Phosphatidylinositol 3-Kinase-Dependent Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase 1/2 and NF-κB Signaling Pathways Are Required for B Cell Antigen Receptor-Mediated Cyclin D2 Induction in Mature B Cells

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*J Immunol* 2004; 172:2753-2762; doi: 10.4049/jimmunol.172.5.2753

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Phosphatidylinositol 3-Kinase-Dependent Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase 1/2 and NF-κB Signaling Pathways Are Required for B Cell Antigen Receptor-Mediated Cyclin D2 Induction in Mature B Cells

Michael J. Piatelli,* Carrie Wardle,† Joseph Blois,‡ Cheryl Doughty,† Brian R. Schram,* Thomas L. Rothstein,* and Thomas C. Chiles‡‡

Phosphatidylinositol 3-kinase (PI-3K) has been linked to promitogenic responses in splenic B cells following B cell Ag receptor (BCR) cross-linking; however identification of the signaling intermediates that link PI-3K activity to the cell cycle remains incomplete. We show that cyclin D2 induction is blocked by the PI-3K inhibitors wortmannin and LY294002, which coincides with impaired BCR-mediated mitogen-activated protein/extracellular signal-related kinase kinase (MEK1/2 and p42/44ERK phosphorylation on activation residues. Cyclin D2 induction is virtually absent in B lymphocytes from mice deficient in the class IA PI-3K p85α regulatory subunit. In contrast to studies with PI-3K inhibitors, which inhibit all classes of PI-3Ks, the p85α regulatory subunit is not required for BCR-induced MEK1/2 and p42/44ERK phosphorylation, suggesting the contribution of another PI-3K family members in MEK1/2 and p42/44ERK activation. However, p85α−/− splenic B cells are defective in BCR-induced IκB kinase β and IκBα phosphorylation. We demonstrate that NF-κB signaling is required for cyclin D2 induction via the BCR in normal B cells, implicating a possible link with the defective IκB kinase β and IκBα phosphorylation in p85α−/− splenic B cells and their ability to induce cyclin D2. These results indicate that MEK1/2-p42/44ERK and NF-κB pathways link PI-3K activity to Ag receptor-mediated cyclin D2 induction in splenic B cells. The Journal of Immunology, 2004, 172: 2753–2762.

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ignaling by the B cell Ag receptor (BCR)1 leads to distinct cell fates, including survival, proliferation, or apoptosis. The outcome is dependent, in part, on the developmental stage of the B lymphocyte at the time of BCR engagement and on the signals provided by coreceptors (1). BCR ligation is followed by the activation of several src-family protein tyrosine kinases, Bruton’s tyrosine kinase (Btk), Syk, and the lipid kinase, phosphatidylinositol 3-kinase (PI-3K) (1, 2). These kinases coordinate the activation of signal transduction cascades, including phospholipase C-γ (PLC-γ), Vav, Akt/glycogen synthase kinase 3, and Ras/mitogen-activated protein kinase (MAPK) pathways (3). Efforts continue to focus on the contribution of individual signaling pathways in regulating cell fate responses following Ag binding. Notably, PI-3K has been shown to interface BCR signaling with B cell survival (4–9).

PI-3K members are classified into three primary groups according to their structure and substrate specificity. The most important group for B cell responses are the class IA PI-3Ks, which comprised a catalytic subunit (p110α, β, γ) and a regulatory subunit (p85α, p85β, p55γ) (8, 10). The p85 subunit is recruited to the plasma membrane and activates the p110 subunit in response to receptor ligation. Activation of class IA PI-3Ks results in the rapid phosphorylation of membrane phosphatidylinositol lipids on the D3 position of the inositol ring to generate phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-biphosphate, or phosphatidylinositol 3,4,5-trisphosphate (8–12). The resulting 3-phosphorylated phosphoinositides act to recruit to the plasma membrane and facilitate activation of pleckstrin homology domain-containing proteins (9, 13).

Pharmacologic inhibition of PI-3K activity abolishes BCR-dependent proliferation in murine and normal human B cells and human B lymphoma cells (14–17). Genetic studies of mice deficient for individual PI-3K catalytic and regulatory subunits point to profound defects in B cell function (18, 19). The p85α subunit is the most abundantly expressed class IA regulatory subunit (20, 21). Mice lacking only the p85α regulatory subunit exhibit a developmental block at the pro-B cell stage and show a reduction in the peripheral mature B cell compartment (18). p85α-null splenic B cells cultured ex vivo have pronounced proliferation defects in response to BCR cross-linking (18, 19). Similarly, mice expressing a catalytically inactive form of the p110α catalytic subunit exhibit a block in pro-B cell development and impaired proliferation in response to BCR engagement (22, 23).

D-type cyclins (D1, D2, and D3) function as positive regulatory subunits for cyclin-dependent kinase (cdk)4 and cdk6, and are rate limiting for G1 phase progression (24–29). Mitogen receptor-coupled signal transduction pathways regulate D-type cyclin accumulation, whereas cyclin E, which associates with cdk2, is regulated autonomously, peaking near the G1/S transition (25).

