Syk and Bruton’s Tyrosine Kinase Are Required for B Cell Antigen Receptor-mediated Activation of the Kinase Akt*

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Activation of Akt by multiple stimuli including B cell antigen receptor (BCR) engagement requires phosphatidylinositol 3-kinase and regulates processes including cell survival, proliferation, and metabolism. BCR cross-linking activates three families of non-receptor protein tyrosine kinases (PTKs) and these are transducers of signaling events including phospholipase C and mitogen-activated protein kinase activation; however, the relative roles of PTKs in BCR-mediated Akt activation are unknown. We examined Akt activation in Lyn-, Syk- and Btk-deficient DT40 cells and B cells from Lyn−/− mice. BCR-mediated Akt activation required Syk and was partially dependent upon Btk. Increased BCR-induced Akt phosphorylation was observed in Lyn-deficient DT40 cells and Lyn−/− mice compared with wild-type cells suggesting that Lyn may negatively regulate Akt function. BCR-induced tyrosine phosphorylation of the phosphatidylinositol 3-kinase catalytic subunit was abolished in Syk-deficient cells consistent with a receptor-proximal role for Syk in BCR-mediated phosphatidylinositol 3-kinase activation; in contrast, it was maintained in Btk-deficient cells, suggesting Btk functions downstream of phosphatidylinositol 3-kinase. Calcium depletion did not influence BCR-induced Akt phosphorylation/activation, showing that neither Syk nor Btk mediates its effects via changes in calcium levels. Thus, BCR-mediated Akt stimulation is regulated by multiple non-receptor PTK families which regulate Akt both proximal and distal to phosphatidylinositol 3-kinase activation.

The B cell antigen receptor complex (BCR) is composed of antigen-binding membrane Ig and Igα/Igβ heterodimers, which are the signaling components of the BCR. BCR engagement induces rapid activation of three different families of non-receptor protein tyrosine kinases (PTKs) (1–4). These include Lyn, Fyn, and Blk of the Src family PTKs, Syk of the ZAP-70/Syk family PTKs, and Btk of the Tec family PTKs. BCR ligation subsequently leads to the induction of downstream signaling events including stimulation of phospholipase C-γ2 (PLC-γ2), Ras, and at least three subfamilies of mitogen-activated protein kinases, phosphorylation of Vav and HS-1 and activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) (1–4). The PTK requirements for some of these signaling events including PLC-γ2 tyrosine phosphorylation, calcium signaling, and the activation of various serine/threonine kinases including mitogen-activated protein kinases (e.g. ERK, JNK, p38 MAPK), p70S6K, p90Rsk, and PKC(θ) have been characterized in some detail (5–9). However, the role of non-receptor PTKs in BCR-dependent stimulation of components of the PtdIns 3-kinase pathway remains to be defined in detail.

CD19 is necessary for BCR-mediated PtdIns 3-kinase activation, and this requirement probably involves binding of the p85 regulatory subunit of PtdIns 3-kinase to two YXX motifs in the cytoplasmic region of CD19 (10, 11). The Src family PTK Lyn also appears to play a role in BCR-induced PtdIns 3-kinase stimulation. Upon BCR cross-linking, Lyn associates via its Src homology 3 (SH3) domain with PtdIns 3-kinase, albeit a small percentage of total cellular PtdIns 3-kinase. This interaction may augment lipid kinase activity at least in vitro (12, 13). On the other hand, both Syk and the PtdIns 3-kinase p85 regulatory subunit have been shown to associate upon BCR cross-linking with the adaptor c-Cbl (14). However, whether Syk is necessary for PtdIns 3-kinase activation remains unclear.

Akt is a protein serine/threonine kinase that can be activated by ligand binding to a diverse range of cell-surface receptors including the BCR (15, 16). Akt phosphorylates cellular targets involved in multiple biological processes including apoptosis (17, 18), glycerogen metabolism (19), and gene transcription (20, 21). The activation of Akt appears to require type-1 PtdIns 3-kinases and their lipid products and/or metabolites, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) (22, 23). According to one model, binding of PtdIns(3,4,5)P3/PtdIns(3,4)P2 to the pleckstrin homology domain of Akt causes a proportion of Akt to translocate to the plasma membrane, whereupon it undergoes conformational changes and is subsequently phosphorylated on Thr308 and Ser473 by PtdIns(3,4,5)P3-dependent kinase-1 and integrin-linked kinase/PtdIns(3, 4, 5)P3-dependent kinase-2, respectively, in a PtdIns 3-kinase-dependent manner (24–26). Phosphorylation of both residues is necessary for maximal activation of Akt.

