Phosphorylation of CD19 Y484 and Y515, and Linked Activation of Phosphatidylinositol 3-Kinase, Are Required for B Cell Antigen Receptor-Mediated Activation of Bruton's Tyrosine Kinase[1]

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Bruton's tyrosine kinase (Btk) plays a critical role in B cell Ag receptor (BCR) signaling, as indicated by the X-linked immunodeficiency and X-linked agammaglobulinemia phenotypes of mice and men that express mutant forms of the kinase. Although Btk activity can be regulated by Src-family and Syk tyrosine kinases, and perhaps by phosphatidylinositol 3,4,5-trisphosphate, BCR-coupled signaling pathways leading to Btk activation are poorly understood. In view of previous findings that CD19 is involved in BCR-mediated phosphatidylinositol 3-kinase (PI3-K) activation, we assessed its role in Btk activation. Using a CD19 reconstituted myeloma model and CD19 gene-ablated animals we found that BCR-mediated Btk activation and phosphorylation are dependent on the expression of CD19, while BCR-mediated activation of Lyn and Syk is not. Wortmannin preincubation inhibited the BCR-mediated activation and phosphorylation of Btk. Btk activation was not rescued in the myeloma by expression of a CD19 mutant in which tyrosine residues previously shown to mediate CD19 interaction with PI3-K, Y484 and Y515, were changed to phenylalanine. Taken together, the data presented indicate that BCR aggregation-driven CD19 phosphorylation functions to promote Btk activation via recruitment and activation of PI3-K. Resultant phosphatidylinositol 3,4,5-trisphosphate probably functions to localize Btk for subsequent phosphorylation and activation by Src and Syk family kinases. ***The Journal of Immunology*****, 1999, 162: 4438–4446.**

B cell activation induced by aggregation of the B cell Ag receptor (BCR)[3] is a complex process known to involve the proximal activation of Src-family kinases, the Tec family kinase Btk, and Syk (reviewed in Refs. 1–3). Src-family kinases and Syk are activated by virtue of their association with immunoreceptor tyrosine-based activation motifs found in the cytoplasmic tails of receptor Ig-α and Ig-β subunits, and subsequent tyrosyl phosphorylation. Btk, however, does not appear to bind Ag receptors, and spacio-temporal factors that regulate its activation and function are poorly defined.

Mutations in the Btk gene are responsible for X-linked immunodeficiency (Xid) in mice and X-linked agammaglobulinemia (XLA) in humans (4–6). In mice a mutation of amino acid 28 from cysteine to arginine results in the Xid phenotype, while in humans multiple mutations including deletions and insertions in various domains of Btk result in XLA (7). XLA patients exhibit severely reduced numbers of peripheral B cells, small lymph nodes and a lack of germinal centers. In XLA patients this reflects arrest at an early stage of B cell development and cells that escape this arrest exhibit unusually high levels of surface IgM. Additionally, serum Igs are decreased in concentration in XLA (reviewed by Ref. 8). Xid mice exhibit a similar, but less extreme, phenotype. Production and analysis of Btk knockout mice have confirmed that the Xid phenotype is solely a result of the Btk mutation (9, 10).

Btk is composed of a pleckstrin homology (PH) domain, an SH3 domain, an SH2 domain, and a catalytic region. Activation of Btk occurs by transphosphorylation of tyrosine 551 in the catalytic domain, resulting in a dramatic increase in the catalytic activity of the kinase (11–13). This allows for autophosphorylation at tyrosine 223 in the SH3 domain (14). Both Lyn and Syk have been demonstrated to be involved in BCR-mediated Btk activation (11), but processes that drive colocalization of these kinases are ill-defined. Recently, it was suggested that phosphatidylinositol 3-kinase (PI3-K) is also involved in Btk activation (15). Additionally, several groups have described the ability of the Btk PH domain to bind inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol-1,3,4,5,6-pentakisphosphate (IP_5), inositol-1,2,3,4,5,6-hexakisphosphate (IP_6) (16), and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) (17, 18). Biochemical data suggest that the Xid mutation decreases the affinity of the PH domain for IP_4 and $PI(3,4,5)P_3$ (16, 17). Structural analysis of the N-terminal portion of Btk by x-ray crystallography has confirmed these results and supports the possibility that the PH domain mutations inactivate the Btk pathway by reducing the affinity for inositol lipids (19). Thus, Ag-induced PI3-K activation and resultant BCR-localized production of $PI(3,4,5)P_3$ may drive translocation of Btk to the plasma membrane

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[3] Abbreviations used in this paper: BCR, B cell Ag receptor; Btk, Bruton's tyrosine kinase; Xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia; PH, pleckstrin homology domain; PI3-K, phosphatidylinositol 3-kinase; IP_2, inositol 1,3,4,5-biskisphosphate; IP_3, inositol 1,3,4,5-triskisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_5, inositol-1,3,4,5,6-pentakisphosphate; IP_6, inositol-1,2,3,4,5,6-hexakisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PLCγ2, phospholipase Cγ2; IMDM, improved modified Dulbecco's medium; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PVDF, polyvinylidene difluoride.

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where it is phosphorylated by Src and/or Syk family kinases. Consistent with this possibility, targeting of Btk to the plasma membrane has been shown to potentiate its activation (20).

Previously two mechanisms have been defined by which BCR aggregation increases PI3-K activity. These involve interaction of Src-family kinase SH3 domains with proline-rich sequences found in the p85 subunit of PI3-K (21). Inhibition of this interaction with proline-rich peptides derived from p85 has been shown to block BCR activation of PI3-K (21). The second mechanism involves BCR-mediated phosphorylation of CD19 tyrosines Y484 and Y515 (22). CD19 is a BCR-associated membrane protein that functions as both an accessory in receptor signaling and a BCR coreceptor (23–25). CD19 Y484 and Y515 occur in YXXM motifs known to bind to PI3-K SH2 domains. Phosphorylation of these sites following BCR aggregation leads to the recruitment and activation of PI3-K. The roles of these mechanisms in BCR-mediated activation of Btk are unknown.

In this report we have assessed the contribution of CD19 to BCR-mediated Btk activation. We used the previously described J558L myeloma system in which CD19 can be reconstituted (23) as well as splenic B cells from $CD19^{-/-}$ and Xid animals to address this question. Our data demonstrate that expression of CD19 containing Y484 and Y515 is required for BCR activation of Btk. Further BCR-mediated phosphorylation of these CD19 tyrosyl residues and consequent recruitment and activation of PI3-K are required for receptor-mediated Btk activation. Therefore, changing expression of CD19 during B cell development (26) may cause changes in the biologic response to Ag by modulating receptor activation of Btk.

