CD19 is rapidly phosphorylated upon B-cell antigen receptor (BCR) cross-linking, leading to the recruitment of downstream signaling intermediates. A prominent feature of CD19 signaling is the binding and activation of phosphoinositide 3-kinase (PI3K), which accounts for the majority of PI3K activity induced by BCR ligation. Recent findings have implicated activation of the serine/threonine kinase Akt as imparting survival signals in a PI3K-dependent fashion. Using CD19-deficient B-lymphoma cells and mouse splenic B-cells, we show that CD19 is necessary for efficient activation of Akt following cross-linking of surface immunoglobulin or IgM. In the absence of CD19, Akt kinase activity is reduced and transient. In addition, coligation of CD19 with surface immunoglobulin leads to augmented Akt activity in a dose-dependent manner. Thus, CD19 is a key regulator of Akt activity in B-cells; as such it may contribute to pre-BCR or BCR-mediated cell survival in vivo.

Signaling through the B-cell receptor (BCR) complex effects differing cellular fates depending upon the stage of differentiation, nature of the antigen, and the contribution of surface coreceptors and accessory molecules. During early B-cell maturation, signaling through the pre-BCR induces proliferation and differentiation, tantamount to the production of IgM-positive immature cells. However, as newly formed B-cells transit from the bone marrow to become mature peripheral B-cells they become subject to regulation by additional surface markers that can modulate the nature or degree of signaling through the BCR. Collectively, these inductive signals act to direct further B-cell differentiation and antibody production.

Prominent among the B-cell coreceptors is CD19, a 95-kD transmembrane protein expressed throughout B-cell development. CD19 is a proximal substrate for tyrosine phosphorylation following surface immunoglobulin cross-linking and is thought to be the primary signaling component of the CD19/CD21/CD81/Leu13 B-cell coreceptor complex. CD19 has also been implicated as a signaling partner for several other surface receptors including CD40 (1), CD38 (2), CD72 (1), VLA-4 (3) and FcγRIIB (4, 5). Mice deficient for CD19 show reduced B-cell survival and/or survival capacity of Akt in B-cells is regulated by CD19.

We sought to determine the biochemical bases of these phenotypes as they may relate to in vivo proliferation and/or survival of B-lymphocytes.

One of the key functional attributes of CD19 signaling is the recruitment and activation of phosphoinositide 3-kinase (PI3K) following sIg cross-linking (8, 9). CD19 is recruited to the BCR complex via its membrane-proximal cytoplasmic domain and binds the p85 subunit of PI3K following phosphorylation of dual YXXM motifs in the cytoplasmic tail of CD19 (9, 10). Through the generation of 3'-phosphorylated inositides, PI3K can regulate the membrane localization and activation of numerous downstream effector molecules. Recent work has lead to the elaboration of a key cell survival pathway activated by the PI3K products phosphatidylinositol-3,4 bisphosphate and -3,4,5 triphosphate. Newly generated phosphatidylinositol-3,4 bisphosphate and phosphatidylinositol-3,4,5 triphosphate bind and activate 3'-phosphoinositide-dependent protein kinase-1 and -2 (11, 12), which in turn phosphorylate the Ser/Thr kinase Akt, (13), on residues Thr308 and Ser 473, respectively.

Once activated, Akt can inhibit apoptosis by phosphorylating the proapoptotic factor Bad (14), caspase-9 (15), or other uncharacterized substrates. Most recently, it has been shown that BCR engagement leads to activation of Akt that can be terminated by coligation of the FcγRIIB receptor (16). Here we investigate the regulation of Akt activity in primary and transformed mature B-cells. Our findings demonstrate that CD19 is critical for BCR-induced Akt activity, suggesting that the pro-survival capacity of Akt in B-cells is regulated by CD19.

EXPERIMENTAL PROCEDURES

Cell Lines and Isolation of CD19-deficient A20 Variant—The A20 B-lymphoma cell line and A20.4 CD19-deficient A20 variant were grown in complete media (RPMI supplemented with 10% fetal bovine serum, penicillin, streptomycin, l-glutamine, sodium pyruvate, nonessential amino acids (Cellgro; Mediatech, Herndon, VA), and 50 mM 2-mercaptoethanol (Life Technologies, Inc.)). CD19-deficient A20 variants were isolated by depletion of CD19-positive A20 cells using rat anti-mouse CD19 (1D3) antibody coupled to Magna Bind goat anti-rat IgG magnetic beads (Pierce). Clones were expanded and stained for surface CD19, IgG, and CD21 levels and were analyzed by fluorescence-activated cell sorting (anti-CD19-phosphatidylethanolamine, anti-CD21-fluorescein isothiocyanate, biotinylated anti-IgG, streptavidin-Cychrome; Pharmingen, San Diego, CA). A20.4 is representative of several clones isolated in this manner.

