We have recently demonstrated that the D3-phosphoinositide phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P$_3$) is critical for producing sustained calcium signals through its role in promoting the function of TEC family tyrosine kinases such as Bruton’s tyrosine kinase. Although PtdIns-3,4,5-P$_3$ can potentially be synthesized by any of several types of phosphoinositide 3-kinases (PI3Ks), B cell receptor (BCR)-induced PtdIns-3,4,5-P$_3$ production is thought to occur primarily through the activation of the class Ia (p85/p110) PI3Ks. This process has been proposed to be mediated by an interaction between the Src family kinase LYN and the p85 subunit of PI3K and/or through p85 membrane recruitment mediated by CBL and/or CD19. However, calcium signaling and other PI3K-dependent signals are relatively preserved in a LYN kinase-deficient B lymphocyte cell line, suggesting that an alternative pathway for PI3K activation exists. As SYK/ZAP70 kinases are upstream from many BCR-initiated signaling events, we directly analyzed SYK-dependent accumulation of both PtdIns-3,4,5-P$_3$ and PtdIns-3,4-P$_2$ in B cell receptor signaling using both dominant negative and genetic knockout approaches. Both methods indicate that SYK is upstream of, and necessary for, a significant portion of BCR-induced PtdIns-3,4,5-P$_3$ production. Whereas CD19 does not appear to be involved in this SYK-dependent pathway, the SYK substrate CBL is likely involved as the dominant negative SYK markedly attenuates CBL tyrosine phosphorylation and completely blocks the BCR-dependent association of CBL with p85 PI3K.

Engagement of surface immunoglobulin (BCR)$^1$ on B cells results in early signaling events including tyrosine phosphorylation of ITAM motifs, activation of the non-receptor protein tyrosine kinases LYN and SYK, and calcium mobilization. The D3-phosphoinositides PtdIns-3,4,5-P$_3$ and PtdIns-3,4-P$_2$ are also produced after engagement of surface immunoglobulin and have been linked to a multitude of downstream signaling events, including cell survival/AKT activation, membrane ruffling, the activation of the SOS GEF, and TEC kinase-dependent activation of phospholipase C$_{1,2}$ (1–6). D3-phosphoinositide production occurs through the action of members of the phosphoinositide 3-kinase (PI3K) family of lipid kinases. This family now consists of four distinct subtypes of enzymes, including the p85/p110 heterodimeric isoforms (designated class Ia, and reviewed in Refs. 7 and 8), the class Ib G$\beta$$\gamma$-responsive isoforms (9, 10), the class II C2 domain containing isoforms (11–15), and the class III PtdIns-specific isoforms (16–18). The different classes of PI3K enzymes show distinct substrate preferences. Type Ia and type Ib enzymes exhibit a clear preference for PtdIns-4,5-P$_2$ over PtdIns-4-P (see Refs. 19 and 20 and reviewed in Ref. 21). In contrast, type II enzymes show a preference for PtdIns and PtdIns-4-P over PtdIns-4,5-P$_2$ (11), whereas the type III enzymes strongly prefer PtdIns over either PtdIns-4-P or PtdIns-4,5-P$_2$ (16, 22). Based on these preferences, PtdIns-3,4,5-P$_3$ is thought to be the major product of class Ia and Ib enzymes, whereas PtdIns-3-P is thought to be produced by all three classes of enzymes. PtdIns-3,4-P$_2$, on the other hand, can be synthesized in three possible ways as follows: 1) by direct 3'-phosphorylation of PtdIns-4-P by class I or II enzymes, 2) by direct 4'-phosphorylation of PtdIns-3-P by a PtdIns-3-P 4-kinase (23–25), and 3) finally by 5'-dephosphorylation of PtdIns-3,4,5-P$_3$ through the action of many types of inositol-5-phosphatases (26–28). In BCR systems, PI3K signaling has been linked so far only to activation of class Ia PI3Ks (29–31).