Materials and Methods

Reagents and Abs

The murine B cell plasmacytoma J558Lμm3 was provided by M. Reth (Max Planck Institute, Freiburg, Germany). We previously transfected this cell line to obtain a mouse full-length (B220) CD45-expressing variant (27) and variants expressing human CD19 or human CD19Y484, Y515 (23). Rabbit polyclonal Abs against CD19, p85 of PI3-K, Syk, Lyn, and Btk, were prepared using glutathione *S*-transferase fusion proteins produced in bacteria, purified by glutathione-Sepharose chromatography, and cleaved with factor Xa. Immunogens included the unique domain for Btk, residues 1–131 for Lyn, the linker region for Syk, the SH3 domain for p85 of PI3-K, and residues 411–547 of the CD19 cytoplasmic tail. The anti-phosphotyrosine Ab (AB-2) was purchased from Oncogene Science (Cambridge, MA), and rabbit polyclonal to PLCγ2 from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit $F(ab')_2$ Ab to mouse IgG (H+L) ($F(ab')_2$RAMIG) was purchased from Zymed (San Francisco, CA). Horseradish peroxidase-conjugated protein A (Zymed) and rat anti-mouse IgG (Zymed) were used for detection with the enhanced chemiluminescence detecting system (ECL, Amersham, Arlington Heights, IL). Indo-1/AM was obtained from Molecular Probes (Eugene, OR). Geneticin G418 was purchased from Life Technologies (Gaithersburg, MD). Wortmannin was obtained from Sigma (St. Louis, MO). NP_9BSA was prepared by coupling of BSA (10 mg/ml) in 3% $NaHCO_3$ to NP-CAP-OSu (Cambridge Research Biochemicals, Cambridge, U.K.; 40 mg/ml) in dimethylformamide. After dialysis in $NaHCO_3$ and PBS the molecular ratio of NP to BSA was determined by measurement of the absorbance at OD_{430} with an extinction coefficient of 4230, pH 8.5 (NP molarity), and by Bradford analysis (BSA molarity). $CD19^{-/-}$ mice (28) were provided by R. C. Rickert (San Diego, CA).

Cell culture

Cells were propagated in improved modified Dulbecco's medium (IMDM) supplemented with 5% heat-inactivated FCS (HyClone, Logan, UT), 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mg/ml G418 (J558Lμm3CD45$^+$ CD19$^+$) at 37°C with 7% CO_2. Surface expression levels of IgM, CD45, and CD19 were monitored by FACS analysis.

Isolation of splenic B cells

Splenic B cells were prepared as previously described (23). Briefly, spleens were excised from mice, and cells were dispersed through a 100-μm pore size mesh in IMDM. RBCs were lysed using Guy's solution. Total nucleated spleen preparations were depleted of T lymphocytes by complement-mediated lysis using HO13.4 and T24 Abs and dense cells ($\rho \geq 1.066$) isolated by discontinuous Percoll density gradient centrifugation.

Flow cytometric analysis of $[Ca^{2+}]_i$ mobilization

Cells were loaded with indo-1/AM (Molecular Probes), and $[Ca^{2+}]_i$ was monitored by flow cytometry (model 50H, Ortho Diagnostic Systems, Raritan, NJ) as previously described (23). The mean $[Ca^{2+}]_i$ and percentage of cells responding were determined with an appended data acquisition system and MultiTIME software (Phoenix Flow Systems, San Diego, CA).

Analysis of protein tyrosine phosphorylation

Cells were harvested, washed once in IMDM, and resuspended at 10^7 cells/ml in IMDM. After 5-min incubation at 37°C, they were stimulated with NP_9BSA (5 μg/ml), pelleted in a picofuge, resuspended in lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 1% Nonidet P-40, 1 mM PMSF, and 2 μg/ml each of leupeptin, aprotinin, and α_1-antitrypsin), and incubated on ice for 10 min. Lysates were centrifuged for 5 min at 14,000 rpm. Cleared lysates were incubated with appropriate Abs and protein A-Sepharose at 4°C before precipitates were washed four times in lysis buffer, incubated at 95°C for 5 min with reducing SDS sample buffer, and fractionated on 10% SDS-PAGE. SDS-PAGE fractionated proteins were subjected to electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes. Transfers were probed with Abs and developed using the enhanced chemiluminescence detection system. Sequential immunoblotting was performed to assess phosphotyrosine and effector protein levels. For this purpose PVDF membranes were stripped in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) for 30 min at 54°C, washed, and blocked before immunoblotting.

in vitro kinase assay

J558Lμm3 plasmacytoma cells (10×10^6/ml/sample) were assayed for Btk kinase activity following stimulation at 37°C with 5 μg of NP_9BSA. For similar experiments 20×10^6 splenic B cells/ml/sample were stimulated with 36 μg of $F(ab')_2$RAMIG. Lysates were immunoprecipitated using anti-Btk, anti-Lyn, or anti-Syk Ab and protein A-Sepharose beads. Bead immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer (20 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 2 μg/ml each of leupeptin, aprotinin, and α_1-antitrypsin). They were then divided into two equal fractions, one of which was subjected to SDS-PAGE, transfer to PVDF membrane, and immunoblotting with anti-kinase Ab to assure that equal amounts of kinase were present in each kinase assay. The remaining half of the sample was subjected to an in vitro kinase assay (50 μl final volume) at 30°C for 10 min in the presence of 10 mM ATP, 10 μCi [γ-^{32}P]ATP, and 2 mM of the exogenous peptide substrate RRLIEDAEYAARG. This peptide is derived from the autophosphorylation site of Lck. In data not shown, using baculovirus-expressed kinases, we have determined that this procedure is an effective substrate for Src-family kinases, Syk and Btk. Unfortunately, no specific Btk substrates have yet been described. The reactions were terminated by addition of TCA (6% final), peptides were captured on P81 filter paper (Whatman, Clifton, NJ), and filters were washed in 75 mM H_3PO_4 and dried with acetone. Incorporation of ^{32}P into substrates was quantitated by liquid scintillation counting on a Beckman beta scintillation counter (model LS5801, Fullerton, CA). The stimulation index was calculated by determining the fold increase in counts per minute of kinase activity from stimulated cells over that from unstimulated cells. This was performed separately for the different cell lines.

