing both death and rescue signals.<sup>12,19</sup> JNK activation by anti-CD40 antibody was not affected in the presence of dbcAMP. Furthermore, dbcAMP failed to activate JNK and JNK activation by anti-CD40 antibody was not affected in the presence of H89 (data not shown). These findings demonstrate that the regulation of the PKA and JNK pathways are independent of one another in Ramos cells; the rescue from apoptosis by PKA activation therefore appears independent of JNK.

### **cAMP and anti-CD40 inhibit activation of CPP32**

IL-1 $\beta$ -converting enzyme cysteine proteases or caspases appear to be required for apoptosis to proceed in a normal fashion.<sup>20</sup> One of the cysteine proteases, CPP32, is highly expressed in B-lymphoblastoid cell lines.<sup>21</sup>

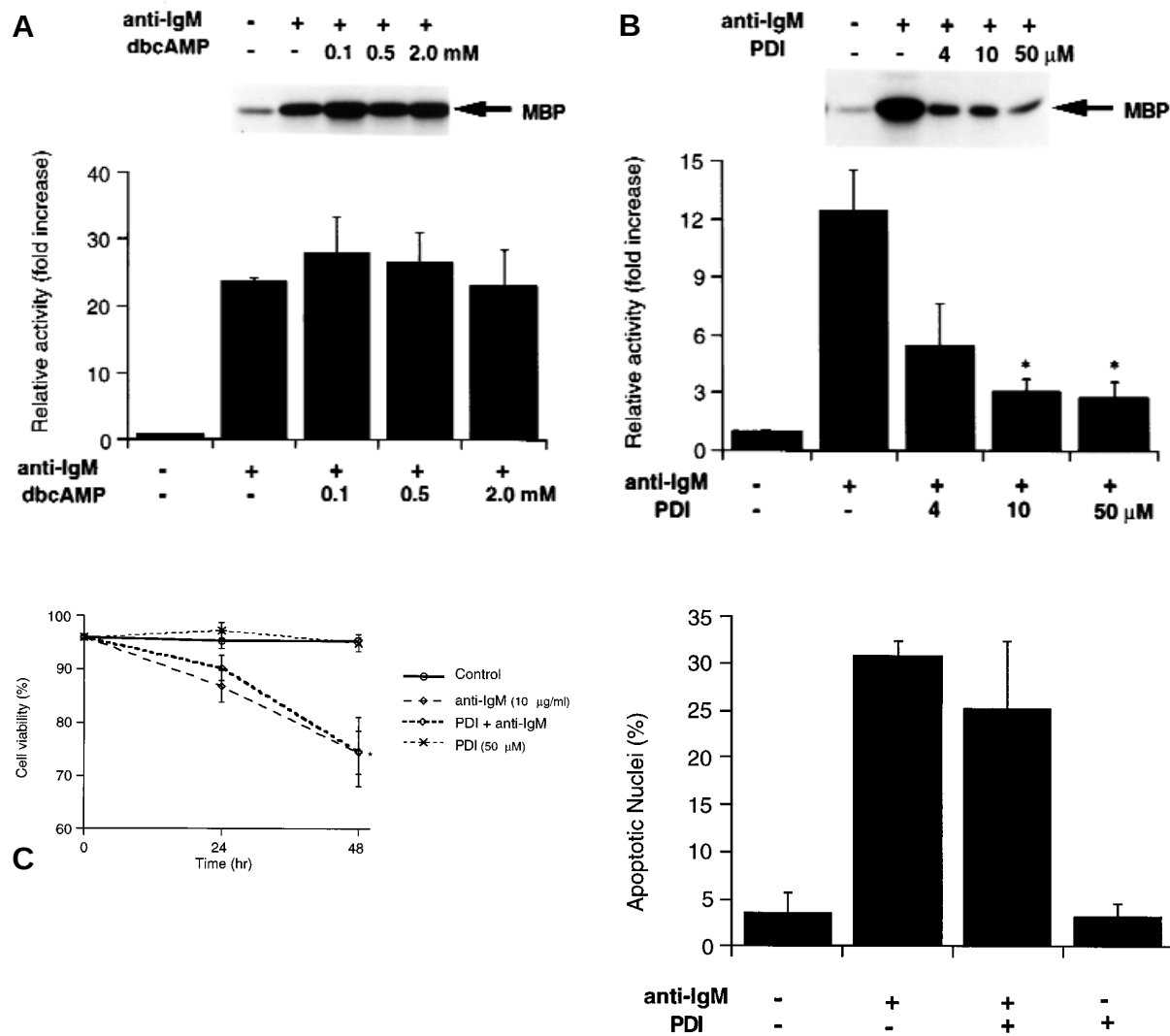
Addition of anti-IgM induced a 20-fold increase, compared with untreated samples, in CPP32 protease activity assayed by monitoring a fluorogenic substrate selective for CPP32 (or CPP32-like activity; Fig 4). Addition of increasing concentrations of dbcAMP resulted in a progressive inhibition of anti-IgM-induced CPP32 levels; in the presence of 2 mmol/L dbcAMP levels of anti-IgM-induced CPP32 were reduced by more than 60%. The progressive decrease in CPP32 activity paralleled the ability of these concentrations of dbcAMP to rescue the cells from apoptosis (Fig 1). Anti-CD40 antibody also suppressed CPP32 activity in keeping with its ability to rescue the cells from apoptosis.<sup>9</sup> Thus PKA activation and ligation of CD40 both suppress caspase activation in response to IgM cross-linking.