How is the BCR linked to class Ia PI3K activation? The lipid kinase activity of class Ia PI3Ks is thought to be activated directly by interactions of Ras-type small G-proteins with the p110 catalytic domain (32) or indirectly via interactions between tyrosine-phosphorylated proteins or SH3 domains and the p85 regulatory subunit (33–39). In the case of the BCR, class Ia PI3K activation has been proposed to occur as the result of an interaction between the SRC family tyrosine kinase LYN SH3 domain and a proline-rich region of the class Ia PI3K p85 subunit (30). Although consistent with in vitro binding data, this mechanism has difficulty accounting for why calcium responses in LYN-deficient DT40 B cells are of near normal maximal magnitude (40), why BTK tyrosine phosphorylation and activation, recently shown to be a PtdIns-3,4,5-P$_3$-dependent process (4, 5), is slower but relatively maintained in LYN-deficient cells (41), or why activation of AKT is not attenuated in LYN-deficient cells (42). These findings suggest that whereas BCR-inducible PI3K activation may occur through p85 PI3K association with SRC family kinases, it can also proceed through SRC kinase-independent routes.

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* The abbreviations used are: BCR, B cell receptor; ITAMs, immunoreceptor tyrosine-based activation motifs; PtdIns-3,4,5-P$_3$, phosphatidylinositol 3,4,5-trisphosphate; PtdIns-3,4-P$_2$, phosphatidylinositol 3,4-bisphosphate; PI3K, phosphoinositide 3-kinase; HPLC, high pressure liquid chromatography; pfu, plaque-forming units.

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SYK Is Upstream of Phosphoinositide 3-Kinase in B Cell Receptor Signaling*

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The tyrosine kinase SYK is a 72-kDa tyrosine-phosphorylated protein activated early in BCR signaling. SYK is an important mediator of BCR-induced protein tyrosine phosphorylation and has been shown both genetically and in dominant negative studies to be necessary for receptor-induced calcium mobilization (5, 40). Because it is upstream from many types of BCR-dependent signaling events, we hypothesized that SYK could provide either an alternative or convergent link for antigen receptor-mediated PI3K activation. We therefore analyzed BCR-mediated PtdIns-3,4,5-P3 and PtdIns-3,4-P2 accumulation using TLC and HPLC analyses of 32P-labeled B lymphocytes with either wild type SYK or mutant SYK. Our initial results indicated that a significant portion of BCR-induced PtdIns-3,4,5-P3 and PtdIns-3,4-P2 accumulation is dependent on SYK function. We therefore undertook studies to address whether loss of SYK function impairs BCR-induced PI3K activity through CBL or CD19. We found that dominant negative SYK blocks association of p85 PI3K with CBL and attenuates CBL tyrosine phosphorylation but that CD19 tyrosine phosphorylation and CD19/p85 PI3K association after receptor engagement were unchanged.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, cDNAs, and Recombinant Virus Production—**A20 B cells were maintained in RPMI 1640 with 10% fetal bovine sera (Biofluidics Inc., Rockville, MD) and 10−4 M 2-mercaptoethanol (Bio-Rad). DT40 cell lines (40, 43) were maintained in the same media as above but with the addition of 1% chicken sera (Sigma). GB and SYKT vaccinia virus vector constructs and production for the A20 experiments were previously described (44).

**Cell Infections and Stimulations—**A20 infections were performed by adding 10 pfu/cell of recombinant virus to ~20% confluent adherent 75-cm2 flasks of cells and allowing the infection to proceed for 15 h. Control infection was performed in parallel samples such that paired samples were all exposed to 10 pfu of vaccinia virus/cell, as described previously (44). Stimulations were performed in calcium buffer as described previously (4) at 30 °C with the following: 1) DT40, 10 μg/ml goat anti-chicken IgM (Bethyl Labs, Montgomery, TX); 2) A20, 30 μg/ml rabbit anti-mouse Fab'2 IgG H + L (Jackson Immunoresearch, West Grove, PA) (44).

**Calcinim Assays—**Cytoplasmic calcium concentration was monitored by using Fura-2 (Molecular Probes Inc, Eugene, OR) bulk spectrofluorimetry (Photon Technology Inc, Ashland, MA) as described previously, with identical stimuli and conditions as those used in the phosphoinositide analysis (4).

**Cell Labeling, Lysis, and Phosphoinositide Analysis—**A20 32P cell labeling and lipid extractions were performed as described previously (4), and DT40 labeling and lipid extractions were performed in an identical fashion. Radiolabeling of all cells was done in calcium buffer, at 37 °C with rocking, and 1 mCi of [32P]orthophosphate/106 cells (NEN Life Science Products) for 1 h. Cells were then spun down and resuspended in calcium buffer at 1 × 107/ml, warmed to 30 °C, and stimulated as above. Lipid extractions for TLC and lipid extraction and deacylation for HPLC were all performed as described previously (4).