Analysis of PLCγ2 phosphorylation in vitro

J558Lμm3CD45$^+$ CD19$^+$ cells (5×10^6 cells/ml) were immunoprecipitated with various combinations of Abs and protein A-Sepharose beads for 1 h at 4°C as described above. After washing in lysis buffer and kinase buffer, immunoprecipitates were incubated in the presence of 10 μCi of [γ-^{32}P]ATP for 10 min at 30°C. Reactions were terminated by washing in ice-cold lysis buffer and adding SDS sample buffer. Samples were fractionated by SDS-PAGE, and the incorporation of ^{32}P was determined by phosphorimaging the gel as well as by autoradiography. The fractionated proteins were transferred electrophoretically to a PVDF membrane and probed for PLCγ2 by immunoblotting.

Results

Although requirements for Btk tyrosyl phosphorylation in BCR signaling are well documented (13, 29, 30), other parameters that

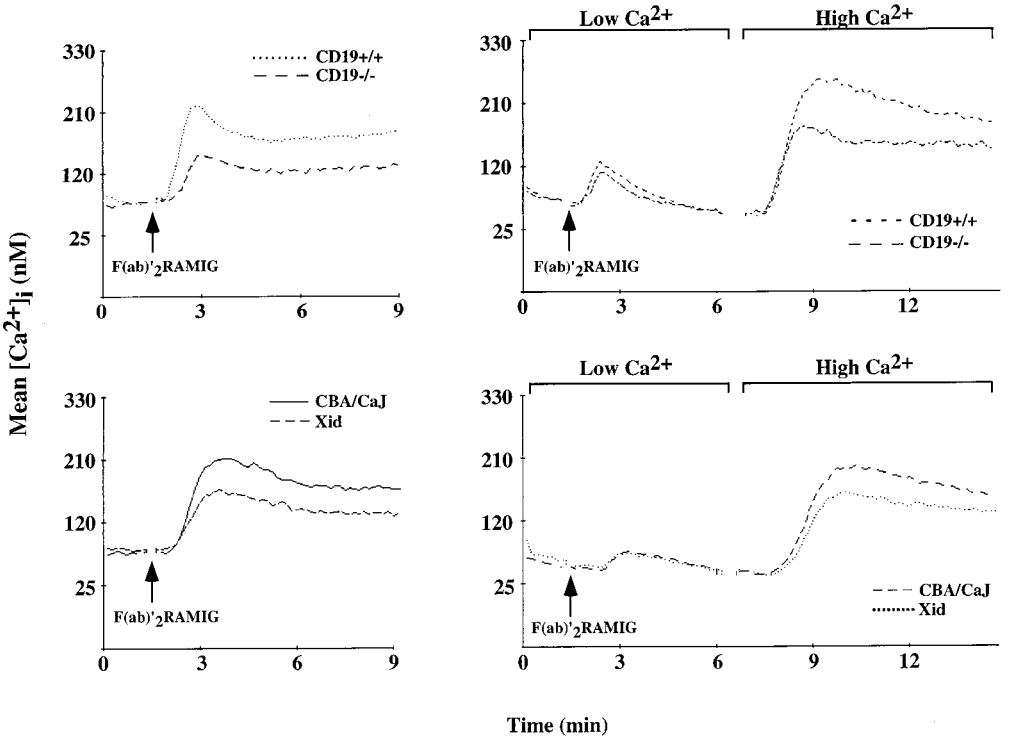

FIGURE 1. BCR-mediated $[Ca^{2+}]_i$ mobilization is similarly reduced in splenic B cells from $CD19^{-/-}$ mice and Xid mice compared with that in cells from normal littermates. B cells ($\rho \geq 1.066$) were isolated from spleens of $CD19^{-/-}$ mice, Xid mice, or normal littermates and loaded with indo-1/AM, and analysis of $[Ca^{2+}]_i$ was initiated before Ab stimulation (13 μg $F(ab')_2$ RAMIG/10^6 cells/ml). The analysis was conducted under conditions of 60 nM extracellular free calcium initially, and later $CaCl_2$ was added to achieve a final free Ca^{2+} concentration of 1.3 mM. Conditions to obtain these Ca^{2+} concentrations were determined by the CalCalc program (56). $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (57). Approximately 600 cells were analyzed per second. $[Ca^{2+}]_i$ in resting cells is 70 nM.

determine BCR-mediated activation of Btk are not defined. In particular, it was recently suggested that the catalytic subunit p110 of PI3-K may be able to synergize with tyrosine kinases in the activation of Btk (15). These findings suggest that receptor-mediated PI3-K activation may play an important role in BCR-mediated Btk activation. CD19 has been implicated in BCR-mediated activation of PI3-K (22, 23). To investigate formally the role of CD19 in BCR-mediated Btk activation and downstream events, we studied two systems in which the expression of CD19 can be genetically manipulated.

$CD19^{-/-}$ and Xid B lymphocytes have a similar deficiency in BCR-mediated Ca^{2+} mobilization

We reported previously that $F(ab')_2$RAMIG stimulation of Ca^{2+} mobilization is decreased in splenic B cells from $CD19^{-/-}$ animals compared with that in cells from wild-type controls (23). Similarly, it has been shown previously that BCR-mediated calcium responses are defective in Xid B cells (32, 33). We thought it important to compare directly the character of these defects to gain insight into whether CD19 and Btk lie in the same signaling pathway.

The Ca^{2+} mobilization difference is particularly prominent for the influx phase of the response, as shown by experiments in which extracellular Ca^{2+} was buffered to intracellular levels found in resting cells (60 nM) by addition of an EGTA buffer at the time of stimulation with Ab, followed by repletion of Ca^{2+} to physiologic extracellular concentrations of 1.3 mM (Fig. 1). Sato et al. have published contrasting data suggesting that $CD19^{-/-}$ splenic B cells behave as wild-type cells in terms of Ca^{2+} mobilization (31). We have reproduced these authors' findings and discovered that the Ca^{2+} mobilization differences between $CD19^{-/-}$ animals and normal littermates are dependent on the stimulating ligand. When an anti-IgM (such as b-7-6 or $F(ab')_2$ goat anti-mouse IgM) is used for stimulation, there is no detectable difference in the Ca^{2+} mobilization pattern between the two types of cells. In contrast, when anti-IgD Abs (such as JA12.5) or reagents that ligate all BCR, e.g., $F(ab')_2$RAMIG, anti-Ig-β, or Ag, are used, there is a marked deficiency in the ability of $CD19^{-/-}$ splenic B cells to mobilize Ca^{2+} (Fig. 1 and data not shown). When we compared $F(ab')_2$RAMIG-mediated Ca^{2+} mobilization in splenic B cells from Xid mice and CBA/CaJ controls, we saw, as has previously been published (32, 33), a difference primarily in the influx response (Fig. 1). The Xid mouse Ca^{2+} mobilization defect was similar to that seen in $CD19^{-/-}$ animals, consistent with the possibility that $CD19^{-/-}$ and Btk are intermediaries in the same pathway leading to Ca^{2+} mobilization. Since CD19 is required for most BCR-mediated PI3-K activation, IP_3 production, and Ca^{2+} mobilization (23), and Btk is required for IP_3 production and Ca^{2+} mobilization (34), we hypothesize that CD19 phosphorylation, PI3-K activation, and Btk activation are sequential events required for BCR-mediated phosphoinositide hydrolysis and calcium mobilization.