Purification of Primary Splenic B-cells—Splenic B-cells from 6–12-week-old wild-type BALB/c and CD19 null mice (BALB/c background) were isolated by depletion using the MidiMACS system with anti-CD43 (Ly-48) microbeads (Miltenyi Biotech, Auburn, CA) as per the manufacturer’s instructions. Isolated B-cells were greater than 97% pure by fluorescence-activated cell sorting (anti-B220-phosphatidylethanolamine, anti-CD3-fluorescein isothiocyanate; Pharmingen). Cells were kept at room temperature at all times with the exception of the antibody/magnetic bead binding step, which was done at 4 °C for 15 min.

Stimulations and Immunoblotting—A20 B-lymphoma cells were

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1 The abbreviations used are: BCR, B-cell receptor; PI3K, phosphoinositide 3-kinase; GSK, glycogen synthase kinase; sIg, surface immunoglobulin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.
grown overnight in complete RPMI supplemented with 1% fetal bovine serum followed by 3–5 h of serum starvation before stimulations. Cells were adjusted to 1–2 × 10^6/ml in PBS at room temperature. Stimulations were allowed to proceed for the indicated times and were stopped by the addition of an equal volume of 2× Nonidet P-40 lysis buffer (1× = 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10 mM sodium fluoride, 1 mM pyrophosphate, 1 mM EDTA, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM microcystin). Antibodies used for stimulations were goat anti-mouse IgG F(ab')2, minimal cross-reactive rat anti-mouse IgG F(ab')2 (Jackson ImmunoResearch, West Grove, PA), and hamster anti-mouse CD79b (HM79b; Pharmingen). 15 μg of total cell lysate were loaded onto 7.5% SDS polyacrylamide gels, and separated proteins were transferred to PVDF (Millipore, Bedford, MA). Primary antibodies for Western blots were rabbit anti-phospho Akt (Thr308 and Ser 473), rabbit anti-Akt (New England Biolabs, Beverly, MA), and rabbit anti-mouse CD19 (a generous gift from Dr. John Cambier, National Jewish Center, Denver, CO). Western blots were probed with horseradish peroxidase-conjugated secondary antibodies (either goat anti-mouse IgG or goat anti-rabbit IgG) for 1 h at room temperature and developed with ECL (Amersham Pharmacia Biotech). Quantitation was performed by comparing densitometry readings using NIHImage software; numbers (arbitrary units) represent values corrected for loading (reprobes for total Akt).

Purified splenic B-cells were stimulated, and protein lysates were prepared and analyzed as described above for A20 cells. Stimulations were performed with biotinylated goat anti-mouse IgM F(ab')2 (Jackson ImmunoResearch), biotinylated rat anti-mouse CD19 (1D3), and hamster anti-mouse CD79b (HM79b) (Pharmingen). For synergistic activation of splenic B-cells, cells at 2 × 10^6/ml in PBS were preincubated with 5 μg/ml FcBlock (anti-CD16/CD32; Pharmingen) for 10 min at room temperature. Cells were washed once with PBS before being resuspended in PBS at 3 × 10^6 cells/ml containing varying amounts of biotinylated goat anti-mouse IgM F(ab')2 fragments with or without varying amounts of biotinylated rat anti-mouse CD19. Antibodies were allowed to bind for 10 min at room temperature, after which 10 μg/ml avidin was added, and cells were stimulated at 37 °C for 2 min. Cell lysates were prepared and analyzed as described above.