### **Expression of Bcl-2 protein is enhanced in the presence of rescue agents**

Several reports demonstrate that Bcl family proteins, including Bcl-2 or Bcl-xL, have antiapoptotic functions in B cells.<sup>22-24</sup> Twenty-four hours after treatment of the cells, low levels of Bcl-2 protein were detected in the untreated cells or cells treated with dbcAMP or anti-CD40 antibody (Fig 5). The expression of Bcl-2 protein increased in the cells treated with anti-IgM antibody, a proapoptotic stimulus. Exposure of cells to dbcAMP or anti-CD40 antibody in the presence of anti-IgM antibody further increased the expression of Bcl-2 protein levels relative to that observed for cells treated with anti-IgM antibody alone (Fig 5). Similar results were observed in 3 independent experiments in which a 2.5- to 3-fold increase in Bcl-2 levels was quantitated by densitometry with anti-IgM costimulation with either dbcAMP or anti-CD40 antibody. These findings suggest a compensatory Bcl-2 induction during treatment of B cells with anti-IgM antibody that could serve as a protective response. The induction of Bcl-2 in response to anti-IgM is potentiated by either PKA activation or anti-CD40 antibody, neither of which alone measurably increase Bcl-2 expression. Thus costimulation of PKA or CD40 with anti-IgM potentiates the expression of an antiapoptotic regulatory protein, Bcl-2.

## **DISCUSSION**

In this study we demonstrate that cell-permeable cAMP analogs rescue B cells from apoptosis after surface antigen receptor ligation. Alterations in cytosolic cAMP levels have numerous effects in different cells. One major function involves the regulation of the transcriptional activation of genes that contain a corticosteroid response element in the promoter region.<sup>25</sup> cAMP exhibits a dual role on cell proliferation; in some cell types it is mitogenic, promoting G1- to S-phase transition in the cell cycle.<sup>26</sup> In other cell types it is inhibitory, arresting cells in the G1 phase.<sup>27</sup> In the Ramos B-lymphoblastoid cell line, dbcAMP at concentrations up to 2 mmol/L did not affect cell proliferation, cell cycle kinetics, or cell viability. Importantly, we observed that



**D** Figure 3; Sakata N et al.

**FIG 3.** ERK2 activation is not inhibited by dbcAMP and is not required for apoptosis. **A**, Ramos cells ( $3 \times 10^6$  cells/sample at a concentration of  $10^6$ /mL) were treated with 10  $\mu$ g/mL anti-IgM antibody alone or treated with 0.1, 0.5, or 2.0 mmol/L dbcAMP for 15 minutes before the addition of 10  $\mu$ g/mL anti-IgM antibody. ERK2 activity was measured 20 minutes after addition of the antibody as described in the "Methods" section.  $^{32}$ P incorporation into the substrate (MBP) was measured by autoradiography (upper panel). MBP was excised, and the level of  $^{32}$ P incorporation from 3 independent experiments was measured by liquid scintillation counting. Results show the relative activity (mean  $\pm$  SE) compared with the basal activity from untreated samples (lower panel). The basal activity was  $1.46 \pm 0.75$  mU/ $10^6$  cells (mean  $\pm$  SE) from 3 independent experiments. Statistically significant differences are identified by an asterisk ( $P < .05$ ). **B**, Ramos cells ( $3 \times 10^6$  cells/sample at a concentration of  $10^6$ /mL) were treated with 10  $\mu$ g/mL anti-IgM antibody alone or treated with 4, 10, or 50  $\mu$ mol/L PD 98059 (PDI) for 1 hour before the addition of 10  $\mu$ g/mL anti-IgM antibody in a 24-well culture plate. All samples contained the same concentration of DMSO (0.1%) as a vehicle.  $^{32}$ P incorporation into the substrate (MBP) was measured 20 minutes after addition of the antibody by autoradiography (upper panel). MBP was excised and the level of  $^{32}$ P incorporation from 3 independent experiments was measured by liquid scintillation counting. Results show the relative activity (mean  $\pm$  SE) compared with the basal activity from untreated samples. The basal activity of ERK2 was  $2.36 \pm 0.38$  mU/ $10^6$  cells (mean  $\pm$  SE) in 3 independent experiments. The basal activity slightly increased in the presence of 0.1% DMSO; however, the response of ERK2 activation after treatment with anti-IgM antibody was not affected by 0.1% DMSO. Statistically significant differences are identified by an asterisk ( $P < .05$ ). **C** and **D**, Ramos cells (at a concentration of  $5 \times 10^5$  cells/mL) were treated with 10  $\mu$ g/mL anti-IgM antibody alone, 50  $\mu$ mol/L PDI alone, or treated with 50  $\mu$ mol/L PDI for 1 hour before the addition of 10  $\mu$ g/mL anti-IgM antibody in a 24-well culture plate. After 48 hours, cell viability and the percentage of hypodiploid nuclei were evaluated as described in the "Methods" section. Data represent mean  $\pm$  SD from 6 independent experiments. Statistically significant differences were analyzed as described in the "Methods" section.