**Cell Lysis, Immunoprecipitations, and Immunoblotting—**In SYKT dominant negative experiments for CBL precipitations, two individual experiments were done for anti-phosphotyrosine and anti-p85 PI3K blotting with the same conditions except for cell number. In the case of antiphosphotyrosine, 5 × 107 infected cells were split evenly and stimulated or left unstimulated. For the anti-p85 PI3K experiment, 1 × 107 infected cells were split 1/3 for no stimulation and 2/3 for stimulation in order to conserve limited reagents and allow appropriate comparisons of non-stimulated GB versus non-stimulated SYKT-infected cells, and stimulated GB versus stimulated SYKT-infected cells. In the CD19 experiments, 2 × 106 cells were split evenly and either stimulated as above or left unstimulated. Cells were then lysed in buffer containing 0.5% Triton X-100 (Bio-Rad), 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM NaF, 2 mM sodium orthovanadate, 5 mM EDTA, and 5 μg/ml leupeptin, aprotinin, and pepstatin. Lysates were then subjected to precipitation with anti-CBL C-15 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD19 (PharMingen, San Diego, Ca) for 2–4 h at 4 °C, washed three times with lysis buffer, separated by SDS-polyacrylamide electrophoresis, and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). Membranes were blocked in 4% bovine serum albumin and probed with monoclonal anti-phosphotyrosine (4G10, Upstate Biotechnology Inc., Lake Placid, NY), polyclonal anti-p85 PI3K P103030 (Transduction Laboratories, Lexington, KY), or polyclonal anti-CD19 (a gift of Dr. John Cambier, Denver, CO).

**RESULTS**

In order to analyze SYK-dependent PI3K signaling, we chose to utilize two different B cell receptor signaling systems where SYK function can be inhibited (Fig. 1). In the A20 B cell system, we expressed a dominant negative form of SYK (SYKT) that blocks the interaction of endogenous SYK with BCR ITAMs, resulting in a substantial, but not complete, inhibition of SYK functions such as calcium mobilization (Fig. 1A, this represents a typical degree of inhibition for the samples used in the D3-phosphoinositide analyses). In the DT40 system, we compared wild type DT40 B cells with a DT40 cell line in which the sole SYK gene has been disrupted, resulting in a complete loss of SYK-dependent functions including the calcium signal (40) and Fig. 1B). These systems provide complementary information because the dominant negative SYK not only blocks the interaction of endogenous SYK with the BCR ITAMs but also other molecules such as SHC which are potentially involved in PI3K signaling. In contrast, in the SYK-deficient DT40 system, ITAM-interacting proteins will have enhanced access because SYK is not present to interact with the BCR ITAMs, potentially resulting in antifactual signaling processes. In this way, information from both systems controls for potentially confounding problems with either system used alone.

**Inhibition of SYK Function Blocks D3-phosphoinositide Accumulation—**For assessment of PI3K activation, we chose to measure the in vivo accumulation of both PtdIns-3,4,5-P3 and PtdIns-3,4-P2. Measurement of the level of accumulation of PtdIns-3,4,5-P3 provides the most direct measure of the activation of class Ia PI3Ks, the PI3K subtype linked to BCR-signaling pathways. However, PtdIns-3,4,5-P3 typically accumulates only to a limited extent after antigen receptor activation in a variety of systems (including the A20 system), with the level of its accumulation approaching the detection limits of HPLC analysis in the A20 system (4). Because of this, it is informative to additionally monitor the accumulation of PtdIns-3,4-P2. PtdIns-3,4-P2 typically accumulates to higher and therefore more easily detectable levels than does PtdIns-3,4,5-P3, and is accordingly both less variable and a better measure of small differences in signal magnitude (19). In addition, as it is formed in part as a breakdown product of PtdIns-
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FIG. 2. Inhibition of SYK function blocks D3-phosphoinositide accumulation in A20 B cells. 1 × 10⁷ cells/time point were loaded with 1 μCi of [32P]orthophosphate/10⁷ cells for 1 h, resuspended in calcium buffer at 1 ml per time point, and stimulated as above. Cells were lysed at the given times and lipids extracted. Six experiments were combined at this point, and samples were deacylated and analyzed by HPLC. Accumulations of PtdIns-3,4,5-P³ and PtdIns-3,4-P² are shown. (Note: Two additional experiments each confirmed the 50% block in PtdIns-3,4-P² accumulation, data not shown.)