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findings contribute to the view that cdk4/6 and cdk2 function to drive cells through G1 phase and initiate S phase entry, respectively (30, 31). The principal substrates of cdk4/6 and cdk2 are members of the retinoblastoma gene product (pRb) family (p107, p110, and p130) (25, 32). In quiescent and early G1 phase cells, unphosphorylated and hypophosphorylated pRb members bind transcription factors, such as E2F, thereby negatively regulating gene transcription (32). Sequential phosphorylation of pRb members by cdk4/6 and cdk2 disrupts the association with E2F, leading to the coordinated transcription of genes involved in G1/S transition and DNA metabolism.

Cyclin D2, and to a lesser extent, cyclin D3 are induced in response to BCR cross-linking (33–35). The principal target of cyclin D2 in mature splenic B lymphocytes is cdk4 (33). Definitive evidence in support of the need for cyclin D2 in BCR-induced proliferation has come from studies of mice deficient for cyclin D2 (36). Although recent studies have identified individual components necessary for cyclin D2 induction, our understanding of the signal transduction pathways that link the BCR to cyclin D2 remains far from complete. Notably, studies point to a requirement for the adaptor protein, B cell linker protein, and the proto-oncogene, Vav, in BCR-mediated cyclin D2 protein induction (37, 38). Interestingly, neither B cell linker- nor Vav-mediated cyclin D2 induction involve Akt or MAPK activation. Despite these findings, we have implicated mitogen-activated protein/extracellular signal-regulated kinase (MEK)1/2 activity in BCR-mediated cyclin D2 induction, consistent with the recent reported role for MEK1/2 in ex vivo splenic B cell proliferation (39, 40).

To gain insight into the regulation of cyclin D2 by PI-3K, we used both pharmacologic inhibitors of PI-3K and mice deficient in the class I δ PI-3K p85α regulatory subunit to identify signaling pathways that link PI-3K activity to cyclin D2 induction. Results of this study provide the first reported evidence that MEK1/2-p42/44ERK and IκB kinase (IKK)-inhibitory κBα (IκBα) pathways function, in part, to couple PI3K activity to BCR-mediated cyclin D2 induction.

Materials and Methods

Abs and reagents

- Anti-cyclin D2, anti-mouse cdk4, anti-JunB (N17), anti-c-Myc, anti-IκBα, anti-rabbit IgG, and anti-mouse IgG-HRP (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-MEK1/2, anti-IKKα, anti-phospho-IKKα (S180/181), anti-phospho-p44-p42 MAPK (202/204), anti-phospho-p44/p42 MAPK, anti-Akt, anti-phospho-Akt (S473) Abs, and the anti-phospho-IκBα (S32/36) mAb were purchased from Cell Signaling Technology (Beverly, MA). Human pRb mAb (clone G3-245) was obtained from BD PharMingen (San Diego, CA). The anti-PI3K p85α Ab was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-cyclin D3 Ab was obtained from NeoMarkers (Fremont, CA). Anti-phospho-pRb (T281) and anti-phospho-pRb (S807/811) were obtained from BioSource International (Camarillo, CA). F(ab)2 Fragment of anti-mouse IgM were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-β-actin mAb, PMA, and ionomycin were obtained from Sigma-Aldrich (St. Louis, MO). NF-κB p50 subunit cell-permeable inhibitory peptide (SN50), corresponding inactive control peptide (SN50M), and Wortmannin were obtained from Biomol (Plymouth Meeting, PA). ECL reagents were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Phosphatidylinositol substrate was supplied by Avanti Polar Lipids (Alabaster, AL) and TLC plates impregnated with 1% potassium oxalate were obtained from Alltech Associates (Deerfield, IL). U0126 was obtained from Calbiochem-Novabiochem International (San Diego, CA).

Preparation of splenic B lymphocytes

BALB/c mice, p85α-deficient mice and the corresponding wild-type mice (Balb/AnNTac-Pik3r1 N12) at 8–12 wk were obtained from Taconic Farms (Germantown, NY). Mice were housed, cared for and handled at all times in accordance with National Institutes of Health and Boston College institutional guidelines. Spleens were removed and dissociated in HBSS (Atlanta Biologicals, Norcross, GA) supplemented with 2% heat-inactivated FCS (Atlanta Biologicals). Macrophages (and other adherent cells) were removed by plastic adherence. RBC and nonviable cells were removed by sedimentation on Lympholyte M (Accurate Chemical and Scientific, Westbury, NY). B lymphocytes were then isolated by negative selection using MACS B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). The cells (107) were incubated with 10 μl biotin-Ab mixture at 4°C (10 min) and then with 20 μl anti-biotin microbeads for 4°C (15 min). Cells were washed and resuspended in ice cold PBS containing 0.5% FCS. The cell suspension was then applied onto an LS magnetic separation column (Miltenyi Biotec). The effluent was collected as a pooled fraction containing unlabeled B cells. The B cells were then washed in PBS plus 0.5% FCS and then cultured in RPMI 1640 (Atlanta Biologicals) supplemented with 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 5 × 10−5 M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS. B cells were maintained in a 37°C humidified incubator at 5% CO2.