## Suppression of Caspase Activation

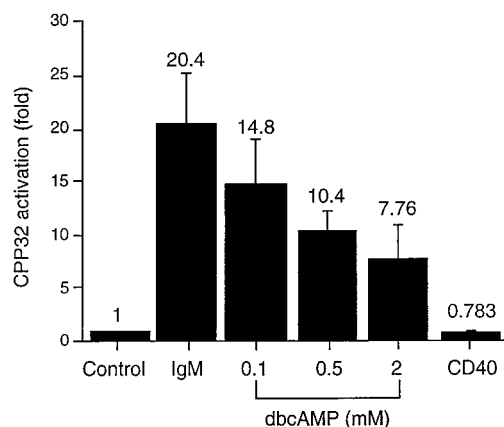


**FIG 4.** Anti-IgM-induced CPP32 activation is suppressed by dbcAMP and anti-CD40 antibody. Cells were treated with increasing concentrations of dbcAMP or 1  $\mu$ g/mL anti-CD40 for 30 minutes before the addition of 10  $\mu$ g/mL anti-IgM antibody. After 24 hours, CPP32 activity was determined as described in the "Methods" section. The activity is shown as fold activation compared with control samples. Data are means  $\pm$  SE from 3 independent experiments.

dbcAMP at concentrations up to 2 mmol/L had demonstrable protective effects against IgM-induced apoptosis in tonsillar B cells. The role of PKA regulation of apoptosis is therefore physiologically relevant in primary human B cells.

The effect of reagents that increase cytosolic cAMP levels was the prevention of apoptosis mediated through the B-cell antigen receptor. These effects were dose-dependent, similar to what has been observed in some T-cell hybridomas and T lymphoblasts.<sup>28</sup> They differ from what has been reported in resting B cells and thymocytes, where apoptosis may have been enhanced by elevation of cAMP levels.<sup>13,29,30</sup> The state of lymphocyte differentiation or activation may be a major determinant if cAMP promotes survival or death. Modulation of cAMP levels in certain cell types, such as germinal center B cells, indicates that PKA regulates apoptosis.<sup>14</sup> The relevance of the cAMP pathway in regulating B-cell apoptosis was further demonstrated by the action of an adenosine A2 agonist. Adenosine is proposed to play a role in protecting organs under stress, such as ischemia. Adenosine is a major factor suppressing inflammation in myocardial ischemia<sup>16</sup> and reduces the oxygen burst and cytokine production, such as TNF- $\alpha$  in neutrophils.<sup>31</sup> In leukocytes and Ramos cells the adenosine A2 receptor is predominant, and addition of adenosine increases cAMP synthesis after activation of the receptor. The increase in cAMP induced by the adenosine analog parallels the rescue of Ramos cells from apoptosis induced by anti-IgM antibody. Thus adenosine is predicted to physiologically regulate B-lymphocyte survival through the cAMP pathway.

Our findings demonstrate that PKA does not alter several signaling responses stimulated by ligation of surface IgM, including Ras and ERK activation and Ca<sup>2+</sup> mobi-