3,4,5-P³, the level of its accumulation in comparison with the level of PtdIns-3,4,5-P³ accumulation provides a rough index of changes in the rate of PtdIns-3,4,5-P³ breakdown.

SYKT Blocks D3-phosphoinositide Accumulation when Expressed in A20 B Cells—A20 B cells were infected with either a control virus (GB) or the SYKT construct and then harvested and loaded with [32P] for lipid analysis. Loaded cells were then resuspended and stimulated as for the calcium studies in Fig. 1. Cells were lysed at the indicated times, and lipids were extracted, deacylated, and subjected to HPLC. As PtdIns-3,4,5-P³ accumulates in such low quantities after stimulation in the A20 system as to be difficult to differentiate from baseline unstimulated cells by HPLC analysis, the extracts of six individual experiments were combined for D3-phosphoinositide accumulation. As can be seen in Fig. 2, PtdIns-3,4,5-P³ accumulation is attenuated by approximately 40% and PtdIns-3,4-P² accumulation by approximately 50% after BCR engagement. Two additional experiments for PtdIns-3,4-P² accumulation both confirmed the 50% attenuation of PtdIns-3,4-P² accumulation of GB versus SYKT constructs after BCR engagement (data not shown). These results suggest SYK is necessary for normal PI3K activation after BCR engagement or that the dominant negative SYK was interfering with access to the BCR ITAMs of PI3K activation after BCR engagement or that the dominant

SYKT on two proximal signaling molecules known to associate with p85 PI3K and PI3K lipid kinase activity after BCR engagement (45–48). GB- or SYKT-infected A20 B cells were stimulated, and cell lysates were subjected to immunoprecipitation with anti-CBL antibodies. The resultant proteins were separated by polyacrylamide gel electrophoresis, immunoblotted, and probed with anti-phosphotyrosine or anti-p85 PI3K antibodies (two separate experiments with same conditions of infection and stimulation). As can be seen in Fig. 4, A and B, overexpression of SYKT results in attenuation of CBL tyrosine phosphorylation and complete block in association of p85 with CBL, indicating a role for CBL in this pathway. In contrast, as can be seen in Fig. 4C, the association of CD19 with p85 is not altered in SYKT-overexpressing cells, nor is the tyrosine phosphorylation state, making CD19 an unlikely contributor to the SYK-dependent PI3K activation pathway.

DISCUSSION

Previous studies have suggested that BCR-mediated PI3K activation is driven by the action of the Src family kinase LYN, the CD19 coreceptor, and the p85 PI3K regulatory subunit (30, 45, 48–52). In contrast, the data we have presented demonstrate that SYK plays a major role in linking the BCR to PtdIns-3,4,5-P³ and PtdIns-3,4-P² accumulation and therefore...
PI3K activation and that this role is evident in two different BCR-signaling systems.

Although SYK may mediate PI3K activation by a number of different pathways, one likely involves the adaptor molecule CBL. Our data support a model of SYK-dependent PI3K activation (see Fig. 5) wherein after SYK is recruited to Iga/Igβ ITAMs and activated, it then tyrosine phosphorylates CBL, allowing CBL to associate with p85 PI3K. This would in turn activate PI3K lipid kinase activity, resulting in PtdIns-3,4,5-P3 and subsequent PtdIns-3,4,5-P3 accumulation. In this model, SYK-dependent phosphorylation of CBL would therefore play a role in BCR-dependent PI3K activation, thereby accounting for the upstream role of SYK in BCR-induced PtdIns-3,4,5-P3 accumulation. Although distinct from previous analysis of BCR PI3K signaling, our results are consistent with previous reports that SYK tyrosine phosphorylates CBL in a receptor-dependent manner (53) and that SYK phosphorylates CBL at or in the vicinity of the p85 PI3K-binding site (53–55). This model is also consistent with CBL binding to p85/PI3K (45, 47, 56) and associating with PI3K lipid kinase activity after engagement of the BCR (45), with the augmentation of PI3K activity seen with overexpression of CBL, and abolition of this effect when the CBL p85 PI3K site is mutated in an interleukin-4 receptor-dependent model (57). Therefore, one receptor-dependent pathway in which SYK may regulate PI3K activity is through the tyrosine phosphorylation of CBL at the p85 PI3K-binding site (tyrosine 731).

