Role of Phosphatidylinositol 3-Kinase in Anti-IgM- and Anti-IgD-Induced Apoptosis in B Cell Lymphomas

Gregory B. Carey and David W. Scott

*J Immunol* 2001; 166:1618-1626; doi: 10.4049/jimmunol.166.3.1618

http://www.jimmunol.org/content/166/3/1618

---

**References**  This article cites 60 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/166/3/1618.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Role of Phosphatidylinositol 3-Kinase in Anti-IgM- and Anti-IgD-Induced Apoptosis in B Cell Lymphomas\textsuperscript{1,2}

Gregory B. Carey and David W. Scott\textsuperscript{3}

Cross-linking of surface Ig receptors with anti-IgM (anti-\(\mu\) heavy chain, anti-\(\mu\)), but not anti-IgD (anti-\(\delta\) heavy chain, anti-\(\delta\)), Abs leads to growth arrest and apoptosis in several extensively characterized B cell lymphomas. By poorly understood mechanisms, both Abs transiently stimulate c-Myc protein expression. However, ultimately, only anti-\(\mu\) causes a severe loss in c-Myc and a large induction of p27\textsuperscript{Kip1} protein expression. Because phosphatidylinositol 3-kinase (PI3K) has been established as a major modulator of cellular growth and survival, we investigated its role in mediating anti-Ig-stimulated outcomes. Herein, we show that PI3K pathways regulate cell cycle progression and apoptosis in the ECH408 B cell lymphoma. Anti-\(\mu\) and anti-\(\delta\) driven c-Myc protein changes precisely follow their effects on the PI3K effector, p70\textsuperscript{S6K}. Upstream of p70\textsuperscript{S6K}, signaling through both Ig receptors depresses PI3K pathway phospholipids below control with time, which is followed by p27\textsuperscript{Kip1} induction. Conversely, anti-\(\delta\), but not anti-\(\mu\)-stimulated PI3K-dependent phospholipid return to control levels by 4–8 h. Abrogation of the PI3K pathway with specific inhibitors mimics anti-\(\delta\) action, potentiates anti-\(\mu\)-induced cell death and, importantly, converts anti-\(\delta\) to a death signal. Transfection with active PI3K kinase construct induces anti-\(\mu\) resistance, whereas transfection with dominant negative PI3K augments anti-\(\mu\) sensitivity. Our results show that prolonged disengagement of PI3K or down-regulation of its products by anti-\(\mu\) (and not anti-\(\delta\)) determines B cell fate. The Journal of Immunology, 2001, 166: 1618–1626.
time activation of a latent cellular apoptotic program induced in B cell lymphomas by IgD.

Materials and Methods

Cell lines and culture conditions

The murine B cell lymphoma line, ECH408, was derived from the CH3 clone transfected with a murine b-chain gene construct (2). The ECH408 line has been defined as functionally immature based on its sensitivity to anti-µ-induced growth arrest and apoptosis and its µb/µg/µh surface Ig expression profile (4, 25, 26). Some studies also used CH2 cells, which are µb/µg/µh and resistant to anti-µ-mediated growth arrest and apoptosis (27). The characteristics and functional behavior of all of the above cell lines have been extensively characterized (vide infra, Ref. 25, 26). All cell lines were maintained at 37°C in a humidified 7% CO2 atmosphere as previously described (7, 8, 25, 26) in supplemented RPMI 1640 with 5% FCS and 50 μg 2-ME (BioWhittaker, Walkersville, MD and Sigma, St. Louis, MO, respectively). Briefly, cells were maintained in exponential growth phase (0.1–0.6 × 106 per ml), and all experiments used single lot cells passaged <25 times to control for possible phenotypic drift. Cells were pretreated or cotreated with or without signaling inhibitors as specified in accompanying figure legends.

Reagents

PI3K inhibitors included LY294002 (LY, 2-4-morpholinyl-8-phenyl-4H-1-benzopyran-4-one) (28), used at 0–30 μM, and wortmannin (0–1 μM, Ref. 29). Wortmannin was obtained from Calbiochem-Novabiochem (San Diego, CA) and LY was obtained both from Calbiochem-Novabiochem and LICLabs (Woburn, MA). There were no differences in the effects of the products. Identical results were observed with either specific PI3K inhibitor (wortmannin or LY). LY was extensively used because of its greater stability. Rapamycin, a specific inhibitor of p70S6K activation (20) was obtained from Calbiochem-Novabiochem (San Diego, CA) and LY was obtained both from Calbiochem-Novabiochem and LICLabs (Woburn, MA). There were no differences in the effects of the products. Identical results were observed with either specific PI3K inhibitor (wortmannin or LY). LY was extensively used because of its greater stability.

Ab treatment of lymphoma lines

Maximal anti-µ-mediated growth arrest and apoptosis occurs at ~1 μg Ab per ml and between 20 and 32 h, depending on the cell line (see above). Maximal effects on c-Myc expression also occur with 1 μg/ml at appropriate times. The following Abs were purified by protein G affinity chromatography: B7.6, a monoclonal rat IgG1 anti-mouse µ-chain and Ja12.5, a monoclonal rat IgG1 anti-mouse δ-chain. All Ab batches were titrated for biologically effective doses and confirmed in the range of 0.01–3 μg/ml. Similar results were obtained with monoclonal and polyclonal Abs obtained from commercial sources. Typical FACS analysis used 0.5–1 × 106 cells and was performed essentially as described by Donjerkovic et al. (8, 30).