While BCR engagement has also been recently reported to activate Akt in a PtdIns 3-kinase-dependent manner (16), the individual PTKs necessary for BCR-mediated Akt stimulation
BCR-induced Akt Activation Requires Syk and Btk

have not been delineated. In this study we define the PTKs involved in regulating Akt after BCR cross-linking.

EXPERIMENTAL PROCEDURES

Reagents—Mouse anti-chicken IgM (M4) mAb was prepared as described previously (27). F(ab')2 fragments of goat anti-mouse IgM and IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Murine Ig and Akt substrate peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human Akt phosphoserine 473-specific sera, rabbit anti-mouse Akt phosphothreonine 308-specific sera, rabbit anti-Akt mouse polyclonal antibodies, and rabbit anti-human phospho-ERK serum were purchased from New England Biolabs (Beverly, MA). Polyclonal sheep anti-rat Akt serum (catalog numbers 06-558 and 06-608), polyclonal anti-PtdIns 3-kinase p85 sera (catalog number 05-212, for Western blotting and catalog number 06-195, for immunoprecipitation) and anti-phosphoarginine (4G10) mAb were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-human Btk mAb was purchased from Pharmingen (San Diego, CA). Histone 2B (H2B) was obtained from Roche Molecular Biochemicals. Wortmannin and LY294002 were purchased from Calbiochem (La Jolla, CA). U0126 was obtained from Promega (Madison, WI).

Cells and Cell Lines—Murine WEHI-231 cells were cultured at a density of 106 cells/ml in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, 2 mM l-glutamine, 1 mM pyruvate, and 50 μM 2-mercaptoethanol at 37 °C and 5% CO2. Parental chicken DT40 cells and DT40 mutant cell lines with deletions of Syk, Lyn, Btk, or PLC-γ2 have been described and were cultured in the same medium as described above supplemented additionally with 1% heat-inactivated chicken serum (5, 6, 28). Cells were grown at a density of 3 × 106 cells/ml or less. Splenic B cells were isolated from age-matched young (≤7 weeks old) normal mice or Lyn−/− mice following lysis of erythrocytes with Gey’s solution and depletion of T cells with a mixture of anti-CD3, -CD4, and -CD8 antibodies and complement lysis.

Western Blot Analysis of Akt/PKB Phosphorylation—DT40, WEHI-231, or isolated splenic B cells from wild-type or Lyn−/− mice were resuspended at 106 cells/ml in ice-cold PBS supplemented with 1% heat-inactivated fetal bovine serum and 5 μM Akt inhibitor-1 (PKI) and 2.5 μl of MK-2206 (H2B) exogenous substrate and reactions initiated by the addition of 10 μl of [γ-32P]MgATP solution (75 μM MgCl2, 0.5 mM ATP, 10 μCi of [γ-32P]ATP). Kinase reactions were performed for 15 min at 30 °C and terminated by the addition of 55 μl of 2 × Laemmli buffer and subsequent boiling for 5 min. Following brief centrifugation, samples were analyzed by 15% SDS-PAGE and transferred to nitrocellulose. The upper portion of the gel was transferred to a nitrocellulose membrane and immuno-blotted with rabbit polyclonal anti-Akt antibody to detect immunoprecipitated Akt. The lower portion of the gel was stained with Coomasie Brilliant Blue G-250 to visualize H2B, destained and dried prior to autoradiography—at 70 °C for various times.

Alternatively, in vitro kinase assays were performed using 30 μM Akt substrate heptapeptide (RPRAATF). Following incubation for 15 min at 30 °C, reactions were briefly centrifuged and half of the supernatant was spotted onto phosphocellulose paper (2.5 × 2.5 cm; Whatman P81). After washing papers 3–4 times with 0.75% (v/v) phosphoric acid for 10 min per wash, followed by one wash with acetone, papers were dried and 32P incorporation into the peptide was quantified by liquid scintillation analysis. The remaining portion of the reaction mixture was analyzed by SDS-PAGE, in order to assess the recovery of Akt in immunoprecipitates, as described above.