In vitro PI-3K assay

B cells (106) were resuspended in 1 ml of PI-3K lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, and 1% Triton X-100) supplemented with 1 mM PMSF, 1 μg/ml leupeptin, and 20 mM β-glycerophosphate. Cell lysates were treated with 5 μg of isotype-matched control rabbit IgG or rabbit anti-p85α Ab (Upstate Biotechnology) for 5 h at 4°C. The immune complexes were captured by incubation with 50 μl of 1:1 protein A- to protein G-agarose (1 h), washed six times with 1 ml PI-3K lysis buffer, and twice with PI-3K reaction buffer (30 mM HEPES, pH 7.4, 1 mM EDTA, 0.1% Triton X-100). Kinase reactions were conducted in 40 μl of PI-3K reaction buffer supplemented with 10 μCi of [γ-32P]ATP (250 Ci/mmol; PerkinElmer Life Sciences, Boston, MA), 10 μM ATP and 0.2 μg/ml phosphatidylinositol substrate (15 min, 30°C). The reactions were terminated with 100 μl of 1 N HCl, followed by sequential extraction in 200 μl 1:1 chloroform to methanol and 200 μl 1:1 N HCl to methanol. The organic phase was then removed, lyophilized, dissolved in 10 μl 1:1 chloroform to methanol, and spotted on Silica TLC plates impregnated with 1% potassium oxalate. The plates were developed by ascending chromatography in a 9:7:2 chloroform to methanol to 4 M NH4OH solvent system, dried, and subjected to phosphoimaging using a Molecular Dynamics PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA).

RNA isolation and RT-PCR

Total cellular RNA was isolated from 2 × 108 splenic B cells using Ultraspec Reagent (Biotex Laboratories, Houston, TX) denaturing solution following the manufacturer’s protocol. Purified RNA was treated with 1 U RNase I using DNA-free (Ambion, Austin, TX). First-strand cDNA synthesis was conducted with 2 μg of total RNA using RETROscript First-Strand Synthesis kit (Ambion). The concentration of cDNA in each sample was adjusted to produce equal amounts of product following quantitative PCR with primers specific for β2-microglobulin gene (β2-microglobulin sense primer 5'-gggctctcggactctgctgaac-3' and antisense primer 5'-cagctgccggtcttggtcgg-3') The PCR were performed in 25 μl final reaction volumes each consisting of template cDNA using reagents from the GeneAmp PCR kit (Roche Molecular Systems, Branchburg, NJ) supplemented with 10 μl of [α-32P]dCTP (6000 Ci/mmol; PerkinElmer Life Sciences). Amplification was conducted in an Eppendorf MasterCycler gradient (Brinkmann Instruments, Westbury, NY) as described (41). PCR products (20 μl) were separated through an 8% polyacrylamide gel and visualized by autoradiography. Under these conditions, the number of PCR cycles and the amount of cDNA used were such that increasing amounts of cDNA template yielded proportionately increasing amounts of β2-microglobulin-specific PCR product (data not shown). The variation of β2-microglobulin PCR product between adjusted samples was ±5%, as judged by densitometric analysis of the resulting autoradiograms using a Molecular Dynamics PhosphorImager. The number of cycles and amount of cDNA used was then adjusted accordingly to yield the same signal output. Cyclin D2 cDNA was amplified from the normalized β2-microglobulin cDNA samples using the identical PCR and temperature cycling parameters. The sequence of the cyclin D2 primer set corresponds to cyclin D2 sense primer 5'-agctggctcggactctgctgaac-3' and antisense primer 5'-gtaacatcggctgctgg-3'.

TAT-coupled IκBα dominant negative (TAT-IκBα-DN) purification

The pTAT-HA expression vector contains a six-histidine tag and the TAT transduction domain (provided by Dr. S. Dowdy, University of California).
at San Diego, La Jolla, CA) (42). The construction of the pTAT-HA expression vector containing IκBα-DN has been previously described (43). The pTAT-IκBα-DN was used to express the TAT-IκBα-DN fusion protein in the BL21 (DE3) strain of Escherichia coli. In brief, the cells were induced for 6 h with 0.1 mM isopropl β-D-thiogalactosidase, sonicated in 1 M urea, and then the TAT-IκBα-DN protein was purified by sequential chromatography on a nickel-Sepharose column (Qiagen, Valencia, CA) and ion exchange column (Mono Q) in 4 M urea. Shock-misfolding of the protein was conducted by replacing the 4 M urea with aqueous buffer (20 mM HEPES, pH 8.0) during the last ion exchange chromatographic step. TAT-IκBα-DN was eluted with a gradient from 50 mM to 1 M NaCl, followed by desalting on a PD-10 column (Amersham Pharmacia Biotech, Sunnyvale, CA) into PBS and storage in 10% glycerol at −80°C. The pTAT-green fluorescence protein (GFP) control plasmid used in these experiments was the gift of Dr. S. Dowdy and was prepared in a similar manner as the TAT-IκBα-DN.