Gel electrophoresis and Western blotting

For Western blots, 3–5 × 106 cells were harvested and washed twice with cold PBS. Western blot analysis of cellular proteins was essentially as described by Donjerkovic et al. (8, 30), except that the Nonidet P-40 lysis buffer was slightly modified to include the following reagents at the indicated final concentrations: 100 mM NaCl, 10 mM β-glycerophosphate, 1 mM calcium A, 1 mM Na2VO3, and 1 mM DTT, all obtained from Sigma. Detergent-soluble proteins were prepared by incubation of the lysates on ice for 10 min, followed by centrifugation at 10,000 × g for 10 min at 4°C. Soluble protein concentrations in the resulting supernatants were measured by the bichinoninic acid protein assay kit (Pierce, Rockford, IL). Fifty micrograms (50 μg) cellular proteins were loaded per lane and separated by SDS-PAGE as previously described (7, 8), using 4–12% or 10% SDS-polyacrylamide gels (Novex, San Diego, CA) or 10% polyacrylamide gels after the method of Laemmli et al. (31). Resolved proteins were transferred either to nitrocellulose (Transblot; Bio-Rad, Richmond, CA) or polyvinylidene difluoride (Millipore, Bedford, MA) membranes by electroblotting in Tris/methanol/glycine/SDS buffer (8, 30). Filters were blocked for at least 1 h at room temperature with 2% BSA in TBS/Tween 20 (TBST). TBST included 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20. Specific proteins were probed with primary Abs for 2 h at room temperature or overnight at 4°C with gentle shaking. Primary Abs included rabbit polyclonal anti-p70S6K (C-18, catalog no. SC-230, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho Thr389-p70S6K (activation-specific) Ab (catalog no. 9205S at 1:500 dilution; New England Biolabs, Beverly, MA), polyclonal rabbit anti-mouse p27Kip (C-19, catalog no. SC-528, at 1:200 dilution; Santa Cruz Biotechnology), and rabbit anti-mouse c-Myc (rabbit polyclonal, catalog no. SC-764, at 1:200 dilution; Santa Cruz Biotechnology). Following probing with the primary Abs, the filters were washed five times for 5 min with 10 ml TBST, then blocked with fresh 2% BSA/TBST for at least 1 h. Finally, proteins were detected with secondary Ab: HRP-conjugated, polyclonal goat anti-rabbit IgG (Boehringer-Mannheim, Indianapolis, IN), which was added at room temperature at a 1:5000 dilution for 1–2 h. Excess secondary Ab was removed by three 5-min washes using TBST, followed by two 5-min washes using TBS. Secondary Abs were detected by chemiluminescence (Boehringer-Mannheim) according to kit specifications. Denitrographic analysis of specific bands on resulting exposed films was performed using Un-Scanit software (Silk Scientific, Orem, UT).

Transfections

For transient transfections, 2.5 × 106 exponentially growing ECH408 cells were harvested, washed, and resuspended in RPMI 1640 medium (without serum) in a final volume of 0.2 ml. The cell suspension was transferred to chilled (4°C) BTX model 640 electroporation cuvettes, which contained 10 μg PI3K pCDNA3 construct or pCDNA3 vector alone in 50 μl RPMI 1640. The cells were incubated with the DNA for 5 min at 4°C. The DNAs were transduced into the cells by electroporation at 250 V and 960 μF using the Gene Pulser (Bio-Rad). The murine cDNA/expression vector constructs encoding for p110α/p110δ and variants were provided by Dr. Astrid Brown, University of Texas, M.D. Anderson Cancer Center (Houston, TX). Transfections included 10 μg of the following: empty pCDNA3 cassette, p110α/p110β/p110γ/pcDNA3 (constitutively active myristoylated wild-type src-p110γ/pcDNA3 catalytic subunit, containing an N terminus myristoylation sequence: Refs. 32, 33) and Δp110α/p110β (a kinase dead mutant of the former), both containing C-terminal human Myc tags. After electroporation, the cells were transferred to 10 ml RPMI 1640 medium containing 10% FBS and were allowed to recover for 24 h. Successfully transfected cells were diluted into ‘selection medium’ (culture medium containing 0.6 mg/ml G418, Life Technologies, Rockville, MD). After the first 48 h in selection medium, live cells were purified using Lymphocyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) following standard protocols and, the selection medium was changed every 48 h. Selected cells were then tested for anti-µ and anti-δ responses.

Phospholipid analysis

One hundred milliliters of exponentially growing ECH408 cells, initially at 0.1 × 106 per ml were metabolically labeled for 48 h in the presence of 2.5 μCi/ml [32P]PO4, essentially as described by other investigators (34). The labeled cells, which achieved a final, linear density of 0.7 × 106 per ml, were transferred into fresh medium supplemented with 2.5 μCi/ml [32P]PO4 and separated into 16 175-cm2 flasks. The cells were then treated with either monoclonal anti-µ or anti-δ Abs for 0, 10, 20, 30, 60, 120, 240, and 480 min, respectively. Cells were harvested (7 × 106 cells) and, at appropriate times, excess [32P]PO4 was removed by three washes with 4°C RPMI 1640. Excess medium was quantitatively removed by aspiration. The resulting cell pellets were resuspended in 100 μl × PBS, pH 7.4, and extracted with 500 μl chloroform/methanol, after gentle vortexing. Cell extracts were centrifuged at 2000 × g for 10 min at room temperature, and 200 μl of the lower phase were removed and transferred to fresh tubes. Ten microliters of this organic phase extract were added to Merck TLC 60 plates (Sigma-Aldrich), and phospholipids were analyzed according to the method of Whitman et al. (35–36). Radiolabeled phospholipids were visualized by autoradiography and identified by comparison to simultaneously run cold standards (12) by standard L, vapor exposure. The fraction of PI3K-dependent phospholipids was determined by pretreating cells with 2 h with 0.1 or 1 μM wortmannin, followed by anti-Ig stimulation. By this method, we found that 25.4 ± 3.2% of the phospholipids analyzed at the appropriate position were wortmannin sensitive (data not shown).