Immunoprecipitation and Western Blot Analysis of PtdIns 3-Kinase Tyrosine Phosphorylation—10 × 106 cells were resuspended in 1 ml of complete RPMI 1640 medium for 5 min at 37 °C prior to stimulation with 10 μg/ml anti-IgM (M4) mAb for the indicated times at 37 °C. Following anti-IgM treatment, incubations were terminated on ice by dilution with 10 volumes of ice-cold PBS and centrifuged at 500 × g for 5 min at 4 °C. Supernatants were aspirated, washed again with 1 ml of ice-cold PBS, and cells lysed by resuspension in 0.5 ml of PtdIns 3-kinase lysis buffer (50 mM Tris-HCl, pH 7.4, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM pepstatin, 1 mM Na3VO4, 1 μM E-64, 1 μM Na3VO4, 0.1% 2-mercaptoethanol) and (ii) 1 ml of Akt lysis buffer. After electrophoresis, the upper portion of the gel was transferred to a nitrocellulose membrane and immuno-blotted with rabbit polyclonal anti-Akt antibody to detect immunoprecipitated Akt. The lower portion of the gel was stained with Coomasie Brilliant Blue G-250 to visualize H2B, destained and dried prior to autoradiography—at 70 °C for various times.

RESULTS

Bcr-induced Akt Activation Is PtdIns 3-Kinase-dependent—Using both murine WEHI-231 and chicken DT40 B cells, we tested whether BCR engagement resulted in Akt activation. Murine WEHI-231 cells were either unstimulated or stimulated with F(ab')2 fragments of anti-mouse IgM while chicken DT40 cells were treated with mouse anti-chicken IgM or mouse Ig as a control stimulus. BCR engagement rapidly and strongly increased Akt activity in both B cell lines with a maximal 6–7-fold activation observed after 5–10 min (Fig. 1 A and B), under conditions where equivalent amounts of Akt were immunoprecipitated in each sample. Treatment of DT40 cells with mouse Ig as a negative control increased Akt activity less than 2-fold (Fig. 1B). Moreover, the increase in Akt activity following BCR stimulation corresponded with Akt phosphorylation on serine 473 in both mouse WEHI-231 (Fig. 1A) and chicken DT40 B cells (Fig. 3B) as detected by immunoblotting using a phospho-specific serine 473 anti-Akt antibody. However, at extended times of stimulation with anti-IgM, the magnitude of BCR-mediated Akt activation decreased while phosphorylation on serine 473 was maintained.

Since the activation of Akt in response to growth factors (including platelet-derived growth factor, epidermal growth
factor, and nerve growth factor), cytokines (interleukin-3, interleukin-4, stem cell factor, and granulocyte/macrophage colony-stimulating factor) and insulin depends upon PtdIns 3-kinase activity (15, 29), we tested whether BCR-mediated Akt activation was also downstream of PtdIns 3-kinase using two potent pharmacological and structurally different PtdIns 3-kinase inhibitors, wortmannin and LY294002 (30, 31). Preincubation of either DT40 or WEHI-231 cells with wortmannin or LY294002, respectively, prior to treatment with anti-IgM blocked BCR-induced Akt stimulation and serine 473 phosphorylation in a dose-dependent manner (Fig. 2, A and B). These results show that BCR engagement activates Akt in a PtdIns 3-kinase-dependent fashion.

BCR-mediated Akt Activation Requires Syk and Btk—BCR cross-linking has been reported to induce association between the Src family PTK member Lyn and a proportion of the cellular PtdIns 3-kinase p85 regulatory subunit (12). As BCR-induced Akt stimulation is PtdIns 3-kinase-dependent (Fig. 2), we tested whether Lyn was required for Akt activation in response to BCR engagement. In addition, we also examined whether other BCR signaling effectors such as the ZAP-70/Syk PTK family member Syk and the Tec PTK family member Btk were required for BCR-mediated Akt activation. We compared BCR-induced Akt stimulation in chicken DT40 wild-type and mutant cells deficient in Lyn, Syk, and Btk that expressed comparable levels of surface IgM (5, 6). Surprisingly, BCR-mediated Akt activation in Lyn-deficient B cells was similar to that in wild-type B cells, suggesting that Lyn is not required for BCR-mediated Akt activation. However, BCR-mediated Akt activation was significantly reduced in Syk-deficient and Btk-deficient B cells, indicating that Syk and Btk are required for BCR-mediated Akt activation. These results suggest that BCR engagement activates Akt in a Lyn-independent, Syk-dependent, and Btk-dependent fashion.