BCR-mediated activation of Btk, but not Lyn or Syk, is dependent on CD19 expression

To investigate the molecular mechanisms underlying Ag activation of Btk we initially used the J558Lμm3 plasmacytoma system (23). As shown in Fig. 2*A*, Ag stimulation of the CD19-negative and CD19-positive J558Lμm3CD45$^+$ plasmacytoma cell lines revealed that CD19 is required for maximal Btk activation. In the

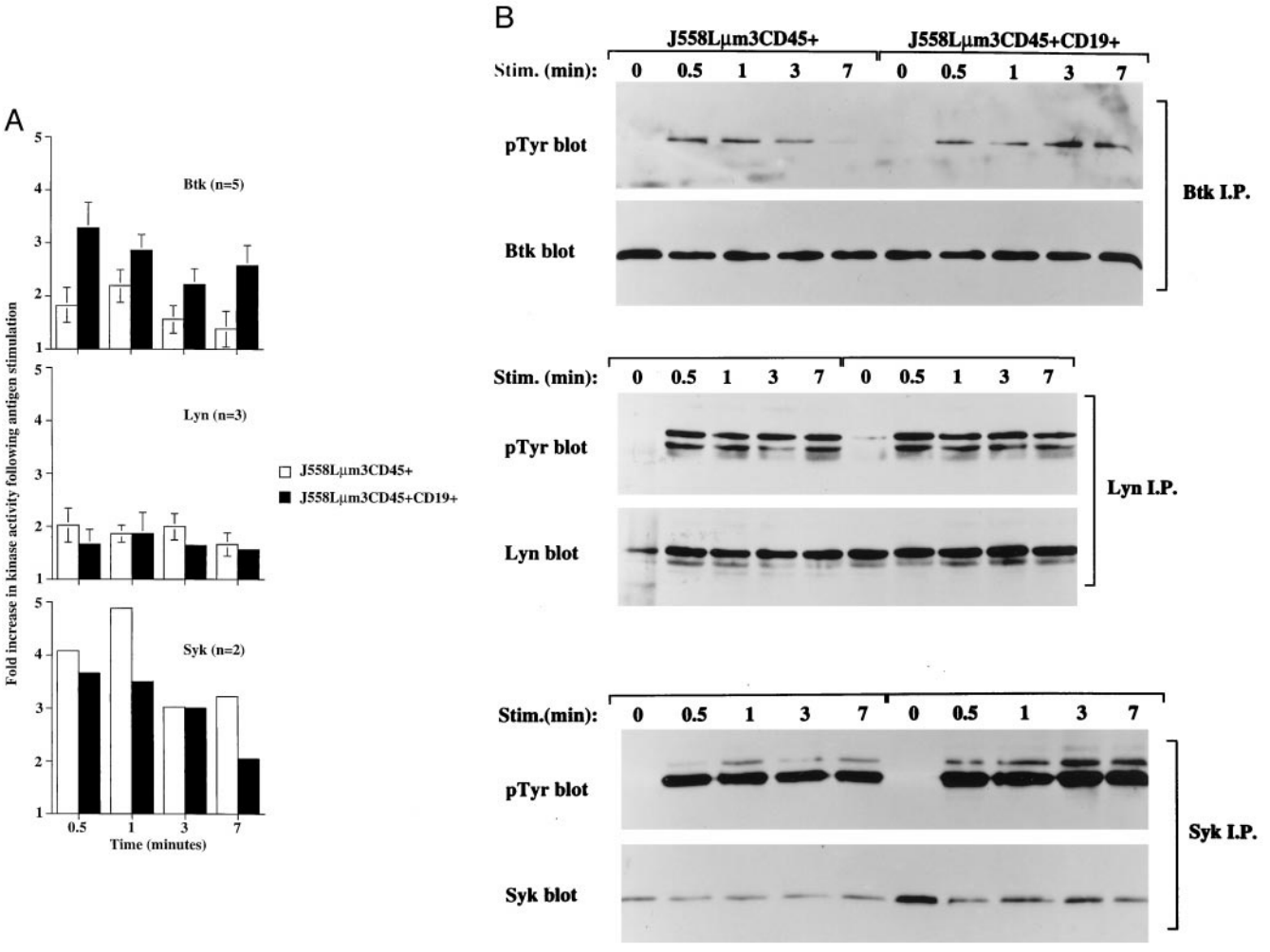

FIGURE 2. The CD19-negative and CD19-positive J558Lμm3CD45$^+$ plasmacytoma variants activate Btk to different extent following Ag stimulation, while Lyn and Syk activations are comparable. J558Lμm3CD45$^+$ CD19$^-$ and J558Lμm3CD45$^+$ CD19$^+$ cells (10×10^6/ml) were stimulated with 5 μg of NP_9BSA for various times and lysed, and Btk, Lyn, and Syk were immunoprecipitated. *A*, The kinase activity of the immunoprecipitates was determined in an in vitro kinase assay using the exogenous Lck peptide substrate, RRLIEDAEYAARG. The fold increase in kinase activity was calculated by determining the fold increase in incorporated counts per minute by kinase from stimulated cells over that from unstimulated cells. An equal kinase content of each sample was confirmed by immunoblotting as described in *Materials and Methods*. Shown are the mean fold increases seen in a number of independent experiments (Btk, $n = 5$; Lyn $n = 3$; Syk $n = 2$) $\pm$ SEM. *B*, The immunoprecipitates were fractionated on 10% SDS-PAGE. After electrophoretic transfer to PVDF membrane, both anti-phosphotyrosine and anti-effector immunoblottings were performed. Data are representative of at least three independent experiments.