Examination of PI3K-dependent phospholipids was chosen to be the best method to examine the contribution of PI3K. Fruman, Myers, and Cantley recently published that PI3K can be inhibited by Nonidet P-40 (37). This raised concerns for a Nonidet P-40 lysis/immunoprecipitation followed by a kinase assay. Our initial experiments also revealed that p85 protein expression did not change in response to anti-µ and anti-δ (p110 Abs unavailable at that time). Hence, immunoprecipitation of constant amounts of free p85/p110 complex also could have resulted in the measurement of constant enzymatic activity following anti-Ig stimulation. Lastly, if there were a biological effect of another cellular protein on PI3K metabolites, and not the kinase itself, the lipid labeling method and subsequent TLC/radiography or TLC/scintillation counting would be the best
method for determining the fate of those products. Based on these considerations, ¹⁴C]PJ0 labeling of cellular lipids would provide the most information.

Cell cycle analysis

Cells treated with appropriate Abs were washed with cold PBS and fixed in 70% ice-cold ethanol for at least 4 h at 4°C. For analysis, the cells were washed with cold PBS, resuspended in 0.5 ml PBS containing 1 µg/ml RNAase (Sigma), and incubated for 15 min at 37°C. Propidium iodide (PI, Sigma) was added to a final concentration of 50 µg/ml, and the cell cycle was assessed with a FACS caliber flow cytometer (Becton Dickinson, San Diego, CA) using standard protocols. The resulting data was analyzed using CellQuest software (Becton Dickinson).

[^H]Thymidine cell proliferation assay

A total of 2 x 10⁴ cells in a final volume of 0.2 ml were plated onto 96-well microtiter plates (Costar, Cambridge, MA) and pretreated with or without various inhibitors and/or specified Abs for the times indicated in accompanying legends for 24 h. The cells were then pulse labeled with a final concentration of 2.5 µCi/ml [³H]deoxythymidine (New England Nuclear, Boston, MA) for the final 4 h of the incubation. The cells were harvested onto Packard, self-aligning glass-fiber filters using a Packard Filtermate 196 cell harvester, following standard protocols. The filters were dried, and radiolabel incorporation was assessed by the Packard Matrix 9600 Direct Beta Counter. Data were processed and analyzed using Microsoft Excel software.

Results

Anti-Ig-mediated regulation of c-Myc and p27⁰⁵⁴¹ protein expression correlates with differential effects on growth arrest and apoptosis

We have used a well established model in which anti-IgM (anti-µ) treatment of murine B cell lymphomas leads to growth arrest and, later, apoptosis (17). In contrast to anti-µ, anti-IgD (anti-δ) treatment causes neither growth arrest nor apoptosis in the ECH408, as well as other surface IgD (slgD) expressing lines (2, 3, 17, 25). The results presented in Table I establish the kinetics of these effects in slgM⁰⁴¹/⁺/slgD⁰⁴¹ ECH408 cells and confirm the results of Tisch et al. (3) in IgD-transfected WEHI-231 cells. Moreover, we and others have shown that anti-µ causes a transient rise, followed by a dramatic loss in c-Myc protein and message, with a subsequent rise in p27⁰⁵⁴¹ protein expression in other B cell lymphoma lines (7, 8, 38, 39). Samples of the cells used to generate the data in Table I were used to precisely establish the kinetics of p27⁰⁵⁴¹ and c-Myc protein expression and their relationship to growth arrest and apoptosis. The ECH408 cells were treated with or without anti-Ig for the indicated times and processed for Western blot analysis. The results (Fig. 1, kinetics Fig. 1A, and corresponding Western blot, Fig. 1B) confirm those obtained from IgD-transfected WEHI-231 by Tisch et al. (3). As seen in the WEHI-231 system, BCR cross-linking with both anti-µ and anti-δ also results in a transient increase followed by a decrease in c-Myc protein expression in ECH408 cells. However, anti-δ-modulated c-Myc protein expression returns to baseline over 8–24 h (Fig. 1).

Table I. Kinetics of growth arrest and apoptosis

<table>
<thead>
<tr>
<th>Stimulation Time (h)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₀/G₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-δ</td>
<td>44%</td>
<td>44%</td>
<td>51%</td>
<td>54%</td>
<td>48%</td>
</tr>
<tr>
<td>Anti-µ</td>
<td>43%</td>
<td>42%</td>
<td>54%</td>
<td>68%</td>
<td>62%</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-δ</td>
<td>4.7%</td>
<td>7.7%</td>
<td>7.3%</td>
<td>7.1%</td>
<td>7.5%</td>
</tr>
<tr>
<td>Anti-µ</td>
<td>5.6%</td>
<td>7.3%</td>
<td>6.0%</td>
<td>13.5%</td>
<td>26.0%</td>
</tr>
</tbody>
</table>

^aSee Fig. 1.