**Akt in Vitro Kinase Assay**—In vitro kinase assays were carried out with A20 or A20.4 cells using the Akt kinase assay kit from New England Biolabs as per the manufacturer’s instructions. In brief, Akt was immunoprecipitated from 0.5–1× 10^6 unstimulated or stimulated cells, and kinase assays using glutathione S-transferase-GSK-3α fusion protein as substrate were performed. Akt-mediated phosphorylation of GSK-3α on Ser21 was determined by Western blot using an antibody that specifically recognizes phospho-Ser21 on GSK-3α (21). Akt-mediated phosphorylation of endogenous GSK-3α was performed. Akt phosphorylation was quantitated by densitometry and was corrected for immunoprecipitated Akt. The degree of induced phosphorylation was quantitated by densitometry and was corrected for immunoprecipitated Akt. These data are representative of at least five experiments. B, Akt was immunoprecipitated from cell lysates following stimulation as in A and was subjected to an in vitro kinase assay using [γ-32P]-ATP and GSK-3α as an exogenous substrate. After transfer to PVDF, incorporation of labeled phosphate into GSK-3α was quantitated by phosphorimager analysis, and standard deviations from three experiments are shown. p values are shown as a measurement of the significance of the difference in Akt activity observed in A20 and A20.4 cells at the indicated time points. C, induced phosphorylation of the Akt substrate GSK-3α was measured as in A. Here, whole cell lysates were probed for phosphorylation of endogenous GSK-3α in primary B-cells.

**RESULTS AND DISCUSSION**

**Akt Kinase Activity Is Reduced in CD19-deficient Cells**—Several groups, including ours, have found induced Akt kinase activity following sIg cross-linking (16–19). This occurs in a PI3K-dependent manner; hence we were interested in determining how Akt activity is induced by the BCR complex. Although several BCR-associated proteins, including Igα and Igβ,
have been implicated as associating with PI3K (20), CD19 is the only coreceptor that has been functionally linked to PI3K activation (8, 9). To assess the role of CD19 in Akt activation, we isolated CD19-deficient variants of A20 murine B-lymphoma cells by immunodepletion of CD19-positive cells. One such line, designated A20.4, was selected for further characterization and was found to be devoid of cell surface and intracellular expression of CD19 protein, yet it retained similar levels of sIg (Fig. 1).

To assess BCR-induced activation of Akt in the presence or absence of CD19, A20 and A20.4 cells were stimulated with anti-IgG F(ab)2 fragments for the indicated time points, and kinase activity was determined by a cold kinase assay to specifically measure Akt-induced phosphorylation of GSK-3 in vitro on position Ser473 as revealed by blotting with a phosphospecific antibody (Fig. 2A). In parallel, [γ-32P]-ATP was incorporated into the kinase assay to directly measure kinase activity levels with the natural substrate GSK-3 (Fig. 2B). A20 cells showed rapid induction of Akt kinase activity, which began to diminish by 15 min. In the absence of CD19, BCR-induced Akt activity is not only reduced (Fig. 2B) but is also more rapidly attenuated (Fig. 2A, compare lanes 4 and 5 with lanes 10 and 11). Remaining Akt kinase activity in the CD19-deficient A20.4 is still PI3K-dependent as seen by sensitivity to Wortmannin treatment (Fig. 2A, lane 12). To assess the effects of reduced Akt activity on downstream substrates in primary cells, phosphorylation of endogenous GSK-3 was measured by Western blots of lysates from nonstimulated and stimulated splenic B-cells from wild-type and CD19-deficient mice (Fig. 2C). Although phosphorylation of GSK-3 was similar at earlier time points (lanes 2 and 8), CD19-deficient splenic B-cells had reduced GSK-3 phosphorylation at later time points (Fig. 2C, compare lanes 4 and 5 with lanes 10 and 11). Hence, CD19 is involved in promoting and sustaining Akt function.

**CD19 Is Necessary to Promote Dual Phosphorylation of Akt**

Full activation of Akt is dependent upon dual phosphorylation at residues Thr308 and Ser473 by 3'-phosphoinositide-dependent protein kinase-1 and 3'-phosphoinositide-dependent protein kinase-2, respectively (11, 12). To explore the enzyme kinetics, activation threshold, and regulation of Akt phosphorylation, we employed phosphospecific antibodies directed against each of these sites. We found that Akt was rapidly phosphorylated at both positions in A20 cells (Fig. 3A). Peak phosphorylation was observed at 1–2 min for Thr308 and 2–5 min for Ser473 post-stimulation and was sustained above background levels for 20–60 min (Fig. 3A and data not shown). In striking contrast, Akt phosphorylation in A20.4 cells showed reduced levels of phosphorylation on both Thr308 and Ser473 residues, which was not sustained beyond 10–20 min. These results are consistent with the differential kinase activity observed in CD19-sufficient and -deficient cells.