EMSA

Nuclei were isolated by hypotonic lysis and extracted in a 450 mM NaCl buffer as previously described (44). Binding reactions were conducted in a final volume of 15 μl and contained 1.5 μg of nuclear protein extract, 0.5 μg of double stranded poly(dI:dC), and 10,000 cpn of a DNA-labeled probe containing an NF-κB binding element derived from the murine Igκ L chain enhancer (45). DNA probes were labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). After 20 min (23°C), the reaction products were electrophoresed through a 5% polyacylamide/TBE gel and subjected to autoradiography.

Results

PI-3K activity is required for BCR-induced early G1 phase progression in ex vivo splenic B cells

Pretreatment of ex vivo splenic B cells with the PI-3K inhibitor, wortmannin inhibited de novo RNA synthesis induced by 10 μg/ml F(ab')2 of goat anti-mouse anti-IgM (anti-Ig), as measured by [3H]uridine incorporation, in a dose-dependent manner (Fig. 1A) (46); maximal inhibition by wortmannin occurred at a concentration of 50 nM. Similar results were obtained with the selective PI-3K inhibitor, LY294002 (data not shown). The inhibition of RNA synthesis coincided with an early block of endogenous pRb phosphorylation (Fig. 1A, inset, top lane); phosphorylation of pRb on T821 by cyclin E-cdk2 complexes was also impaired in the presence of 50 nM wortmannin, a modification that serves as an indicator of late G1 phase progression (Fig. 1A, inset, bottom lane).

To confirm that wortmannin inhibits PI-3K activity under the conditions used, we measured PI-3K activity in vitro by immune complex kinase assay. Preincubation of ex vivo splenic B cells with 50 nM wortmannin inhibited BCR-induced PI-3K activity when compared with parallel B cells treated with the solvent control (Fig. 1B). We also investigated anti-Ig-induced Akt activation, a downstream target of PI-3K, by Western blotting with an Ab that detects phosphorylation on S473, a modification known to correlate with its kinase activity (47). We found that anti-Ig-induced Akt phosphorylation was inhibited following preincubation with 50 nM wortmannin before stimulation with anti-Ig (Fig. 1C, top lane).

Requirement for PI-3K activity in BCR-mediated cyclin D2 induction and phosphorylation of endogenous pRb by D-type cyclin-cdk4/6 complexes

We sought to determine whether PI-3K activity is required for BCR-mediated cyclin D2 induction in ex vivo splenic B cells. RNA was isolated from splenic B cells stimulated with 10 μg/ml anti-Ig for 4 and 18 h, and cyclin D2 gene expression was then measured by RT-PCR as described (39). Cyclin D2 mRNA was
induced ~3- and 7-fold in response to BCR cross-linking at 4 and 18 h, respectively (Fig. 2A). Anti-Ig-stimulated cyclin D2 mRNA accumulation was markedly reduced by wortmannin when measured at the 4 and 18 h time points (Fig. 2A).

The requirement for PI-3K activity was mirrored at the level of cyclin D2 protein induction in that wortmannin led to a nearly complete block of anti-Ig-induced cyclin D2 protein accumulation (Fig. 2B). The inhibition of cyclin D2 protein induction by wortmannin did not arise from changes in the total amount of cellular protein being compared across conditions as evidenced by equal levels of β-actin protein (Fig. 2B). Moreover, there was no detectable induction of cyclin D3 in anti-Ig-stimulated B cells pretreated with wortmannin (Fig. 2B). Although cellular cdk4 levels were not measurably affected by inhibiting PI-3K activity (Fig. 2B), the lack of sustained cyclin D2 (and cyclin D3) accumulation is likely sufficient to prevent activation of endogenous cdk4 necessary for pRb phosphorylation. In support of this, wortmannin-treated splenic B cells exhibited a near complete block in BCR-induced pRb phosphorylation on D-type cyclin-ckd4/6-targeted residues (S807/811) (Fig. 2B) (48). We observed similar results with the PI-3K inhibitor, LY294002. Notably, anti-Ig induced cyclin D2 protein and endogenous pRb phosphorylation on cdk4/6-targeted residues (S808/811) were blocked in comparison to B cells devoid of LY294002 (Fig. 2C).

**BCR-mediated activation of the cyclin D2/cdk4-pRb pathway is impaired in ex vivo splenic B cells from p85α-deficient mice**

To corroborate the findings that pharmacologic inhibition of PI-3K activity results in defective BCR-induced cyclin D2 induction and subsequent pRb phosphorylation by D-type cyclin-ckd4/6 complexes, we examined ex vivo splenic B cells isolated from mice deficient in the p85α subunit of PI-3K (18). Splenic B cells from p85α-deficient mice do not express detectable levels of the p85α subunit (Fig. 3A). In addition, p85α-deficient B cells exhibited markedly reduced BCR-mediated Akt phosphorylation on S473 in comparison to B cells isolated from the corresponding wild-type mice (Fig. 3A). p85α-null splenic B cells exhibited a near complete loss of BCR-mediated cyclin D2 mRNA and protein induction in comparison to wild-type B cells (Fig. 3, B and C, respectively). As a positive control for these experiments, cyclin D2 mRNA and protein levels were induced in the p85α−/− splenic B cells following stimulation with phorbol ester (PMA) plus the calcium ionophore, ionomycin (P/I) (Fig. 3, B and C, end lanes). These reagents are mitogenic for ex vivo splenic B cells and act to circumvent the proximal signaling components of the BCR, including PI-3K (49).