Anti-µ also strongly induces p27⁰⁵⁴¹ protein expression in ECH408 cells, peaking at greater than 20-fold over control, and falling to ~10-fold over control by 24 h (Fig. 2, kinetics Fig. 2A, and corresponding Western blot, Fig. 2B). In contrast, anti-δ slowly and weakly induces p27⁰⁵⁴¹ expression, which returns to control levels by 24 h. Coordinated down-regulation of c-Myc and up-regulation of p27⁰⁵⁴¹ precedes growth arrest and apoptosis (Table I, Figs. 1 and 2), as has been shown in other cell lines by Donjerkovic et al. (8, 30). Anti-δ-treated cells continue to cycle and proliferate normally, as determined by FACS analysis and [³H]deoxythymidine incorporation assay (data not shown). Together, the results presented in Figs. 1 and 2 underscore that joint down-regulation of c-Myc and up-regulation of p27⁰⁵⁴¹ are requisites for Ig-mediated growth arrest (and subsequent apoptosis), confirming and extending previous data (5, 7, 8, 40).

Anti-µ and anti-δ differentially modulate p70⁶⁰⁶K

The question of how cell cycle-regulated or specific mRNAs are ‘found’ among the other hundreds, if not thousands, of other messages has been recently addressed (reviewed by Dufner and Thomas, Ref. 41). The Thomas group has established that accelerated protein synthesis depends on the phosphorylation (activation) state of the 29-kDa ribosomal protein S6 (20, 42), which includes early growth response genes, cyclins, ribosomal elongation factors, and c-Myc (above; 43). Although c-Myc mRNA levels parallel and precede the changes in c-Myc protein expression in anti-Ig-treated B cell lines (5, 40), the kinetics suggest that another mechanism(s) may account for the rapid loss in expression of that protein. Therefore, we examined whether anti-µ and anti-δ differentially affected the phosphorylation (activation) state of p70⁶⁰⁶K and whether this activation paralleled the more
The percentage of activation is plotted against time. For anti-Ig effect on growth arrest and apoptosis in ECH408 B cell lymphomas. ECH408 cells were treated as described above, and p27Kip1 protein expression was examined by Western blot analysis. A, Densitometric analysis of anti-µ- or anti-δ (1 µg/ml)-modulated p27Kip1 protein expression. B, Western blot of p27Kip1 from which A was derived. These results are representative of multiple, independent experiments.

rapid changes in c-Myc protein expression observed. Using anti-phospho p70S6K, which detects phosphorylated Thr389, which is critical for p70S6K activation (p70S6K—pp70S6K; see Ref. 44), we found that both Igs mediate a dramatic decrease in pp70S6K levels (Fig. 3) but do not change p70S6K protein expression (data not shown). Similar to patterns with c-Myc protein expression (Fig. 1), anti-µ-modulated pp70S6K remains below baseline, whereas anti-δ-modulated pp70S6K only transiently falls below baseline, then recovers by 4–8 h (Fig. 3). Notably, this differential effect of the two Igs on pp70S6K parallels their effect on c-Myc protein expression (Fig. 1).

Anti-IgM and anti-IgD differentially modulate the PI3K pathway
PI3K-generated inositol bisphosphates are necessary for activation of phosphatidylinositol-dependent kinases 1 and 2 (PDK1&2), which lie upstream of p70S6K and protein kinase B activation (45). Based on the differential effects of anti-µ and anti-δ on pp70S6K, through measuring PI3K-dependent phospholipids, we investigated whether activation of the respective receptors also differentially modulated PI3K. Cells were metabolically labeled and processed as described in Materials and Methods. Using wortmannin, a specific, irreversible inhibitor of PI3K (29), we assessed the fraction of inorganic [32P]O4−labeled phospholipids contributed by PI3K and found that 25.4 ± 3.2% of the phospholipids analyzed were wortmannin-sensitive (see rationale in Materials and Methods). The kinetics of anti-Ig modulation of wortmannin-sensitive phospholipids are presented in Fig. 4. These results show that anti-µ and anti-δ both caused a decrease in PI3K-dependent phospholipids (i.e., PI3K activity), which was indeed similar to, and paralleled their effects on p70S6K activation (pp70S6K, Fig. 3). Interestingly, loss of PI3K-dependent phospholipids also parallels and antecedes p70S6K inactivation and c-Myc loss (Fig. 1). Beckwith et al. (46) demonstrated stimulation of PI3K activity in human B-lymphoma cells by anti-µ or anti-δ and concluded that stimulation of that activity was required for IgM-mediated growth arrest. By examining longer time points under the experimental conditions used, we found a decrease in anti-Ig-modulated PI3K phospholipids (Fig. 4). Throughout the first 30 min, the kinetic patterns for modulation of PI3K by both Igs were superimposable. Importantly, when extended to 8 h, when hallmark changes in c-Myc and p27Kip1 and changes in cell-cycle profiles become pronounced, PI3K products return to baseline in anti-δ- but not anti-µ-treated

FIGURE 2. Up-regulation of p27Kip1 protein expression correlates with anti-Ig effect on growth arrest and apoptosis in ECH408 B cell lymphomas. ECH408 cells were treated as described above, and p27Kip1 protein expression was examined by Western blot analysis. A, Densitometric analysis of anti-µ- or anti-δ (1 µg/ml)-modulated p27Kip1 protein expression. B, Western blot of p27Kip1 from which A was derived. These results are representative of multiple, independent experiments.