![Fig. 1. BCR engagement activates and phosphorylates Akt on serine 473 in both mouse and chicken B cells.](http://www.jbc.org/)

![Fig. 2. BCR-mediated Akt activation is PtdIns 3-kinase-dependent.](http://www.jbc.org/)
PTK-deficient DT40 cells were treated as described in the legend to Fig. 1. Akt was immunoprecipitated from Triton X-100 cell lysates and its specific Akt (upper panel) determined as described in the legend to Fig. 1. Total cell lysates were prepared using RIPA buffer, resolved by 10% SDS-PAGE, and immunoblotted with either anti-phosphotyrosine (4G10) mAb (lower panel) or anti-IgM (M4) mAb (upper panel). In panel C, arrows indicate the positions of prominent anti-IgM-inducible tyrosine-phosphorylated proteins which were reduced in Lyn-deficient compared with wild-type cells.

D

FIG. 3. BCR-induced Akt activation requires Syk and Btk. A, wild-type or various PTK-deficient DT40 cells (20 × 10^3/sample) were stimulated with 10 μg/ml anti-IgM (M4) mAb for the indicated times. Akt was immunoprecipitated from Triton X-100 cell lysates and its activity determined as described in the legend to Fig. 1A (upper panel). The upper portion of the gel was excised, electrotransferred to nitrocellulose, and the quantity of immunoprecipitated Akt determined by Western blotting using anti-Akt (lower panel). B, wild-type or various PTK-deficient DT40 cells were treated as described in A for 0–90 min. Total cell lysates were prepared using RIPA buffer, resolved by 10% SDS-PAGE, and immunoblotted with either anti-phosphoserine 473-specific Akt (upper panel) or anti-Akt (lower panel). Abs. C and D, wild-type or various PTK-deficient DT40 cells were stimulated with 10 μg/ml anti-IgM (M4) mAb for 0–30 min. Whole cell lysates were prepared using RIPA buffer, separated by 10% SDS-PAGE, and immunoblotted with either anti-phosphotyrosine (4G10) mAb (C) or anti-Btk mAb (D). In panel C, arrows indicate the positions of prominent anti-IgM-inducible tyrosine-phosphorylated proteins which were reduced in Lyn-deficient compared with wild-type cells.

to anti-IgM-induced stimulation of Akt in wild-type B cells, with maximal activation after 7.5 min (Fig. 3A). In contrast, anti-IgM-mediated Akt activation was completely abolished in Syk-deficient cells and strongly reduced in Btk-deficient chicken B cells (Fig. 3A), indicating that both Syk and Btk were required for BCR-induced Akt stimulation. No significant differences in endogenous Akt protein expression levels were observed between the parental and various PTK-deficient DT40 B cell lines (Fig. 3A, lower panel). Moreover, similar recoveries of Akt were observed in immunoprecipitates isolated from wild-type and various PTK-deficient DT40 cells (Fig. 3A, lower panel).

Since stimulus-induced Akt activation requires phosphorylation on both Thr^308 and Ser^473 (24), we also measured Akt activation indirectly by analyzing Akt phosphorylation on Ser^473 using immunoblotting and a phospho-specific Ser^473 anti-Akt antibody. Similar to Akt in vitro kinase assays, anti-IgM treatment induced a strong and rapid increase in Akt phosphorylation on Ser^473 in chicken DT40 cells (Fig. 3B, upper panel). Consistent with our in vitro kinase data, anti-IgM-mediated Akt phosphorylation on Ser^473 was completely abolished in Syk-deficient cells and strongly reduced in Btk-deficient cells (Fig. 3B, upper panel), while comparable amounts of Akt protein were detected in parental and various PTK-deficient cells (Fig. 3B, lower panel). Interestingly, BCR-mediated Akt phosphorylation on Ser^473 was moderately but consistently enhanced in Lyn-deficient cells relative to wild-type cells (Fig. 3B, upper panel), suggesting that Lyn performs a negative role in regulating BCR-induced Akt phosphorylation on Ser^473.