Anti-Ig-stimulated cyclin D3 induction was also impaired in p85α−/− splenic B cells relative to their wild-type counterparts (Fig. 3C). Despite the expression of cdk4 in p85α-deficient B cells (albeit at a level somewhat lower in comparison to wild-type B cells), endogenous pRb phosphorylation and D-type cyclin-ckd4/6-targeted phosphorylation on S807/S811 following BCR cross-linking was not detected under the conditions used (Fig. 3C). However, these cell cycle events were induced in parallel cultures of p85α−/− splenic B cells stimulated with PMA and ionomycin (Fig. 3C). These results indicate that BCR-mediated cyclin D2 induction and subsequent phosphorylation of endogenous pRb by D-type cyclin holoenzymes requires expression of p85α gene products of PI-3K.

**BCR-induced cyclin D2 accumulation requires PI-3K-coupled signaling through MEK1/2-ERK in normal splenic B lymphocytes**

A previous report from our laboratory demonstrated that MEK1/2-ERK activity is required for cyclin D2 mRNA induction in re-
response to BCR ligation (39). Therefore, we investigated whether impaired induction of cyclin D2 caused by PI-3K inhibition may be due to inhibition of MEK1/2-ERK activation. As shown in Fig. 4A, BCR-induced phosphorylation of MEK1/2 on activation residues S217/S221 was inhibited in splenic B cells pretreated with wortmannin. Moreover, anti-Ig-induced phosphorylation of p42/44ERK on activation residues T202/Y204 was equally sensitive to wortmannin (Fig. 4A). Importantly, PMA and ionomycin-induced MEK1/2 and p42/44ERK phosphorylation was not sensitive to wortmannin (P/I, Fig. 4A, right side lanes). Phosphorylation of p42/44ERK was also inhibited by pretreatment of anti-Ig-stimulated B cells with LY294002 (Fig. 4A). Accordingly, pre-incubation of splenic B cells with the highly specific inhibitor, U0126, led to a near complete block of anti-Ig-stimulated cyclin D2 mRNA and protein induction (Fig. 4B). Taken together, these data suggest that MEK1/2-p42/44ERK activity is dependent upon a PI-3K activity. Moreover, the data suggest that MEK1/2 functions, at least in part, to link a PI-3K activity to BCR-induced cyclin D2 expression.

In contrast to the experiments using pharmacologic inhibitors of PI-3K, which inhibit all three classes of PI-3Ks, anti-Ig-induced phosphorylation of MEK1/2 in splenic B cells pretreated with wortmannin was not significantly different from that observed in wild-type mice (Fig. 5A).

**FIGURE 3.** Lack of cyclin D2 induction and impaired activation of endogenous D-type cyclin-cdk4/6 complexes in anti-Ig stimulated p85α−/− ex vivo splenic B cells. A, Whole cell lysates were prepared from p85α−/− and wild-type (WT) splenic B cells and Western blotted for p85α subunit expression using an anti-p85α Ab. Splenic B cells from p85α−/− and wild-type mice were cultured in medium alone (M) or were stimulated for 15 min with 10 μg/ml anti-Ig (αlg). The levels of cellular phospho-S473-Akt (pAkt(S473)) and total Akt were determined by Western blotting. B, Wild-type (WT) and p85α−/− splenic B cells cultured in medium (M) or were stimulated with 10 μg/ml anti-Ig (αlg) for the indicated times. p85α−/− splenic B cells were also stimulated with 300 ng/ml PMA and 400 ng/ml ionomycin (P/I). RT-PCR was conducted as described in Materials and Methods. Cyclin D2 expression is reported as a proportion (fold induction) of expression present in unstimulated (M) B cell samples. The inset shows the corresponding autoradiogram of β2-Microglobulin (β2-MG) and cyclin D2 RT-PCR analysis. C, Wild-type (+/+ ) and p85α−/− (−/−) B cells were cultured in medium alone (M) or were stimulated with 10 μg/ml anti-Ig (αlg) for the indicated times. Whole cell lysates were prepared for Western blotting of the indicated cell cycle proteins. Equal protein loading was controlled by stripping the membranes and probing with anti-β-actin Ab. A representative Western blot of β-actin is shown for the anti-cyclin D2 membrane. These data are representative of three independent experiments.
ex vivo p85α−/− splenic B cells in a manner similar to wild-type B cells (Fig. 5A). There was also no significant difference between the ability of anti-Ig-stimulated p85α−/− and wild-type splenic B cells to induce p42/44ERK phosphorylation (Fig. 5A). These results, taken together with the pharmacologic inhibitor data (Fig. 4A) suggest that BCR-induced MEK1/2-p42/44ERK phosphorylation in splenic B cells is dependent on a PI-3K family member that does not require the p85α subunit.