FIGURE 3. Anti-µ and anti-δ differentially modulate p70S6K activation. ECH408 cells were treated as described above with either anti-µ or anti-δ (1 µg/ml) for up to 24 h as indicated. Cell extracts were processed for Western blot analysis. Filters were then probed overnight with anti-phospho Thr389-specific (activation-specific) pp70S6K Ab and developed. Densitometric analysis was performed on exposed films, and the extent of S6 kinase activation was determined by the formula 100% × [(experimental pixel density − background)/control pixel density − background)]. The percentage of activation is plotted against time. For anti-δ and anti-µ ‘zero time’ controls, these values are 112.71 and 72.41 units, respectively (A). Data shown are representative of at least three separate, independent experiments. B, p70S6K activation as a function of the intensity of a non-specific (NS) band from the same film.

FIGURE 4. Anti-µ and anti-δ differentially modulate PI3K-dependent phospholipids. Exponentially growing ECH408 cells were transferred to fresh medium and cultured for 48 h in the presence of [32P]PO4. Cells were washed and extracted, and thin layer chromatography was run as described in Materials and Methods. PI3K-dependent phospholipids are defined as those phospholipids that are wortmannin sensitive. Software analysis of autoradiograms was performed; spot intensity is represented as densitometric units (DU) as a function of time.
ECH408 cells (Fig. 4). The resultant curves also suggest that sIgM and sIgD might trigger a signal that down-regulates PI3K or its products, which persists in the presence of anti-μ, but is aborted in the presence of anti-δ.

We next examined the notion that if prolonged suppression of the PI3K/pp70^S6K signaling module was required for down-regulation of c-Myc and up-regulation of p27^Kip1, then specific pharmacological inhibitors of PI3K and pp70^S6K should mimic the effect of anti-μ on those proteins.

**Effect of PI3K and pp70^S6K activation inhibitors on c-Myc and p27^Kip1 protein expression**

The results presented above suggested an important role of the PI3K pathway in regulating B cell growth and/or survival. If prolonged suppression of the PI3K/pp70^S6K signaling module were required for down-regulation of c-Myc and up-regulation of p27^Kip1, then specific pharmacological inhibitors of PI3K and pp70^S6K should be mimetics of anti-μ with respect to c-Myc and p27^Kip1 protein expression. To answer this question, we used two specific PI3K inhibitors, LY and wortmannin (vide supra Refs. 28, 29, respectively). Additional studies also used the well characterized inhibitor of pp70^S6K activation, rapamycin (20). The effects of LY and wortmannin were similar, although the latter was 30–70 times more potent than LY (as reported in literature and our data not shown). Because of its greater stability, LY was used more extensively than wortmannin. LY negligibly affects PI4K and other protein kinases (28), and data from intact cells indicate that LY concentrations up to 20 μM and wortmannin concentrations up to 0.2 μM specifically inhibit the PI3K/PKB signaling module (44, 47). Therefore, 2.5–20 μM LY was used to block the PI3K pathway in our system. The results presented in Fig. 5A demonstrate that treatment with LY alone induces p27^Kip1 and decreases c-Myc, which is concomitant with growth arrest and later, apoptosis, in ECH408 (also in CH12, CH31, and WEHI-231; data not shown). We also found these effects to be time- and concentration-dependent (data not shown). The effect of LY on p27^Kip1 presented here is similar to data obtained from melanoma cells (47).

Using suboptimal concentrations of LY and anti-μ, we also observed potentiation of the anti-μ signal, i.e., greater induction of p27^Kip1, greater loss of c-Myc, and greater extent of growth arrest and/or apoptosis (Fig. 5A, also in CH31 and WEHI231, data not shown). The coordinate modulation of c-Myc and p27^Kip1 and growth arrest by LY suggests that down-regulation of PI3K is sufficient, and required for anti-μ-induced growth arrest and subsequent apoptosis. The data clearly show that both anti-δ and anti-μ synergize with LY to further decrease c-Myc and to increase p27^Kip1 protein expression (Fig. 5, A and B). We also observe anti-δ-stimulated cleavage of the proapoptotic marker, poly(ADP-ribose) polymerase (PARP; reviewed by D’Amours et al., Ref. 48) in the presence of 5–10 μM LY (data not shown). Together, these results further suggest that loss of PI3K protection facilitates proapoptotic signaling from the sIgD receptor. We also found that both LY (Fig. 5A) and rapamycin (Fig. 5C) reduced c-Myc protein expression and synergized with anti-μ to induce p27^Kip1 expression in ECH408 and several other B cell lines (G.B.C. and D.W.S., manuscript in preparation). Together, these results strongly support that reduced c-Myc and induced p27^Kip1 expression via down-regulation of the PI3K/p70^S6K signaling module regulates BCR-induced growth arrest and apoptosis in B cell lymphomas.