In contrast, the profiles of anti-IgM-induced protein tyrosine phosphorylation in parental and Lyn-deficient DT40 cells were markedly different: major inducibly tyrosine-phosphorylated bands of approximately 55 and 70–75 kDa were strongly reduced in Lyn-deficient cells (Fig. 3C), showing that the absence of Lyn expression in these cells has profound effects on BCR-mediated signaling. The reduced ability to activate Akt following anti-IgM treatment in Btk-deficient cells was not due to a general defect in signaling in these cells, since there were no obvious differences in the patterns of inducible protein tyrosine phosphorylation in Btk-deficient compared with wild-type cells (Fig. 3C). The absence of Btk expression in Btk-deficient cells compared with parental cells and other PTK-deficient DT40 cells was also confirmed by Western blotting (Fig. 3D).

We also compared Akt phosphorylation on Ser^473 following BCR engagement in splenic B cells from wild-type and Lyn^−/− young mice. Anti-IgM-induced Akt phosphorylation on Ser^473 in B cells from Lyn^−/− mice was consistently increased relative to wild-type mice (Fig. 4A, upper panel). The protein expression levels of Akt in B cells from Lyn^−/− mice did not differ from those in wild-type mice (Fig. 4A, lower panel). In addition, we also compared BCR-mediated Akt phosphorylation on Thr^308 in wild-type and Lyn-deficient chicken DT40 cells using a phospho-specific antibody. Anti-IgM-mediated Akt phosphorylation on Thr^308 was also moderately enhanced in Lyn-deficient cells compared with wild-type cells (Fig. 4B). Similar results were

Fig. 4. Akt is hyperphosphorylated in Lyn^−/− mice compared with wild-type mice. A, splenic B cells from either wild-type (+/+) or Lyn^−/− mice were stimulated with 20 μg/ml anti-IgM (M4), anti-IgM (−IgM, lanes 2, 4, 6, and 8) or 20 μg/ml F(ab')2 anti-IgG control (−IgM; lanes 1, 3, 5, and 7) for 2 min. Whole cell lysates (2.5 × 10^6/sample) were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with either anti-phosphoserine 473-specific Akt (upper) or anti-Akt (lower) Abs. B, wild-type or Lyn-deficient DT40 cells were treated as described in the legend to Fig. 3B for 0–30 min. Total cell lysates were prepared using RIPA buffer, resolved by 10% SDS-PAGE, and immunoblotted with anti-phosphothreonine 308-specific Akt Ab.
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Calcium is not required for BCR-mediated Akt phosphorylation. A, PLC-γ2-deficient DT40 cells were stimulated for 0–90 min with 10 μg/ml anti-chicken IgM (M4) mAb. B, DT40 cells were pretreated with either BAPTA-AM (10 μM) for 10 min, or cyclosporin A (150 ng/ml) for 30 min or EGTA (2 mM) for 15 min prior to stimulation with 10 μg/ml anti-chicken IgM (M4) mAb for 0–60 min. The concentrations of BAPTA-AM and EGTA were titrated to chelate intra- and extracellular calcium, respectively, and thereby prevent calcium mobilization and extracellular calcium influx, respectively (9). In both panels A and B, whole cell lysates were prepared using RIPA buffer, resolved by 10% SDS-PAGE, and immunoblotted with either anti-phosphoserine 473-specific Akt (upper) or anti-Akt (lower) Abs.

Also seen in splenic B cells from Lyn−/− mice, where Thr208 of Akt was hyperphosphorylated upon BCR cross-linking relative to wild-type cells (data not shown). Taken together, these results suggest that BCR-mediated Akt activation requires both Syk and Btk, whereas Lyn may play a negative role in Akt stimulation.

**BCR-induced Akt Activation Does Not Require Intra- or Extracellular Calcium**—The positive role of Syk and Btk, but not Lyn, in BCR-mediated Akt activation is similar to the pattern of anti-IgM-induced calcium fluxes in these PTK-deficient DT40 cells (5, 6), suggesting that either intra- or extracellular calcium may regulate BCR-mediated Akt-stimulated. Since anti-IgM-mediated Akt phosphorylation on Ser473 correlated with Akt activation (Fig. 3), we used immunoblotting with the phospho-specific Ser473 anti-Akt antibody to test the role of calcium in BCR-induced Akt activation. First, wild-type or PLC-γ2-deficient DT40 cells, which do not mobilize intracellular calcium in response to anti-IgM treatment (28), were either left untreated or stimulated with anti-IgM and then, Akt phosphorylation on Ser473 was detected by Western blotting. BCR-induced Akt phosphorylation on Ser473 was still observed in the PLC-γ2-deficient cells (Fig. 5A), although these cells reproducibly had a higher basal level of Akt phosphorylation compared with parental cells.