BCR-induced phosphorylation of IKKβ and IκBα is impaired in p85α-deficient splenic B cells

We interpret the observations in p85α-null splenic B cells to implicate additional signaling components, downstream of p85α, in cyclin D2 induction. We investigated the activation of several known BCR-coupled signaling pathways and, in data not shown, anti-Ig-stimulated phosphorylation of the two remaining classes of mammalian MAPKs, c-Jun NH2-terminal kinase and p38 MAPK in p85α−/− splenic B cells was similar to that of wild-type B cells. Moreover, there was no detectable difference between anti-Ig-stimulated calcium mobilization in p85α−/− splenic B cells and wild-type B cells.

We also examined NF-κB signaling in p85α−/− splenic B cells. BCR ligation results in the phosphorylation of cytosolic IκBα by the IKK complex, which consists of two catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ (50). Phosphorylation of IκBα targets it for degradation via the ubiquitin-dependent proteasome pathway, which releases NF-κB/c-Rel subunits for subsequent translocation into the nucleus (51, 52). Stimulation of the wild-type B cells with 10 μg/ml anti-Ig resulted in

FIGURE 4. BCR-mediated cyclin D2 induction requires PI-3K-coupled MEK1/2 and ERK signaling in normal splenic B cells. A, Splenic B lymphocytes (M) were stimulated with 10 μg/ml anti-Ig (αrg) for the indicated times, with (+ Wtn) or without (− Wtn) a 30 min pretreatment with 50 nM wortmannin (Wtn). Whole cell extracts were prepared and Western blotted for phospho-MEK1/2 and MEK1/2. Cells were pretreated (30 min) with 50 nM wortmannin or 10 μM LY294002 (LY) for 5 and 10 min and whole cell extracts Western blotted for phospho-p42/44ERK. B cells were also stimulated (10 min) with 300 ng/ml PMA and 400 ng/ml ionomycin (PII), following a pretreatment in the absence (−) and presence (+) of 50 nM wortmannin and Western blotted for phospho-p42/44ERK. The membranes were stripped and reprobed with Abs to the nonphosphorylated forms of MEK1/2 and p42/44ERK. B, B cells were cultured in medium alone (M) or stimulated with 10 μg/ml anti-Ig (αrg) for 4 h in the absence (−) or presence (+) of 10 μM U0126. Cyclin D2 and β2-microglobulin (β2-MG) mRNA expression were measured by RT-PCR as described in Materials and Methods. The levels of cyclin D2 and β-actin protein expression were also measured in parallel splenic B cell cultures. These data are representative of four independent experiments.

FIGURE 5. Impaired BCR-induced IKKβ and IκBα, but not MEK1/2-p42/44ERK phosphorylation in p85α−/− splenic B cells. A, Wild-type and p85α−/− splenic B cells were cultured in medium alone (M) or were stimulated with 10 μg/ml anti-Ig (αrg) for the indicated times. Whole cell extracts were prepared and Western blotted for phospho-IKK and phospho-p42/44ERK. B, Whole cell extracts were also Western blotted with Abs specific for phospho-IKKα(S180)/IKKβ(S181) and phospho-IκBα (S32/S36). The corresponding membranes were stripped and reprobed with Abs to the nonphosphorylated forms of MEK1/2 and p42/44ERK. B. Whole cell extracts were also Western blotted with Abs specific for phospho-IKKα(S180)/IKKβ(S181) and phospho-IκBα (S32/S36). The corresponding membranes were stripped and reprobed with Abs that recognize the nonphosphorylated forms of IKKα and IκBα. Equal protein loading of IκBα was confirmed by Western blotting with anti-β-actin. These data are representative of three independent experiments.
increased phosphorylation of IKKα/IKKβ, as measured by Western blotting of cellular extracts with a highly specific Ab that recognizes IKKα and IKKβ phosphorylated on S180 and S181, respectively (Fig. 5B); these modifications are known to correlate with catalytic activation of IKKα and IKKβ (53). In contrast, phosphorylation of IKKβ was virtually absent and IKKα phosphorylation was diminished in anti-Ig-treated p85α−/− splenic B cells (Fig. 5B). As a control, we found that the total cellular levels of IKKβ (and IKKα) were similar in p85α-deficient and wild-type B cells (Fig. 5B). BCR cross-linking induced phosphorylation of IκBα on activation residues, S32/S36 and its degradation was detected at the 10 min time point in the wild-type B cell population, whereas anti-Ig-stimulated phosphorylation of IκBα was impaired in p85α−/− splenic B cells (Fig. 5B).