**Anti-IgD induces growth arrest and apoptosis in the presence of PI3K inhibitor, LY**

If PI3K disengagement were the limiting step for anti-μ-induced growth arrest and apoptosis, then inhibition of PI3K alone should induce growth arrest and apoptosis and should potentiate the effects of anti-μ on the cell cycle. Therefore, FACS analysis was performed on samples of anti-μ-stimulated cells (±10 μM LY) used for Western blot analysis in Fig. 5. Indeed, PI3K inhibitor (LY) alone induces growth arrest and apoptosis (Fig. 6A), greatly potentiates the effect of suboptimal (0.1 μg/ml) anti-μ, and also enhances apoptosis induced by optimal (1 μg/ml) anti-μ.

The results presented above (Figs. 3 and 4) also suggested that the initial signals generated by either anti-μ or anti-δ are similar and, potentially, are both proapoptotic. However, the anti-δ-modulated signals in ECH408 cells diverge from the proapoptotic pathway and recover after 4–8 h. Therefore, we then asked if anti-μ-driven apoptosis was critically dependent on prolonged suppression of PI3K activity, then inhibition of that activity with LY should switch anti-δ to a death signal. FACS analysis of the cell cycle was performed on samples of anti-δ-stimulated cells (±10 μM LY) used for Western blot analysis in Fig. 5. The results (Fig. 6B), which are consistent with the results of Ales-Martinez et al. (2) and Tisch et al. (3), show that anti-δ alone does not affect the ECH408 cell cycle. However, in the presence of LY, anti-δ induces growth arrest and apoptosis (Fig. 6B). It is also important to note that PI3K blockade with LY induces growth arrest and apoptosis in the anti-μ-resistant CH12 line and partially sensitizes those cells to the effect(s) of anti-μ (data not shown, G.B.C. and D.W.S., manuscript in preparation). Together, the conversion of anti-δ signaling by LY, and the potentiation of the effect of suboptimal and optimal (1 μg/ml) anti-μ, suggests that disengagement of PI3K pathway-mediated survival signals is indeed the rate-determining step for anti-μ-mediated B cell growth arrest and apoptosis. Furthermore, these...
results also suggest that anti-δ and anti-μ stimulate a cellular death factor that is antagonized by an active PI3K pathway.

Effect of transfection of PI3K subunits on anti-μ and anti-δ effects in ECH408 cells

It has recently been shown that p85<sup>PI3K</sup>, the regulatory subunit of PI3K, is recruited to the BCR via SH2/SH3 interactions (49, 50). Furthermore, translocation of the p85:p110<sup>PI3K</sup> heterodimer to the plasma membrane provides the p110<sup>PI3K</sup> catalytic subunit access to lipid substrates at that level (29, 37). PI3K phosphorylates myo-inositol at the D3 position, and in concert with PI4- and PI5-kinases, generates Ins<sub>3,4</sub> and inositol 3,4,5 bis- and tris-phosphates, among other products (29). Inositol trisphosphate is critical for calcium fluxing, which is one of the first signals downstream of BCR cross-linking (51), whereas inositol 3,4 bisphosphate is required for the activation and/or translocation of PDKs, which lie upstream of PKB and pp70<sup>60K</sup> (45). Based on the differential effects of sIgM and sIgD receptor signaling on PI3K and pp70<sup>60K</sup> activation, we anticipated that overexpression of constitutively active p110<sup>PI3K</sup> (+p110<sup>ΔPI3K</sup>) should render ECH408 cells resistant to anti-μ-induced growth arrest and apoptosis. In contrast, overexpression of a kinase dead mutant (Δp110<sup>PI3K</sup>) should increase ECH408 sensitivity to anti-μ and possibly allow induction of growth arrest and apoptosis by anti-δ.

The results in Fig. 7 show that, indeed, transfection with constitutively active p110<sup>PI3K</sup> renders ECH408 cells resistant to anti-μ-induced growth inhibition measured at 24 h (Fig. 7A, open squares). Conversely, as predicted, transfection with kinase dead p110<sup>PI3K</sup> (Δp110<sup>PI3K</sup>) synergizes with anti-μ, resulting in a dramatic reduction in [H]<sup>3</sup>thymidine incorporation (open circles). This correlates with a greater than 2-fold increase in anti-μ-induced apoptosis (Fig. 7C), strongly suggesting that regulation of p110<sup>PI3K</sup> couples the IgM receptor to growth arrest and apoptosis. Although constitutively active p110<sup>PI3K</sup> blocked anti-μ-modulated growth inhibition (at 0.1 μg/ml anti-μ, Fig. 7A) at the time point for the apoptosis assay, the effects of vector control and +p110<sup>PI3K</sup> were similar (Fig. 7C). Additional experiments confirmed that active +p110<sup>PI3K</sup> cannot block, but significantly delays, anti-μ-induced apoptosis (data not shown) as it blocks inhibition of proliferation (Fig. 7A). At later time points massive (and less distinguishable) death is observed in vector-transfected (control) cells increased dramatically after >24 h, but was delayed by transfection of the +p110<sup>PI3K</sup> construct (data not shown).

![FIGURE 6](Image)

FIGURE 6. PI3K blockade synergizes with anti-μ to disrupt the cell cycle and converts anti-δ to a death signal. Samples of the cells used in Fig. 5, A and B, were processed for FACS analysis as described above. A, Effects of anti-μ (0.1 or 1 μg/ml) on ECH408 cell cycle in the presence or absence of 10 μM PI3K inhibitor, LY. B, Effects of anti-δ (0.1 or 1 μg/ml) on the cell cycle in the presence or absence of 10 μM LY.