absence of CD19, BCR-mediated Btk activation is small and relatively transient. In CD19-expressing cells, Btk activation is about 3-fold higher and more sustained. This activation difference is also reflected in the Btk tyrosine phosphorylation (Fig. 2*B*). Ag-stimulated Btk tyrosine phosphorylation is greater and more sustained in the CD19-positive cell line. It is currently believed that BCR-mediated Btk activation is a consequence of Src-family kinase-mediated tyrosine phosphorylation of tyrosine 551 in the catalytic domain of Btk (12). This results in a 5- to 10-fold increase in the catalytic activity and leads to Btk autophosphorylation at tyrosine 223 in the SH3 domain (14). The tyrosine kinase Syk is also known to be required for phosphorylation of tyrosine 551 of Btk (11). To address whether the CD19-mediated increase in Btk activation is a consequence of increased Lyn and Syk activation in the presence of CD19, we measured Lyn and Syk activation levels and tyrosine phosphorylation following Ag stimulation of the J558Lμm3 plasmacytoma cell lines. Ag-induced Lyn and Syk activation and tyrosine phosphorylation did not differ significantly in CD19-negative and CD19-positive cell lines (Fig. 2, *A* and *B*), suggesting that CD19 does not promote Btk phosphorylation and activation by promoting Lyn and Syk activation. Rather, CD19 may somehow promote the interaction of Lyn/Syk with Btk.

Splenic B cells from CD19$^{-/-}$ animals show a deficiency in BCR-mediated Btk activation

To investigate the role of CD19 in BCR-mediated Btk activation in a more physiological system, we purified splenic B cells ($\rho \geq 1.066$) from spleens of CD19$^{-/-}$ and normal mice and assessed Btk activation following BCR ligation (Fig. 3). As observed in the J558Lμm3CD45$^+$ CD19$^+$ cell line, the absence of CD19 resulted in a decrease in both the magnitude and the duration of BCR-mediated Btk activation. Thus, BCR-mediated Btk activation in J558Lμm3CD45$^+$ CD19$^+$ and splenic B cells is similarly sensitive to CD19 function.

The kinetics of Btk activation and phosphorylation seen here are more rapid than those reported previously for goat anti-μ-stimulated WEHI 231 cells (maximum at 3–5 min) (29). This apparent discrepancy may simply reflect the use of WEHI 231 vs

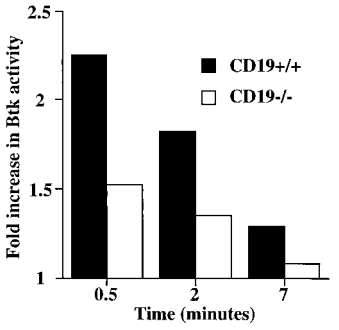

FIGURE 3. BCR-mediated stimulation of Btk activation is diminished in splenic B cells from $CD19^{-/-}$ mice compared with that in cells from $CD19^{+/+}$ littermates. Splenic B cells ($\rho \geq 1.066$) were purified from spleens of $CD19^{-/-}$ or $CD19^{+/+}$ mice, stimulated with $F(ab')_2$ RAMIG (36 μg/20 $\times$ 10^6/ml) for various times, lysed, and immunoprecipitated with an anti-Btk Ab and protein A-Sepharose beads. Immunoprecipitates were washed and assayed for Btk activity and immunoreactive Btk content. The fold increase in kinase sp. act. is calculated by determining the fold increase in counts per minute of the kinase from stimulated cells over that from unstimulated cells. Immunoreactive Btk was equivalent in all samples (data not shown). Shown is a representative experiment of three independent experiments. Basal kinase activities in resting cells were 16,692 cpm in $CD19^{-/-}$ cells and 9,716 cpm in $CD19^{+/+}$ cells.

J558L (Fig. 2*A*) and normal B cells (Fig. 4). Alternatively, it may reflect the use by Saouaf (29) of an intact polyclonal Ab stimulus that should coaggregate FcγRIIB, inhibiting the phosphorylation and thus function of CD19 (23, 35, 36). As shown in Fig. 2*A*, in the absence of CD19 function, the kinetics of residual Btk activation are delayed.

Btk activation is diminished in Xid animals compared with that in CBA/CaJ controls

The PH domain of Btk has been shown to interact with a number of proteins and lipid species. Examples are $\beta\gamma$ subunits of heterotrimeric G proteins (37); the α subunit of G_q (38); protein kinase C (39); BAP-135 (40); the inositol phosphates IP_4, IP_5, and IP_6 (16); and $PI(3,4,5)P_3$ (17, 18). Additionally, it has recently been

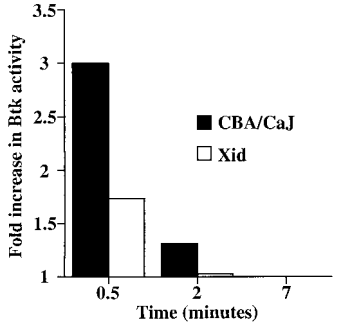

FIGURE 4. Splenic B cells from Xid mice show decreased Btk activation in response to BCR ligation. Splenic B cells ($\rho \geq 1.066$) were purified from spleens of Xid or control mice, prewarmed, stimulated with $F(ab')_2$ RAMIG (36 μg/20 $\times$ 10^6/ml) for various times, lysed, and immunoprecipitated with an anti-Btk Ab and protein A-Sepharose beads. Immunoprecipitates were washed and assayed for Btk activity and immunoreactive Btk. The fold increase in kinase sp. act. was calculated by determining the fold increase in counts per minute of the kinase from stimulated cells over that from unstimulated cells; this was performed separately for Xid and CBA/CaJ cells. Shown is a representative experiment of three independent experiments. The basal kinase activity in resting cells was 5531 cpm in Xid cells and 8477 cpm in CBA/CaJ cells.