We next sought to ascertain whether the impaired IKKβ and IκBα phosphorylation observed in p85α−/− B cells could be recapitulated in normal BALB/c splenic B cells pretreated with wortmannin. Stimulation of normal B cells with anti-Ig resulted in increased phosphorylation of IKKα/IKKβ, whereas phospho-IKKα levels remained relatively unchanged in comparison to unstimulated B cells (Fig. 5C). Interestingly, Saijo et al. (54) recently reported a similar pattern of IKKα/IKKβ phosphorylation in ex vivo splenic B cells from C57BL/6 mice. Anti-Ig induced the phosphorylation of IκBα, and at the 20 min time point, appeared to initiate its degradation (Fig. 5C). In contrast, pretreatment of splenic B cells with wortmannin inhibited anti-Ig-induced IKKβ and IκBα phosphorylation at the time points examined (Fig. 5C).

NF-κB inhibitor SN50 blocks BCR-induced cyclin D2 induction and endogenous pRb phosphorylation in splenic B cells

To investigate whether disruption of NF-κB signaling in normal splenic B cells might lead to impaired cyclin D2 induction following BCR cross-linking, we adopted the approach of Lin et al. (55) to block nuclear import of NF-κB with the in vivo delivery of a synthetic peptide SN50 (55). This peptide contains the nuclear localization sequence (NLS residues 360–369) of NF-κB p50 subunit coupled to the membrane-permeable signal peptide of Kaposi fibroblast growth factor. The Kaposi fibroblast growth factor domain allows dose-dependent uptake into virtually all cells (55). Results in Fig. 6A demonstrate that preincubation of ex vivo splenic B cells with 75 μM SN50 significantly reduced BCR-mediated cyclin D2 induction. Moreover, BCR-induced phosphorylation of endogenous pRb on cdk4/6-targeted S807/S811 was reduced. Pretreatment of parallel B cells with a control mutant peptide (SN50M), which fails to block NF-κB nuclear translocation due to an inactive NLS (55), did not decrease BCR-mediated cyclin D2 induction or pRb phosphorylation by cdk4/6 in comparison to nontreated anti-Ig stimulated B cells. In control experiments, we observed that at the concentration used to inhibit BCR-induced cyclin D2 induction, SN50 peptide blocked the induction of c-Myc, an immediate downstream target of NF-κB activity (Fig. 6B) (56). By contrast, induction of Jun-B in response to BCR ligation was not inhibited by SN50 (44). Note, Jun-B protein migrates as a doublet in SDS-polyacrylamide gels (44). Furthermore, the functional effect of the SN50 peptide on nuclear translocation of NF-κB/Rel complexes in B cells stimulated by BCR cross-linking was determined by EMSA. As shown in Fig. 6C, BCR-induced NF-κB binding activity in nuclear extracts was inhibited by SN50 peptide, whereas the mutant SN50M peptide was without measurable effect.
Ig was not reproduced by incubating B cells with TAT-GFP, which had no effect. Thus, two selective inhibitors of NF-κB activation (SN50 and TAT-IκBα-DN) that target distinct components of the NF-κB pathway inhibit induction of cyclin D2 produced by BCR engagement.

Discussion

A role for PI-3K activity in BCR-mediated cyclin D2 induction is provided by the findings that PI-3K inhibitors, wortmannin and LY294002 block BCR-mediated cyclin D2 induction in splenic B cells. Moreover, a role for PI-3K is further supported by studies in splenic B cells from mice deficient for the p85α gene product. In this study, we provide compelling experimental evidence for the lack of detectable BCR-mediated cyclin D2 mRNA and protein induction in p85α−/− ex vivo splenic B cells. Given the essential role of cyclin D2 in G1-to-S phase progression (36), it is probable that such impairment in BCR-mediated accumulation of cyclin D2 results in the reported defective mitogenic capacity of p85α-null splenic B cells (18, 19).

In dissecting potential mechanisms underlying defective cyclin D2 induction in the absence of p85α gene products, we find that BCR-induced MEK1/2 and p42/44ERK phosphorylation on activating residues is not significantly diminished in p85α-null splenic B cells compared with their wild-type B cell counterparts. This result contrasts with our observations in normal splenic B cells treated with wortmannin in which a requirement for PI-3K is clearly established in BCR-initiated MEK1/2-p42/44ERK phosphorylation. We also observed that LY294004 blocks p42/44ERK phosphorylation following BCR cross-linking, in agreement with a recent report by Jacob et al. (57). The molecular basis underlying this discrepancy is currently unknown; however, because all mammalian class I, II, and III PI-3K members show sensitivity to wortmannin and LY294004, it is plausible that BCR-dependent MEK1/2-p42/44ERK activation requires PI-3K members other than class Iα (58). It is also conceivable that MEK1/2-p42/44ERK activation may rely more heavily on p85β subunit expression. Notably, B cells from p85α-null mice used in this study express p85β, albeit at a relatively low level, in comparison to wild-type B cells (59). Alternatively, it remains possible that a pool of p110 catalytic subunits may be recruited to the membrane and activated by Ras (60). It is interesting to note that we observed a very low level of BCR-induced Akt phosphorylation on S473 in p85α-null splenic B cells, as shown in Figure 8.
suggesting residual PI-3K activity. Notwithstanding, the data suggest that BCR signaling selectively uses a p85α-independent and wortmannin- (and LY294002-sensitive) PI-3K family members to signal MEK1/2-p42/44ERK phosphorylation. These results, taken together with the sensitivity of cyclin D2 induction to wortmannin and the MEK1/2 inhibitor, U0126 (as shown in our study and in Ref. 39), suggest a role for MEK1/2-p42/44ERK in linking PI-3K activity to BCR-mediated cyclin D2 induction.