These results suggested that PLC-γ2 is not required for BCR-mediated Akt activation and also infer that calcium fluxes do not regulate BCR-mediated Akt stimulation. To directly test this possibility, prior to stimulation with anti-IgM, wild-type DT40 cells were pretreated with EGTA, which chelates extracellular calcium, cell-permeant BAPTA-AM, which chelates both intra- and extracellular calcium, or cyclosporin A, a potent inhibitor of calcineurin. The doses of BAPTA-AM and EGTA were titrated to prevent intracellular calcium release and extracellular calcium influx, respectively (9). Neither EGTA, BAPTA-AM, nor cyclosporin A pretreatment had any significant effect on BCR-induced Akt phosphorylation (Fig. 5B), under conditions we previously have shown block BCR-mediated JNK/SAPK activation (9). Thus, BCR-mediated activation of Akt is both calcium- and calcineurin-independent.

**Regulated—Anti-IgM-induced ERK2 activation also required Syk and Btk** (9). To test the possibility that either the PtdIns 3-kinase pathway was required for BCR-mediated ERK2 stimulation or vice versa, we first examined the effect of two pharmacological PtdIns 3-kinase inhibitors, wortmannin and LY294002, on anti-IgM-induced ERK2 phosphorylation, which correlated closely with ERK2 activation in these cells (9). Under conditions where wortmannin or LY294002 completely blocked BCR-mediated Akt phosphorylation, anti-IgM-induced phosphorylation of ERK2 was unaffected (Fig. 6), showing that PtdIns 3-kinase activity was not required for BCR-mediated ERK2 activation. Second, the effect of U0126, an inhibitor of MEK1/2, two kinases upstream of ERK2 (32), was tested on anti-IgM-dependent Akt phosphorylation. The MEK1/2 inhibitor, U0126, did not affect anti-IgM-mediated Akt phosphorylation, under conditions where ERK2 phosphorylation was completely blocked (Fig. 6), demonstrating that activation of the ERK pathway was not necessary for BCR-mediated Akt stimulation.

**BCR-induced Tyrosine Phosphorylation of the PtdIns 3-Kinase p110 Catalytic Subunit Requires Syk but Neither Btk nor Lyn**—While our earlier results showed that BCR-mediated Akt activation was both PtdIns 3-kinase-dependent and required Syk and Btk, it remained to be determined whether Syk and/or Btk were upstream or downstream of PtdIns 3-kinase activation. Recent studies have shown that the activation of other Tec PTK family members such as Etk and Itk require PtdIns 3-kinase activation in response to interleukin-6 treatment and CD28 cross-linking, respectively, and are hence downstream targets of PtdIns 3-kinase (33, 34). Inducible tyrosine phosphorylation of the p110 catalytic and p85 regulatory subunits of PtdIns 3-kinase correlates with anti-IgM-mediated PtdIns 3-kinase activation in various B cell lines (35). Hence, we tested whether Syk, Btk, or Lyn were required for BCR-induced tyrosine phosphorylation of PtdIns 3-kinase subunits in either wild-type or various PTK-deficient DT40 B cells. Immunoprecipitates of the p85 PtdIns 3-kinase regulatory subunit, which also co-precipitated the p110 catalytic subunit of PtdIns 3-kinase, were isolated from either unstimulated or anti-IgM-stimulated DT40 cells and subjected to immunoblotting with anti-phosphotyrosine mAb. BCR cross-linking induced a rapid increase in tyrosine phosphorylation of the p110 catalytic subunit, although tyrosine phosphorylation of the p85 regulatory subunit was undetectable in anti-IgM treated DT40 cells (Fig. 7, upper panel, data not shown).
similar preferential BCR-induced tyrosine phosphorylation of the p110 catalytic subunit, but not the p85 subunit of PtdIns 3-kinase, was also seen in the human Daudi B cell line (data not shown). Anti-IgM-induced tyrosine phosphorylation of the PtdIns 3-kinase p110 catalytic subunit was completely abolished in Syk-deficient cells (Fig. 7, upper panel), showing that Syk was required for BCR-mediated tyrosine phosphorylation of the PtdIns 3-kinase p110 catalytic subunit. In contrast, BCR-induced tyrosine phosphorylation of the PtdIns 3-kinase p110 catalytic subunit was maintained in Btk-deficient cells and enhanced in Lyn-deficient cells relative to wild-type cells (Fig. 7, upper panel). Thus, Btk is not required for BCR-mediated tyrosine phosphorylation of the PtdIns 3-kinase p110 catalytic subunit and Lyn may play a negative regulatory role in PtdIns 3-kinase tyrosine phosphorylation. Comparable levels of PtdIns 3-kinase p85 regulatory subunit were detected in immunoprecipitates from parental and mutant chicken B cell lines (Fig. 7, lower panel).