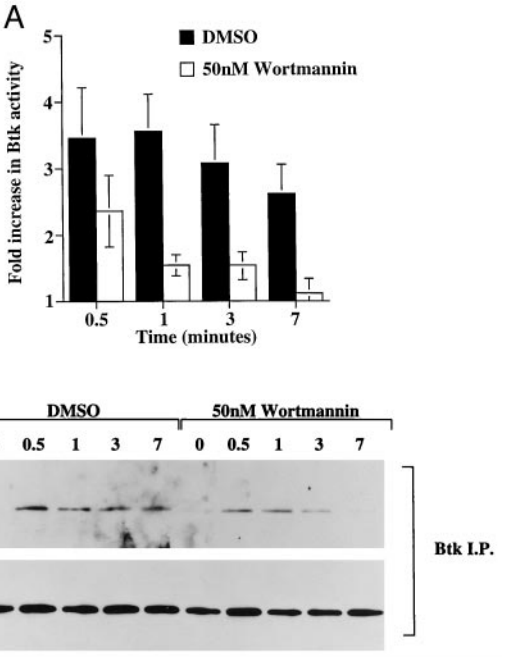

FIGURE 5. Wortmannin inhibits the Ag-induced increase in Btk activity and Btk phosphorylation in the J558Lμm3CD45$^+$ CD19$^+$ plasmacytoma. Cells were preincubated with DMSO (vehicle) or 50 nM wortmannin (in a 0.2% final DMSO concentration) for 25 min at 25°C, prewarmed for 4 min at 37°C, and then stimulated for various times with 5 μg of NP_9BSA/10 $\times$ 10^6 cells/ml before analysis of Btk kinase activity or Btk phosphorylation. *A*, Shown is the mean fold increase in sp. act. over the basal value from four independent experiments ± SEM. *B*, Samples were fractionated by SDS-PAGE, and electrophoretic transfer was performed. In *B*, anti-phosphotyrosine and anti-Btk immunoblots are shown. Results are representative of at least three independent experiments.

shown that membrane translocation of Btk is critical for the transformation efficiency of Btk (20). To investigate whether the Xid mutation influences BCR-mediated activation of Btk, we measured $F(ab')_2$RAMIG-induced Btk activation in splenic B cells from Xid mice and CBA/CaJ controls. As shown in Fig. 4, BCR-mediated Btk activation is severely reduced in splenic B cells from Xid mice, suggesting that the PH domain, presumably via its interaction with regulatory proteins and/or lipids, is required for BCR-mediated Btk activation. Interestingly, while Btk autophosphorylation is not affected by the Xid mutation (4, 5), BCR-mediated Btk phosphorylation is absent in splenic B cells from Xid mice (41). This suggests that the PH domain mutation does not influence the intrinsic kinase activity of Btk, but somehow interferes with Btk phosphorylation and activation by other kinases, such as Lyn and Syk.

BCR-mediated Btk activation is dependent on PI3-K activation

Since the Btk PH domain is known to interact with $PI(3,4,5)P_3$, a product of PI3-K activation, we investigated whether BCR-mediated Btk activation requires PI3-K activation. Preincubation of J558Lμm3CD45$^+$ CD19$^+$ cells with the PI3-K inhibitor wortmannin (42) severely reduced Ag-induced activation of Btk (Fig. 5*A*). Interestingly, the sensitivity of wortmannin was only partial at the 30 s point but was complete at later time points. This phenomenon may be explained by the presence of a basal level of $PI(3,4,5)P_3$ in the cells, and only when this intrinsic pool of $PI(3,4,5)P_3$ has been depleted is wortmannin sensitivity maximal. Sensitivity to wortmannin inhibition was also seen in Ag-induced Btk phosphorylation (Fig. 5*B*). Finally, wortmannin also inhibited residual Ag-induced Btk activation in $CD19^{-/-}$ cells (data not shown). These

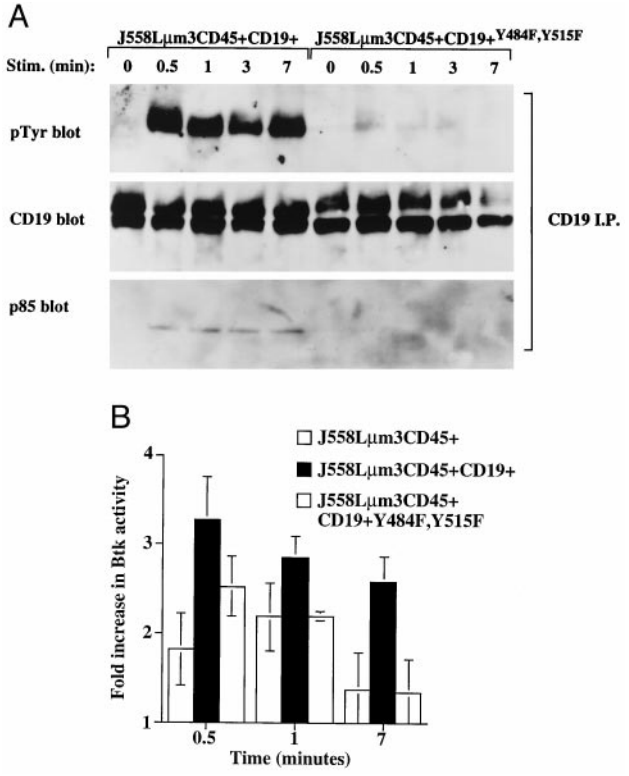

FIGURE 6. Mutant CD19 (CD19 Y484F,Y515F) does not support Ag-induced CD19 tyrosine phosphorylation or Btk activation. *A*, J558Lμm3CD45$^+$ CD19$^+$ and J558Lμm3CD45$^+$ CD19$^+$ Y484F,Y515F cells were stimulated with Ag (5 μg of $NP_9BSA/10 \times 10^6$ cells/ml) for various times, cells were lysed, and CD19 was immunoprecipitated with an anti-CD19 Ab and protein A-Sepharose beads. Immune complexes were washed three times, fractionated on 8% SDS-PAGE, and transferred onto PVDF membrane for subsequent immunoblotting. Tyrosine phosphorylation was assessed using an anti-phosphotyrosine Ab, and the blot was stripped and reprobed with anti-CD19 and anti-p85. *B*, J558Lμm3CD45$^+$ CD19$^-$, J558Lμm3CD45$^+$ CD19$^+$, and J558Lμm3CD45$^+$ CD19$^+$ Y484F,Y515F were assayed for Ag-induced Btk activation. Shown is the mean fold increase in sp. act. (normalizing for immunoreactive Btk content) over the basal value in three independent experiments ± SEM.

results indicate that PI3-K activation is required for maximal BCR-mediated Btk phosphorylation and activation. They are consistent with the possibility that Btk PH domain interactions with $PI(3,4,5)P_3$ are required to position Btk so that it can be phosphorylated by Src family kinases and thereby activated.