![FIGURE 7](Image)

FIGURE 7. Transfection of PI3K subunits alters the anti-μ response in ECH408 cells. Highly viable ECH408 cells were transfected with empty pcDNA3 vector, pcDNA3/constitutively active p110<sup>PI3K</sup> (+p110<sup>ΔPI3K</sup>) or pcDNA3/kinase dead p110<sup>PI3K</sup> (Δp110<sup>PI3K</sup> -), as described in Materials and Methods. Stable transfectants were selected in G418-containing medium, and the cells were treated with or without varying concentrations of anti-μ or anti-δ for 24 h. A and B, Effects of anti-μ or anti-δ on [H]<sup>3</sup>thymidine incorporation in transfected ECH408 cells, respectively. cpm in controls: pcDNA3, 76,198 ± 4,564; +p110<sup>ΔPI3K</sup>, 66,572 ± 3,084; and Δp110<sup>ΔPI3K</sup>, 68,192 ± 3,975, respectively. C and D, Effect of varying anti-μ or anti-δ concentrations on apoptosis in transfected cells (as measured by FACS analysis). For C, % apoptosis in ‘0’ Ab controls were as follows: pcDNA3, 13.5; +p110<sup>ΔPI3K</sup>, 17.9; and Δp110<sup>ΔPI3K</sup>, 13.9. For D, % apoptosis in ‘0’ Ab controls were as follows: pcDNA3, 11.4; +p110<sup>ΔPI3K</sup>, 17.8; and Δp110<sup>ΔPI3K</sup>, 13.9. Note that anti-μ-mediated apoptosis in vector-transfected (control) cells increased dramatically after >24 h, but was delayed by transfection of the +p110<sup>ΔPI3K</sup> construct (data not shown).
Discussion

It is well established that anti-μ induces growth arrest and apoptosis in B cell lymphomas, which is always associated with down-regulation of c-Myc and increased p27\textsuperscript{Kip1} protein expression (Refs. 2–8, 39, 52, 53, and results herein). We and others have also established that cross-linking the sIgD receptor on immature B cell lymphomas 1) transiently modulates c-Myc protein expression; 2) ultimately does not result in a dramatic loss of c-Myc protein expression; and finally 4) does not lead to growth arrest and apoptosis. Although mechanisms of anti-μ signaling in B cell lines have been extensively studied, the role of the sIgD receptor is largely unknown (54). Furthermore, although the fundamental differences in these anti-μ vs anti-δ-stimulated outcomes have been known for some time, the intervening signals involved in eliciting the observed responses are unknown. Therefore, our objectives were 2-fold: firstly, to explore the mechanism of anti-μ-mediated regulation of c-Myc and p27\textsuperscript{Kip1}, and secondly, to examine the point(s) of divergence of anti-δ-modulated signals. The results clearly show that modulation of PI3K by the two Ig receptors accurately predicts death or survival outcomes in the ECH408 B cell lymphoma. Anti-μ stimulation appears to initially provide a pro-growth signal, as evidenced by an initial stimulation in c-Myc protein expression and p70\textsuperscript{S6K} activation (Figs. 1 and 5, respectively). However, the loss of PI3K product(s) (i.e., down-regulation of the PI3K pathway) appears to signal the conversion of the pro-growth anti-μ signal to a growth arresting and apoptotic one. Although the total phospholipid labeling method we used did not reveal an anti-Ig-mediated increase in PI3K products, Beckwith et al. (46) demonstrated that both anti-δ and anti-μ stimulate PI3K activity in a human B cell lymphoma in very early time courses in human B lymphomas. In extended time courses, we initially observe down-regulation of PI3K products in response to BCR cross-linking. This difference could either be due to differences in either cell types or experimental conditions. However, eventually, anti-μ uncouples the PI3K cellular survival program (Fig. 4) and growth arrest and apoptosis ensue (Table 1). Our data clearly show that continued down-regulation of PI3K, as well as one of its effectors, p70\textsuperscript{S6K}, lies upstream of growth arrest and apoptosis in B cell lymphoma lines. Furthermore, transfection with active or mutant PI3K constructs can predictably inhibit or synergize the anti-μ effect, providing molecular evidence to support this notion.

We also provide data that demonstrate how the anti-δ signal deviates from this pathway. The PI3K-regulated p70\textsuperscript{S6K} pathway phosphorylates and thereby activates the ribosomal translational complex, causing accelerated protein synthesis (19, 20, 41, 44). Activated S6 biases protein synthesis toward translation from mRNAs containing a polypyrimidine-rich 5′ untranslated region (Refs. 11, 21, 41, 43, and see introduction). Anti-μ and anti-δ differentially regulate pp70\textsuperscript{S6K} (and c-Myc) protein expression, which is directly related to their eventual effect on ECH408 cells (Figs. 1 and 3). Therefore, the results presented herein are in agreement with a growing body of evidence showing differential modulation of protein translation and ultimately protein expression of c-Myc and other mRNAs by the PI3K/p70\textsuperscript{S6K} pathway.

Treatment with either anti-μ or anti-δ does not significantly affect expression of the regulatory p85α PI3K subunit (data not shown), suggesting that regulation of this protein at the expression level is not responsible for the loss in PI3K products. We are currently examining whether expression of the p110\textsuperscript{π3K} catalytic subunit parallels the changes observed in PI3K products. These results will provide insight to the point(s) of regulation of PI3K.