To address whether CD19 was promoting the activation of

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**Fig. 3.** A, CD19-deficient cells show reduced and transient Akt phosphorylation on residues Thr308 and Ser473. A20 and A20.4 cells were stimulated with 1 μg/ml goat anti-mouse IgG F(ab)2 for the indicated periods of time. Cell lysates were separated on a 7.5% SDS polyacrylamide gel, transferred to PVDF, and immunoblotted with antibodies that specifically recognize Akt phosphorylated on residues Thr308 (p-T308 Akt) or Ser473 (p-S473 Akt) as indicated by arrows. The lower panel shows relative amounts of Akt in cell lysates. The graph shows levels of induced phosphorylation as measured by densitometry and corrected for loading. Data are representative of at least five experiments. B, A20 and A20.4 cells were stimulated with 1 μg/ml goat anti-mouse IgG F(ab)2 for 2 min, and further activation of Akt was halted by the addition of 100 nM wortmannin (wort) and 5 μM LY294002 (LY). Thereafter, kinetics of Akt dephosphorylation on Thr308 and Ser473 was monitored with phosphospecific antibodies as in A. The lower panel shows relative amounts of Akt in cell lysates. The graph shows levels of induced phosphorylation as measured by densitometry and corrected for loading. These data represent one of three separate experiments. C, A20.4 cells were transfected with a murine CD19 expression construct, and stable lines were generated. Transfected A20.4, as well as parent A20.4 and A20 cells, were stimulated with 0.5 μg/ml anti-IgG F(ab)2 for the indicated time points. Akt phosphorylation on Thr308 and Ser473 was monitored as in A. The graph shows levels of induced phosphorylation as measured by densitometry and corrected for loading. These data represent one of three separate experiments.
CD19-dependent Activation of Akt Kinase

**Fig. 4. CD19 promotes Akt activation in splenic B-cells.** A, purified splenic B-cells from wild-type (wt) or CD19-deficient (CD19−/−) mice were stimulated with 1 or 10 µg/ml goat anti-mouse IgG Fab'2 fragments in the presence or absence of 100 nM wortmannin (wort) for the indicated periods of time. Cell lysates from CD19−/− and wild-type B-cells were resolved on separate 7.5% SDS polyacrylamide gels, cotransferred to PVDF, and incubated with antibodies that specifically recognize Akt phosphorylated on residues Thr308 (p-T308 Akt) or Ser473 (p-S473 Akt) as indicated by arrows. The lower panel shows relative amounts of Akt in cell lysates. Western blots from CD19−/− and wild-type mice were developed by enhanced chemiluminescence and are coexposed for identical time periods. Densitometry readings were made with NIHImage software and are corrected for loading controls. These data represent one of four separate experiments. B, synergistic activation of Akt by CD19 and sIg was assessed using suboptimal (0.01 or 0.05 µg/ml) doses of biotinylated goat anti-mouse IgM F(ab')2 fragments in the presence of varying levels of biotinylated anti-CD19 antibody and 10 µg/ml avidin. sIgM/CD19 coligation-induced phosphorylation on Thr308 and Ser473 was compared with sIgM or CD19 cross-linking alone. The lower panel shows relative amounts of Akt in cell lysates. The graph displays densitometry readings of p-Ser473 (solid line) and p-Thr308 (dotted line) of anti-CD19 stimulations without (open symbols) or with (solid symbols) 0.01 µg/ml anti-IgM. These data represent one of three experiments.

Akt or preventing its inactivation, we performed pulse-chase experiments on activated Akt. A20 and A20.4 cells were stimulated with anti-IgG F(ab')2 fragments for 2 min; further PISK-mediated activation of Akt was blocked with wortmannin, and the amount of Akt phosphorylation at positions Thr308 and Ser473 was measured at the indicated time points (Fig. 3B). Although BCR-induced Akt phosphorylation on Thr308 and Ser473 was reduced in A20.4 cells, pulse-chase studies showed comparable rates of dephosphorylation in both cell lines (Fig. 3B). The similar rates of dephosphorylation in A20 and A20.4 cells suggest that CD19 acts to promote Akt kinase activity by induced phosphorylation of Akt, as opposed to preventing dephosphorylation or degradation. To show that reduced Akt phosphorylation in the A20.4 cells was due solely to the absence of CD19, A20.4 cells were transfected with a murine CD19 cDNA construct and stimulated as described above. Phosphorylation of Akt on both sites was restored in A20.4 cells expressing CD19 (Fig. 3C).