Although our data demonstrate that CBL-mediated PI3K activation likely accounts for at least part of the upstream role of SYK in PI3K activation in BCR systems, we were unable to show a SYK kinase-dependent function of CD19 to BCR-mediated PI3K activation. Since others have suggested SYK may tyrosine-phosphorylate CD19 (58) thereby mediating association of CD19 with SH2 domains of other signaling molecules, we tested the hypothesis that SYK may be upstream of PI3K activity via CD19. However, we were unable to demonstrate a significant change in CD19 phosphorylation or p85 PI3K association in the A20 system with dominant negative SYK. This finding does not exclude the possibility that CD19 may be involved in a BCR-induced SYK-independent PI3K-signaling pathway. In fact, several investigators (48, 59) have demonstrated BCR-dependent CD19/p85 PI3K association, and splenic cells of CD19-deficient mice show decreased PI3 kinase activity with BCR engagement (60). This SYK-independent pathway would explain the partial block in D3-phosphoinositide accumulation by dominant negative SYK in the A20 system versus the almost complete block in the DT40 system, as a chicken homologue of CD19 has not been described. Alternatively, the difference in the two cellular systems could be explained by SYKT having a kinase-independent function in PI3K activation or in SYKT having an incomplete dominant negative effect in the A20 system.

Finally, although SYK-dependent BCR-induced PI3K activation likely involves CBL, whether this interaction is important in a qualitative or quantitative manner remains undefined. Certainly other SYK-dependent pathways for PI3K activation, for example RAS, PtdIns-3,4,5-P3, and PtdIns-3,4-P3 in particular membrane domains are in turn available to bind downstream effectors. PtdIns-4,5-P2, PtdIns-3,4,5-P3 PtdIns-4,5-P3, PtdIns-3,4-P3, PtdIns-3,4,5-P3, PtdIns-4,5-P3, PtdIns-3,4-P3, PtdIns-4,5-P3, PtdIns-3,4-PI, inositol-5-phosphatase, SGS, SHC/GRB2/SOS pathway.

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Fig. 4. SYK overexpression blocks BCR-dependent CBL tyrosine phosphorylation and CBL association with p85 PI3K but does not alter BCR-dependent CD19 tyrosine phosphorylation or p85 PI3K association. 5 × 10⁶ A20 B cells (anti-phosphotyrosine A) or 1 × 10⁶ A20 cells (anti-p85 PI3K-B) were infected for 16 h with GB or SYKT at 10 pfu/cell for BCR experiments. Cells were split evenly (A) or 1/3 for no stimulation and 2/3 for stimulation (B). Stimulations were done as described, and lysates were precipitated with anti-CBL antibodies. Immunoblots were prepared as described under “Experimental Procedures” and blotted with the indicated antibodies. For the CD19 experiments, 2 × 10⁶ cells were infected as above, split evenly, and stimulated as described (C). Lysates were precipitated with anti-CD19 antibody, immunoblots were prepared as described under “Experimental Procedures,” and membranes were probed with indicated antibodies.

Fig. 5. Model of SYK-dependent BCR-induced PI3K activation. Upon engagement of the BCR, SYK is recruited to the phosphorylated Ig ITAM motifs and activated. SYK tyrosine phosphorylates CBL at the p85 PI3K-binding site (amino acid 731), whereby CBL binds p85 PI3K leading to activation of PI3K lipid kinase activity and production (??) localized of D3-phosphoinositides. D3-phosphoinositides may also be produced by alternative SYK-dependent pathways, for example via RAS, PtdIns-3,4,5-P3, and PtdIns-3,4-P3 in particular membrane domains are in turn available to bind downstream effectors. PtdIns-4,5-P3, PtdIns-3,4,5-P3, PtdIns-3,4-PI, inositol-5-phosphatase, SGS, SHC/GRB2/SOS pathway.