CD19 residues Y484 and Y515 are required for maximal BCR-mediated Btk activation

The cytoplasmic tail of CD19 contains two YXXM motifs that become phosphorylated upon BCR stimulation and interact with the p85 subunit of PI3-K (22). To further investigate the roles of CD19 and PI3-K in Btk activation, we used a mutant CD19-expressing cell line in which the two YXXM tyrosines were substituted with phenylalanine J558Lμm3CD45$^+$ CD19$^+$ Y484F,Y515F. Ag stimulation of the wild-type CD19$^+$ J558Lμm3CD45$^+$ cells led to very significant CD19 tyrosyl phosphorylation. In contrast, virtually no CD19 tyrosyl phosphorylation was detected in the J558Lμm3CD45$^+$ CD19 Y484F,Y515F cells upon Ag stimulation (Fig. 6*A*). This suggests that although nine potential sites of tyrosyl phosphorylation exist within CD19, the two YXXM tyrosines are the major sites of phosphorylation following Ag stimulation. It is possible that only these sites are phosphorylated upon BCR aggregation; alternatively, phosphorylation of Y484 and/or Y515 may be required for phosphorylation of other tyrosine residues within CD19. Consistent with the previously reported PI3-K interaction with these sites (22), the p85 subunit of PI3-K coprecipitated with CD19 from stimulated J558Lμm3CD45$^+$ CD19$^+$ cells but not from J558Lμm3CD45$^+$ CD19$^+$ Y484F,Y515F cells (Fig. 6*A*). We previously demonstrated that BCR-mediated PI3-K activation in the J558Lμm3CD45$^+$ CD19$^+$ Y484F,Y515F is similar to the Ag-induced PI3-K activation in J558Lμm3CD45$^+$ CD19$^-$ cells and is much reduced compared with that seen in cells expressing wild-type CD19 (23). To investigate whether the YXXM tyrosines of CD19 are required for BCR-mediated Btk activation, we measured the Ag-induced Btk activity in the J558Lμm3CD45$^+$ variants. As shown in Fig. 6*B*, Ag-induced Btk activation was severely diminished in the CD19 Y484F,Y515F mutant cell line compared with that in the CD19 wild-type variant, further supporting the hypothesis that CD19 phosphorylation and the resultant binding and activation of PI3-K are required for Ag-induced Btk activation.

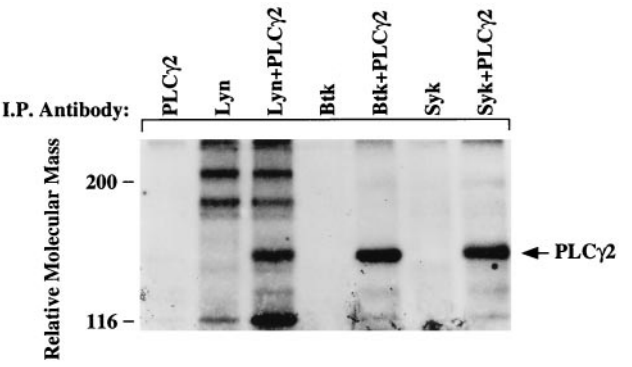

FIGURE 7. PLCγ2 is phosphorylated in vitro by Lyn, Syk, and Btk. J558Lμm3CD45$^+$ CD19$^+$ cells (5×10^6 cells/ml) were lysed, and postnuclear lysates were immunoprecipitated with combinations of Abs and protein A-Sepharose beads. Immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer followed by incubation with 10 μCi of [γ-^{32}P]ATP for 10 min at 30°C. Reactions were terminated by washing in ice-cold lysis buffer, samples were fractionated on SDS-PAGE, and the incorporation of ^{32}P into PLCγ2 was visualized by autoradiography.

Lyn, Syk, and Btk can phosphorylate PLCγ2 in vitro

The defective BCR-mediated calcium mobilization response seen in splenic B cells from Xid and CD19$^{-/-}$ mice may be the consequence of failed activation of PLCγ2. PLCγ2 activation may depend on Btk translocation to the plasma membrane, where it is phosphorylated and activated, and subsequently phosphorylates PLCγ2. To explore the possibility that PLCγ is a Btk substrate, we immunoprecipitated PLCγ2 alone or in combination with Lyn, Syk, or Btk and performed an in vitro kinase reaction in the presence of [γ-^{32}P]ATP to assess phosphorylation of PLCγ2. As expected, both Lyn and Syk phosphorylated PLCγ2. Importantly, however, Btk was also able to phosphorylate PLCγ2 under these circumstances (Fig. 7). Fluckiger et al. recently published data supporting this conclusion; when expressing Lyn, Btk, or both tyrosine kinases in A20 lymphoma cells, they observed tyrosine phosphorylation of PLCγ2 (34). Our in vitro experiment extends the findings of Fluckiger by showing that PLCγ is a direct substrate of Btk. Thus, Btk may phosphorylate PLCγ2 following BCR aggregation, and this phosphorylation may be required for PLCγ activation. Confirmation of this hypothesis will require mapping of Btk tyrosine phosphorylation sites in PLCγ2 and analysis of the roles of these sites in PLCγ activation following BCR aggregation.

Discussion

In this report we have defined a previously unrecognized linkage between BCR-mediated phosphorylation of CD19 at tyrosine residues 484 and 515 and the phosphorylation and activation of Btk. B cells from Xid mice and those from $CD19^{-/-}$ mice were shown to have similar defects in BCR-mediated mobilization of calcium. CD19 expression was found to be necessary for optimal BCR-mediated phosphorylation and activation of Btk. Since CD19 was not required for optimal activation of Lyn and Syk, these data suggest that CD19 somehow functions to position Btk for phosphorylation by these kinases. A potential structural basis for this effect is provided by the findings that 1) Btk activation requires that its PI(3,4,5)P_3-binding PH domain be functional; 2) Btk activation and phosphorylation are blocked by the PI3-K inhibitor wortmannin; and 3) the phosphorylated sites within CD19 that are responsible for PI3-K recruitment and activation, i.e., Y484 and Y515, are essential for its support of Btk activation. These data reveal a circuitry in which BCR-activated kinases phosphorylate CD19 residues Y484 and Y515; this leads to recruitment and activation of PI3-K and generation of PI(3,4,5)P_3. PI(3,4,5)P_3 functions to recruit Btk by binding its PH domain. PH domain-mediated localization of Btk promotes its tyrosyl phosphorylation by colocalized Src- and Syk-family kinases. A further experiment provides a potential explanation for the previously documented role of Btk in BCR-mediated activation of PLCγ. Specifically, it is shown that Btk is capable of phosphorylating PLCγ2 in vitro. Ongoing studies in our laboratory seek to determine the site of this phosphorylation and its effect on PLCγ2 activity. It seems likely that this phosphorylation may serve to activate the lipase.