**Discussion**

The chicken DT40 B cell line does not express detectable levels of many Src family PTK members including Src, Lck, Fyn, Blk, Yes, and Hck or the ZAP-70/Syk family PTK member, ZAP-70 (5). The predominant Src and ZAP-70/Syk PTK family members expressed in these cells are Lyn and Syk, respectively (5). In addition, DT40 cells express the Tec family PTK member, Btk. The high efficiency at which homologous recombination occurs in the DT40 cell line has facilitated the generation of Lyn-, Syk-, and Btk-deficient DT40 cells to genetically study the roles of these specific PTKs in receptor-mediated signaling events.

The serine/threonine kinase Akt has emerged as a major target of PtdIns 3-kinase and has recently been reported for the first time to be stimulated in response to BCR engagement (16). However, the individual PTK requirements for BCR-mediated Akt activation were not investigated. Our studies show that BCR-induced Akt activation requires both Syk and Btk, but rather surprisingly Lyn does not appear to play a positive role in Akt activation. Instead, Lyn may serve as a negative modulator of Akt function (Figs. 3 and 4). A similar requirement for Syk and Lyn, but not Btk, was also observed for both BCR-mediated Ca\(^{2+}\) signaling and JNK1 activation, which is itself dependent upon intracellular Ca\(^{2+}\) levels (5, 6, 9), suggesting that Ca\(^{2+}\) levels may regulate BCR-induced Akt activation. However, neither EGTA, a chelator of extracellular Ca\(^{2+}\), nor BAPTA-AM, which chelates both intra-and extracellular Ca\(^{2+}\), affected BCR-mediated Akt activation/phosphorylation (Fig. 5B). Furthermore, BCR-induced Akt phosphorylation was not significantly reduced in PLC-γ2-deficient cells compared with wild-type DT40 cells (Figs. 3B and 5A). Thus, not only can we confirm that extracellular Ca\(^{2+}\) is not required for BCR-induced Akt activation (16), but also can conclude that intracellular Ca\(^{2+}\) levels do not regulate Akt activation upon BCR cross-linking. Activation of Akt in response to epidermal growth factor treatment in 3T3 fibroblasts is also calcium independent (36). In addition, the Ca\(^{2+}\)-dependent and cyclosporin A-sensitive protein phosphatase, calcineurin, is not required for BCR-mediated Akt activation (Fig. 5B).

In contrast to Syk and Lyn, which together appear to be required for the majority of inducible BCR-mediated tyrosine phosphorylation of cellular substrates, at least in chicken DT40 B cells (6), Btk does not appear to play a major role in mediating protein tyrosine phosphorylation. Indeed, the patterns of tyrosine phosphorylation in wild-type and Btk-deficient DT40 cells are not obviously different (6) (Fig. 3C). Our studies have defined an additional regulatory role for Btk in BCR-mediated signaling. Previously characterized functions for Btk in BCR-dependent signaling include regulatory roles in the tyrosine phosphorylation of PLC-γ2 and subsequent calcium mobilization, PKC\(\mu\) activation, and ERK2 stimulation (6–7, 9, 37). Our results are consistent with a model in which Syk is an upstream regulator of both BCR-induced PtdIns 3-kinase and Akt activation, whereas Btk regulates BCR-mediated Akt activation downstream of PtdIns 3-kinase (Fig. 8). Consistent with one prediction of our model, the activation of other Tec family members such as Itk in response to Src- or CD28-induced activation and Etk upon interleukin-6 stimulation also requires PtdIns 3-kinase activity (33–34, 38). Indeed, the phenotypes of Btk\(^{-/-}\) and xid mice are strikingly similar to ps5α mice, with reduced numbers of mature peripheral B cells and diminished proliferative responses to anti-IgM, anti-CD40, and lipopolysaccharide, supporting the concept that PtdIns 3-kinase and Btk are components of a common signaling pathway (39, 40). Nevertheless, our observation that BCR-mediated ERK2 activation was not blocked by either wortmannin or LY294002 (Fig. 6), whereas ERK2 activity was partially diminished in anti-IgM-treated Btk-deficient DT40 cells (9), suggests that both PtdIns 3-kinase-dependent and -independent pathways for Btk activation may exist (Fig. 8).