**Reduced Phosphorylation of Akt in CD19−/− Splenic B-cells—**Akt phosphorylation and activity varies widely in transformed B-cell lines representing various stages of B-cell differentiation (21) and may in fact be a contributor to, or a consequence of, the transformed state. We therefore examined regulation of Akt activity in primary splenic B-cells from wild-type and CD19−/− mice. Wild-type splenic B-cells showed rapid induction of Akt phosphorylation on Thr308 and Ser473 at both optimal (10 µg/ml) and suboptimal (0.1 µg/ml) levels of sIg cross-linking, whereas CD19-deficient cells exhibited minimal Akt phosphorylation at either Thr308 or Ser473 even at the higher level of sIg cross-linking (Fig. 4A). Moreover, whereas wild-type B-cells achieved a peak state of phosphorylation within 1 min of induction, CD19−/− cells showed delayed kinetics of 2–5 min. This was not attributed to inefficient engagement of the BCR as both cell populations displayed similar extracellular signal-regulated kinase 1/2 phosphorylation at 2 min post-stimulation (data not shown). These data correlate with the loss of Akt-dependent GSK-3 phosphorylation seen at later time points in CD19−/− splenic B-cells (Fig. 2C).

**CD19 Coligation with the BCR Augments Akt Phosphorylation—**Much of the attention to CD19 has focused on its ability to augment signaling through the BCR (22). As CD19 is also thought to be the primary signal transducing component for the CD21(CR2)/CD19/CD81 coreceptor complex, this effect may be attributed to the recruitment of downstream signaling intermediates following B-cell recognition of C3d-bearing antigen (23). In addition, CD19 appears to be a nominal component of the BCR complex as it is rapidly phosphorylated upon cross-linking of sIg alone. To address whether CD19 can synergize with sIg in promoting Akt activation, splenic B-cells were stimulated with suboptimal doses of sIg cross-linking in the pres-
CD19-dependent Activation of Akt Kinase

Fig. 5. Igβ-induced activation of Akt is CD19-dependent. A, purified splenic B-cells from wild-type (wt) or CD19-deficient (CD19−/−) mice were stimulated with 1 μg/ml of anti-Igβ antibody for the indicated periods of time. Cell lysates were separated on a 7.5% SDS polyacrylamide gel, transferred to PVDF, and immunoblotted with antibodies that specifically recognize Akt phosphorylated on residues Thr308 (p-T308 Akt) or Ser473 (p-S473 Akt) as indicated by arrows. The lower panel shows relative amounts of Akt in cell lysates. Densitometry readings were analyzed using the NIHimage software and were corrected for loading controls. B, A20 and A20.4 cells were stimulated with anti-Igβ and were assessed for Akt phosphorylation as in A. The lower panel shows relative amounts of Akt in cell lysates. These data represent one of three experiments.

In summary, our findings support a prominent role for the B-cell coreceptor, CD19, in the regulation of BCR-induced Akt kinase activity in normal B-cells. As a group, the B-cell coreceptors (e.g., CD19, CD21, CD22, and FcyRIIB) are receiving increased attention because of their pivotal roles as contextual molecules involved in determining cell fate decisions at distinct stages of B-cell differentiation. CD19 has been implicated as a proximal downstream target to effect both positive and negative regulation of BCR-mediated signals. In the latter context, several groups have noted decreased tyrosine phosphorylation of CD19 following coligation of slg and FcγRIIB (4, 16, 18). This is likely because of FcγRIIB-mediated recruitment and activation of SHP-1, resulting in impaired SH2-mediated binding of the p85 subunit of PI3K to the cytoplasmic tail of CD19. Recent reports have also shown that FcγRIIB coligation with slg results in reduced Akt activation (16–18, 24), most probably through the activation of the SH2-containing inositol-5-phosphatase, which acts on phosphatidylinositol-3,4,5-triphosphate. Thus, CD19 is likely a central target for inducing and later down-regulating Akt activity. This function may contribute to its key role in promoting B-cell lymphopoiesis and antibody responses in vivo.

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