To identify why cyclin D2 induction is defective in p85α−/− splenic B cells, we investigated the activation of NF-κB signaling, which is known to regulate genes involved in cell proliferation and survival (61–64). We found that BCR-induced IKKβ and IκBα phosphorylation is impaired in p85α−/− splenic B cells. It is therefore likely that p85α expression is required for activation of these key components of the NF-κB pathway in splenic B cells. In keeping with the defective IKKβ and IκBα phosphorylation in p85α-null B cells, analysis of these signaling intermediates in normal BALB/c splenic B cells treated with wortmannin reveals markedly diminished Ag receptor-mediated phosphorylation of IKKβ and IκBα. Based on these results, we investigated whether impaired NF-κB signaling might plausibly explain the defect in cyclin D2 induction in p85α−/− splenic B cells as well as wortmannin-treated normal B cells. In support of this, we find that blocking nuclear translocation of NF-κB/Rel complexes in normal splenic B cells with a cell permeable SN50 peptide carrying a functional NF-κB domain NLS (55), markedly reduces BCR-mediated cyclin D2 induction and endogenous pRB phosphorylation by D-type cyclin-cdk4/6. Similar results are obtained following transduction of splenic B cells with a TAT-IκBα-ΔN that blocks NF-κB activation. Taken together, these results suggest that components of NF-κB pathway function to link PI-3K family members to cyclin D2 induction in response to BCR ligation.

Our results raise an important question as to how PI-3K activation initiates IKKβ-IκBα signaling. An important consideration is the downstream effector of PI-3K, Akt, which has been reported to directly interact with and phosphorylate IκB (65, 66). However, the protein kinase C (PKC) inhibitor, Gö6983 blocks BCR-induce IκBα degradation without affecting Akt phosphorylation, suggesting that PKC may represent an upstream regulator of IκK in splenic B cells (64, 67). Of note, inhibition of BCR-mediated capacitative calcium entry and PKC activation blocks cyclin D2 induction (37, 64). A direct role for PKC in NF-κB signaling is supported by a recent report that BCR-induced IKKα/IKKβ-IκBα phosphorylation is impaired in PKC-β−/− splenic B cells (54). Accordingly, BCR-induced phosphorylation of IκBα is defective in phospholipase C-γ2-null and X-linked immunodeficient B cells, the latter of which expresses a mutant Btk (68–70). It is widely accepted that PI-3K activity is responsible for the activation of Btk by recruiting it to the plasma membrane through interactions between the pleckstrin homology domain of Btk and phosphatidylinositol 3,4,5-trisphosphate (71). Alternatively, PI-3K might initiate NF-κB activation via the downstream effector serine/threonine kinase, PDK1, which has been shown to phosphorylate and activate conventional PKCs and PKC-ζ and -δ (72, 73).

The molecular mechanism underlying NF-κB-dependent regulation of cyclin D2 induction in response to BCR ligation may involve Myc inasmuch as c-myc transcription is regulated by NF-κB/Rel factors and inhibition of PI-3K activity blocks nuclear import of NF-κB/β1c-Rel dimers concomitant with a failure to up-regulate c-myc (56, 61, 73, 75). Likewise, expression of a conditional Myc-estrogen receptor fusion protein is sufficient to induce cyclin D2 (76). In contrast, the mechanism by which PI-3K-coupled MEK1/2-ERK signaling regulates cyclin D2 induction in response to BCR ligation has not been defined. It does not appear however, that MEK1/2-ERK is directly involved in induction of NF-κB/β1c-Rel, as the MEK1/2 inhibitor, PD98059 does not affect endogenous nor transgenic c-myc expression in murine B cells (61).

In summary, our results indicate that PI-3K-dependent MEK1/2-ERK and NF-κB signaling contribute to BCR-mediated cyclin D2 induction. The results in this study do not preclude a role for PI-3K controlling the activation of other signaling pathways, which in addition to MEK1/2-ERK and NF-κB may be essential in BCR-induced cyclin D2 expression.

Acknowledgments

We thank Fay Dufoort for assistance with the uridine assays.

References

9. Gold, M. R., and R. Aebersold. 1994. Both phosphatidylinositol 3-kinase and PDK1, which has been shown to phosphorylate and acti-
vate IKK (65, 66), are required for the activation of other signaling pathways, which in addition to MEK1/2-ERK and NF-κB may be essential in BCR-induced cyclin D2 expression.

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