Although the mechanism(s) by which Btk contributes to Akt activation require further study, our results are consistent with
a model in which the activation of PtdIns 3-kinase leads to receptor-regulated increases in PtdIns(3,4,5)P$_3$ levels. Either PtdIns(3,4,5)P$_3$ or PtdIns(3,4)P$_2$, the product of PtdIns(3,4,5)P$_3$ metabolism by the inositol polyphosphate 5-phosphatase SHIP, subsequently binds to the pleckstrin homology domain of Btk facilitating its translocation to the plasma membrane and its subsequent autophosphorylation and activation. Membrane-localized complexes of PtdIns(3,4,5)P$_3$/Btk may then serve as adaptors to facilitate close contact between PtdIns(3,4,5)P$_3$ and its putative targets such as PtdIns(3,4,5)P$_3$-dependent kinase-1 and/or Akt or, as proposed recently, proximal associations between PtdIns(4,5)P$_2$ and activated PLC-γ (41). Whether the requirement for Btk in BCR-induced Akt activation involves other known Btk-associated proteins is one important area for future investigation. In addition, mutational analysis of Btk will facilitate identification of the structural domains of Btk which are essential for BCR-mediated Akt activation.

Our results showed that Lyn does not play a positive role in BCR-induced Akt activation. Indeed, in both Lyn-deficient chicken DT40 and mouse Lyn$^{-/-}$ splenic B cells, hyperphosphorylation of Akt on Ser$^{473}$ and Thr$^{308}$ and also tyrosine phosphorylation of the p110 catalytic subunit of PtdIns 3-kinase were observed compared with wild-type cells (Figs. 3, 4, and 7). These findings suggest that Lyn may perform a negative regulatory role in BCR-mediated activation of the PtdIns 3-kinase/Akt pathway. The difference we observed between Akt activity and its hyperphosphorylation on both Thr$^{308}$ and Ser$^{473}$ in Lyn-deficient DT40 cells could reflect an additional role for Lyn as a possible negative regulator of SHIP or PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylate PtdIns(3,4,5)P$_3$ at the 5- and 3-positions, respectively, and are negative regulators of the PtdIns 3-kinase/Akt signaling pathway.

A negative role for Lyn in BCR signaling has been shown previously, as B cells from young Lyn$^{-/-}$ mice are hyper-responsive to anti-IgM-induced proliferation (42–44). At least some of the negative effects of Lyn may be mediated via inhibition of Btk-dependent pathways (44). Indeed, genetic studies in which a transgene expressing low levels of Btk was crossed to Lyn$^{-/-}$ mice, in which a transgene expressing low levels of Btk was crossed to Lyn$^{-/-}$ mice, subsequently binds to the pleckstrin homology domain of Btk facilitating its translocation to the plasma membrane and its subsequent autophosphorylation and activation. Membrane-localized complexes of PtdIns(3,4,5)P$_3$/Btk may then serve as adaptors to facilitate close contact between PtdIns(3,4,5)P$_3$ and its putative targets such as PtdIns(3,4,5)P$_3$-dependent kinase-1 and/or Akt or, as proposed recently, proximal associations between PtdIns(4,5)P$_2$ and activated PLC-γ (41). Whether the requirement for Btk in BCR-induced Akt activation involves other known Btk-associated proteins is one important area for future investigation. In addition, mutational analysis of Btk will facilitate identification of the structural domains of Btk which are essential for BCR-mediated Akt activation.

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