We next tested whether constitutively active p110\textsuperscript{π3K} (+p110\textsuperscript{π3K}) would render ECH408 resistant to anti-μ-induced growth arrest and apoptosis, and whether transfection with a kinase dead mutant (Δp110\textsuperscript{π3K}) would increase anti-μ sensitivity in those cells. Indeed, transfection with well characterized PI3K constructs (32, 33) produced most of the predicted outcomes (Fig. 7) in that anti-μ sensitivity is enhanced in cells transfected with Δp110\textsuperscript{π3K}, whereas anti-μ sensitivity is reduced in cells transfected with the +p110\textsuperscript{π3K} construct.

Interestingly, anti-δ did not induce growth arrest and apoptosis in the cells transfected with the kinase dead PI3K mutant (Δp110\textsuperscript{π3K}, Fig. 7). This could suggest other factor(s), e.g., modulation of a negative regulator such as the dual specificity, inositol 3,4/3,5/3,4,5 phosphatase, called PTEN (55), might be more tightly associated with or recruited to the sIgM and not the sIgD signaling complex. In this scenario, failure of the activated sIgD receptor alone to recruit or induce a critical signaling component or adapter might still render it incapable of mediating apoptosis on its own.

Although the effects of LY are accepted to be highly specific for PI3K, there may also be unknown pharmacological effects of LY that interfere with either or both sIgM and/or sIgD signaling in B cell lymphomas. We propose that anti-δ alone cannot sustain or invoke the necessary elements required for death signaling. Future studies will reveal whether this is indeed the case.

It is important to note that pharmacological interference with PI3K in all tested anti-μ-sensitive B cell lymphomas leads to an increase in p27\textsuperscript{Kip1} and synergy with anti-μ (CH31 and WEHI-231 cell lines, data not shown). In fact, treatment with LY also leads to growth arrest and apoptosis in an anti-μ-resistant CH12 B cell lymphoma (data not shown). Interestingly, there was weaker synergy between LY and anti-μ in this cell line, compared with results with the ‘immature’ B cell lines. One explanation is that a disconnect or block exists between the sIgM receptor and the signals that lead to a down-regulation of the PI3K pathway in CH12. In contrast, anti-μ mimicry by LY in both anti-μ-sensitive and -resistant B cell lines supports that down-regulation of PI3K (and its sequelae) is sufficient for B cell growth arrest and apoptosis.

The results of many investigators, as well as our own results, support a model where eventual modulation of CDC25A, CDK activity, pRB, and E2F by c-Myc, is directly responsible for late G\textsubscript{1} arrest in anti-μ-stimulated B cell lymphomas (5, 40, 55, 56). The results presented herein establish PI3K as a proximal signal to both sIgD and sIgM receptors that directly modulates c-Myc and the cell cycle in the ECH408 cell line, further supporting this model.

A recent model of regulation of p27\textsuperscript{Kip1} protein expression has been established where CDK-mediated p27\textsuperscript{Kip1} phosphorylation results in its ubiquitination and targeting to the proteosomal degradation pathway (57, 58). In data obtained from T lymphocytes, Brennen et al. (24) established that IL-2-driven proliferation was due to loss of p27\textsuperscript{Kip1} protein expression, which was mediated by a PI3K/PKB/CDK cascade (24). Casagranda and coworkers also reported that LY induced p27\textsuperscript{Kip1} and inactivated CDK4 activity in choroidal melanoma cells (47). This group also demonstrated that LY treatment caused a loss in CDK4 protein expression as well as blocking serum stimulation of that protein. Interestingly, recent data (see below) suggest that anti-μ can lead to a down-regulation of CDK4 in ECH408 cells, thus providing an additional control mechanism for growth arrest.

Although the results above have been obtained from diverse cell lines, together they suggest that anti-μ-mediated suppression of PI3K could indeed turn off the p27\textsuperscript{Kip1} degradation pathway while concomitantly down-regulating c-Myc protein expression. Our future experiments will test these possibilities. Furthermore, CDK4
was recently shown to be a direct product of an activated c-Myc transcriptional complex (59). Indeed, using a newly developed technique for Rapid Analysis of Gene Expression (RAGE, 60), we have recently obtained data from both ECH408 and WEHI-231 cells (both anti-μ sensitive), which showed a loss in c-Myc expression, loss of CDC25A expression, and a reduction in CDK4 mRNA, and others, in response to anti-μ stimulation (G. B. Carey, I. Carey, and D. W. Scott, manuscript in preparation). Expression levels of the respective proteins reflected the changes in the respective mRNAs. Conceivably, a rapid drop in c-Myc protein expression could simultaneously result in both losses in CDC25A and CDK4 expression and activities, accumulation of p27Kip1, failure to inactivate pRb and late G₁ cell cycle arrest, as is observed. Interestingly, anti-μ weakly affected CDK4 expression in ECH408 cells (G.B.C. and D.W.S., unpublished observations). Hence, the duration of PI3K disengagement might be a critical trigger for a signaling system that crosses over for apoptosis distal from PI3K but proximal to the modulation of CDK4 activity and expression. Our present focus is the closer examination and rigorous testing of these possibilities.

Acknowledgments

We thank Dr. Astrid Eder (University of Texas, M. D. Anderson Cancer Center, Houston, TX) for providing the PI3K constructs and Drs. Achsah Keegan and Yufang Shi (Holland Laboratory of the American Red Cross) for a critical reading of this manuscript.

References