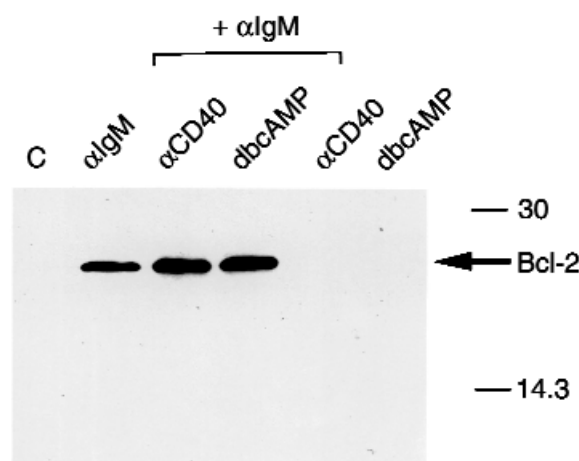


**FIG 5.** Bcl-2 protein levels are increased after treatment with dbcAMP and anti-CD40 antibody. Ramos cells (at a concentration of  $5 \times 10^5$  cells/mL) were treated with 10  $\mu$ g/mL antibody alone or 2 mmol/L dbcAMP or 1  $\mu$ g/mL anti-CD40 antibody in the presence or absence of 10  $\mu$ g/mL anti-IgM antibody for 24 hours. Cell lysates were prepared as described in the "Methods" section. Twenty micrograms of lysates were resolved by 15% SDS-PAGE and transferred to a nitrocellulose membrane. Bcl-2 protein was detected by the ECL system as described in the "Methods" section.

lization, and that anti-IgM induction of ERK2 is probably not required for apoptosis, although involvement of low levels of ERK2 (still detected in the presence of the MEK inhibitor) cannot completely be ruled out at the present time. These findings strongly suggest that it is the integration of multiple signals and not the stimulation or inhibition of a single pathway that commit the cell to apoptosis. Integration of signals rather than a single pathway committing cells to death is similar to the control of cell division involving a series of inputs and checkpoints.<sup>32</sup>

Interestingly, our findings demonstrate two receptor-controlled pathways, one involving cAMP-PKA and the adenosine A2 receptor and the other involving the CD40-stimulated pathway, that protect B cells from apoptosis. The adenosine A2 receptor and CD40-mediated protection responses involving the regulation of cytoplasmic signal transduction pathways seem to be parallel and largely independent. This indicates that the cAMP-PKA- and CD40-mediated survival responses are either through independent mechanisms or they converge downstream of these kinase pathways. A major convergence point is the regulation of cysteine protease activation after B-cell antigen receptor ligation, the commitment step to apoptosis. A role for cAMP-PKA inhibition of caspases has not been previously demonstrated.

The IL-1 $\beta$ -converting enzyme family of cysteine proteases are increasingly recognized as major effectors of apoptotic cell death. This family is comprised of several members, including CPP32. All of the family members are synthesized as proenzymes that are proteolytically processed to form active proteases, which lead to apoptosis by cleaving poly (ADP-ribose) polymerase, inhibiting DNA repair activity. CPP32 is highly expressed in Ramos and other B-lymphoblastoid cells,<sup>20</sup> and the addition of

anti-IgM induces a marked increase in CPP32 protease activity. As previously demonstrated for the processing of the proform of CPP32, anti-CD40 markedly suppressed CPP32 activity. Similarly, dbcAMP, in a dose-dependent fashion, also suppressed CPP32 activity in parallel to the rescue of B cells from anti-IgM-induced apoptosis.

A second potential convergence point in the rescue from apoptosis by cAMP and CD40 ligation may be increases in the levels of Bcl-2 protein. Both treatments increased the levels of Bcl-2 protein over that observed after treatment of the cells with anti-IgM alone. However, the role of Bcl-2 in protecting B cells from surface Ig induced apoptosis has been questioned.<sup>24,33</sup> Bcl-2 (and Bcl-xL) has been shown to interact with Bax to mediate apoptotic rescue.<sup>34,35</sup> Formation of Bcl-2-Bax heterodimers may effectively diminish the ability of Bax to induce cell death, perhaps by preventing Bax from forming homodimers that lead to apoptosis.<sup>35,36</sup> Overexpression of Bcl-2 will suppress caspase activation in response to proapoptotic signals in certain cell types.<sup>37</sup> Further studies are necessary to link increases in Bcl-2 levels and rescue of B-cell apoptosis by cAMP and CD40 ligation.

The significance of our findings is the definition of two receptor-regulated pathways that are capable of rescuing B cells from antigen receptor-mediated apoptosis. The CD40-regulated pathway involves protective responses that could possibly be mediated through the JNK/stress-activated protein kinase pathway, although this is not clear at the present time. The adenosine A2 receptor-protective pathway involves cAMP and PKA and is clearly independent of JNK regulation. Both pathways prevent the execution of the apoptotic program, likely by inhibiting the anti-IgM-induced activation of the cysteine protease CPP32 and cleavage of its substrates including poly (ADP-ribose) polymerase.<sup>15</sup> In addition, the increased levels of Bcl-2 resulting from co-stimulation of PKA or CD40 with anti-IgM could suppress the commitment to apoptosis. Relevant to human diseases, the cAMP-PKA rescue pathway is a strong candidate for a mechanism that allows the presence of normal B-cell numbers in human subjects who fail to express CD40 ligand and are thus incapable of providing a rescue signal by means of CD40.

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