In addition to the similarities reported here, B cells from Xid animals and those from $CD19^{-/-}$ mice show phenotypic similarities, such as poor proliferative responses to anti-BCR-Abs, and these animals exhibit significantly reduced serum Ig levels (28, 43–46). Taken together, these data indicate that Btk and CD19 play important roles in the same or impinging signaling pathways that are critical for B cell development and the immune response. The expression level of these signaling molecules is therefore likely to be tightly regulated. In support of a role for Btk in positive selection during lymphocyte development, peripheral B cells from Xid animals show dramatically increased levels of mIgM (43), which may reflect a compensatory mechanism to obtain an effective tonic BCR signal. A similar increase in surface expression of mIgM is seen on peripheral B cells from $CD19^{-/-}$ mice (31). We recently bred $CD19^{-/-}$ mice onto 3–83 $\mu\delta$ transgenic mice (47) to evaluate the consequence of the CD19 deletion under conditions of a constrained B cell repertoire. The resulting mice have virtually no peripheral B cells, and the few B cells that appear express extremely high levels of sIgM (A. M. Buhl, M. Hertz, and J. C. Cambier, unpublished observations), further emphasizing the importance of CD19 in development and maturation. Interestingly, in both $CD19^{-/-}$ animals and Xid animals there is a total absence of peritoneal B-1 cells, further suggesting that CD19 and Xid may be situated in the same signaling cascade and may be particularly important for the expansion of B-1 cells.

Btk has been shown to be involved in signal transduction by B cell surface proteins other than the BCR. Cross-linking of CD38 or CD72 leads to Btk phosphorylation in splenic B cells (41–48), and B cell proliferation following ligation of either of these surface molecules is impaired in Xid mice (48, 49). Interestingly, both CD38 and CD72 have been suggested to use CD19 in signal transduction. B cell proliferation following CD38 or CD72 ligation is decreased in $CD19^{-/-}$ animals compared with that in wild-type littermates (44) (A. M. Buhl and J. C. Cambier, unpublished observations), and CD38 or CD72 ligation results in tyrosine phosphorylation of CD19 (50, 51). We have analyzed CD19-mediated Ca^{2+} mobilization in Xid B cells and found it decreased compared with that in CBA/CaJ controls (A. M. Buhl and J. C. Cambier, unpublished observations), suggesting that CD19 may be upstream of Btk in this CD19 autonomous signaling pathway. Although we hypothesize that this involves a PI3-K intermediate, it was recently shown that the T cell coreceptor CD28 can directly interact with and activate Itk via an interaction between the Itk-SH3 domain and a proline-rich sequence in CD28 (52). A similar proline-rich sequence is present in CD19. Further studies are therefore required to determine whether Btk interacts directly with CD19.

We and others have recently published data demonstrating that coaggregation of the BCR with FcγRIIB1 leads to decreased phosphorylation of CD19 and failed PI3-K activation (35, 36). FcγRIIB1-mediated inhibition of CD19 phosphorylation therefore appears to prevent the normal contribution of CD19 to BCR signaling, supporting a role for CD19 and its effector PI3-K in BCR-mediated PLCγ activation and calcium mobilization. In A20 lymphoma cells and DT40 chicken B cells IP_3 production is activated by $F(ab')_2$RAMIG stimulation, but not by RAMIG stimulation (35), but overexpression of membrane-targeted Btk has been shown to obviate this FcγRIIB1 inhibitory effect (53). The role of Btk in BCR-mediated IP_3 production is enhanced by overexpressing the p110 catalytic subunit of PI3-K, suggesting that Btk function is dependent on PI(3,4,5)P_3 (54). Indeed, the same group demonstrated that Btk binding to PI(3,4,5)P_3 protects the lipid against degradation. In DT40 cells Btk translocation to the membrane is sensitive to the expression levels of SHIP, the inositol phosphatase that can degrade PI(3,4,5)P_3 (53). It has therefore been proposed that coaggregation of the BCR and FcγRIIB1 allows activation of the inositol phosphatase SH2-containing inositol 5′ phosphatase, which degrades PI(3,4,5)P_3, and thereby inhibits the function of Btk. However, these recent results may also be explained by the fact that PI(3,4,5)P_3 production is inhibited following BCR-FcγRIIB1 coaggregation because of a lack of CD19 phosphorylation and PI3-K activation. Insufficient levels of PI(3,4,5)P_3 in the system because of a lack of PI3-K activation would also contribute to inefficient Btk translocation to the plasma membrane. In recent studies involving analysis of PI(3,4,5)P_3 levels in cells following coaggregation of the BCR and FcγRIIB1 mutants, which lack the ability to engage SHIP, we have found that approximately 40% of the reduced PI(3,4,5)P_3 production seen following receptor coaggregation can be ascribed to failed CD19 phosphorylation, and the remainder is due to SHIP activation (D. Fong, A. Brauweiler, and J.C.C., unpublished observations).

Here we have defined an important accessory function for CD19 in BCR-mediated Btk activation and described a molecular basis for this function of CD19. CD19 was earlier found to influence the magnitude of activation of multiple intracellular signaling pathways following Ag stimulation of the BCR; in particular, CD19 is required to obtain substantial PI3-K activation, IP_3 generation, and maximal calcium mobilization (23). We have now extended these studies to show that CD19 is involved in the activation of Btk in both the J558L plasmacytoma model and primary splenic B cells. Using the PI3-K inhibitor wortmannin and mutational analysis of CD19, we determined that the involvement of CD19 in BCR-mediated Btk activation is a function of PI3-K activation obtained by the presence of CD19. The results presented in this manuscript indicate that CD19 is an intermediary in the signaling cascade between BCR aggregation and Btk activation. This is the first report describing an association between CD19 and Btk activation, and it defines molecular cross-talk that may explain observations made in both transgenic and knockout animals.

The downstream molecular targets of Btk are not well defined. However, Btk has been implicated in the activation of PLCγ2, since B cells from Xid mice exhibit defective BCR-mediated phosphoinositide hydrolysis (33). DT40 B cells in which the Btk gene was ablated by homologous recombination show a total absence of BCR-mediated Ca^{2+} mobilization (55). Btk interacts with additional proteins such as G protein $\beta\gamma$-subunits (37), BAP-135 (40), and protein kinase C (39). The roles of these and other molecules as upstream regulators or downstream effectors are unclear; thus, future research must be directed toward understanding the unique role of Btk in different signaling contexts.

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