The B cell receptor (BCR) initiates three major signaling pathways: the Ras pathway, which leads to extracellular signal-regulated kinase (ERK) activation; the phospholipase C-γ pathway, which causes calcium mobilization; and the phosphoinositide 3-kinase (PI 3-kinase) pathway. These three pathways are important for B cell development and activation. Several model systems show evidence of cross-regulation between these signaling pathways. Here we demonstrate through the use of PI 3-kinase inhibitors and a dominant-negative PI 3-kinase construct that the BCR-induced phosphorylation and activation of ERK is dependent on PI 3-kinase. PI 3-kinase feeds into the Ras signaling cascade at multiple points, both upstream and downstream of Ras. We also show that ERK activation is dependent on phospholipase C-γ, in keeping with its dependence on calcium mobilization. Last, the activation of PI 3-kinase itself is completely dependent on Ras. We conclude that the PI 3-kinase and Ras signaling cascades are intimately connected in B cells and that the activation of ERK is a signal integration point, since it requires simultaneous input from all three major signaling pathways.

Triggers the B cell antigen receptor (BCR) by antigen or polyclonal activators initiates signaling cascades that lead to various cellular responses. Receptor ligation is followed by the phosphorylation of the immunoreceptor tyrosine-based activation motifs (2–4), which recruit cytosolic proteins and enzymes. Receptor recruitment brings enzymes into proximity of their substrates or kinases, which permits the sequential reactions that form the basis of signaling cascades. Three such cascades stimulated by immunoreceptors involve Ras, phosphoinositide 3-kinase (PI 3-kinase), and phospholipase C-γ (PLC-γ) (reviewed in Ref. 1).

The activation of PI 3-kinases results in the rapid accumulation of D-3 phosphatidylinositol 4,5-bisphosphate in the cell membrane (5). PI 3-kinase products bind with high specificity to pleckstrin homology domains of signaling proteins, to recruit pleckstrin homology domain-containing enzymes to the plasma membrane and promote their subsequent activation (6, 7). Class IA PI 3-kinases, the major class induced by tyrosine kinase-dependent receptors (6) consist of an SH2 domain-containing adapter p85 subunit that is constitutively bound to a catalytic p110 subunit. Animals lacking the p85 isoforms p85α show a defect in B cell development, and mature B cells from these animals are impaired in their response to anti-IgM (8, 9).

The activation of Ras requires the membrane localization of a guanine nucleotide exchange factor (GEF) such as Sos (10). In growth factor-stimulated cells, Sos translocation is mediated by the adapter proteins, Grb2 and Shc (11). BCR triggering causes the association of Sos with the adaptors Shc and Grb2 (12–14). The ternary complex of Shc, Grb2, and Sos is associated with the plasma membrane after BCR triggering, consistent with an essential role for the adapter proteins. GTP-bound Ras causes membrane recruitment of the protein kinase Raf, which is followed by its phosphorylation by the kinase PAK (15) and by Src family tyrosine kinases (16). Activated Raf binds to and activates MEK1 and MEK2, which in turn activate the MAP kinases ERK1 and ERK2. The duration of ERK activation induced by receptors can affect signal outcome (17). Thus, growth factor-stimulated fibroblasts require a sustained low level of ERK activation for progression through the G1 phase. Conversely, transient ERK activity of high amplitude causes G1 arrest (17). The activation of MEK and ERK in B cells is also dependent on calcium flux (18), but the mechanism(s) involved are unexplored.

Despite the importance of Ras and ERK activation for B cell development and activation (19, 20), the pathways that lead to the induction of Ras by BCR have not been closely investigated. For example, the requirement for Shc and Grb2 has not been directly tested.

Although signaling cascades involving Ras, PI 3-kinase, and PLC-γ are generally thought to be independent of each other, some reports indicate cross-talk between these pathways. For example, full activation of PI 3-kinase in growth factor-stimulated fibroblasts showed a requirement for an interaction between active GTP-bound Ras and p110 (21–23). Likewise, assembly of a membrane-associated signaling complex containing PLC-γ, the adapter BLNK, and the tyrosine kinases Btk (Bru- ton’s tyrosine kinase) and Syk allows activation of PLC-γ and the subsequent rise in intracellular calcium and the activation of protein kinase C (24). PI 3-kinase products are required for the recruitment and activation of Btk and may also contribute to the recruitment and/or allosteric regulation of PLC-γ itself (25, 26). Consequently, intracellular calcium flux is partially dependent on PI 3-kinase activity (27, 28).

PI 3-kinase might also contribute to the activation of ERK. In fibroblasts, the induction of ERK by low doses of growth factor...
is dependent on PI 3-kinase (29, 30). In Jurkat T cells, ERK activation by T cell receptor is dependent on PI 3-kinase (31). Class IB PI 3-kinase p110γ has been shown to be necessary for ERK activation by G-protein-coupled receptors (32). A mutant of p110γ that lacks lipid kinase activity but retains protein kinase activity (33, 34) was shown to activate ERK but not Akt (33).

Given the central importance of Ras and ERK in B cell antigen-receptor-induced proliferation and development, we examined in greater detail the events that lead to ERK activation. Our broader aim was also to determine whether there is cross-talk between the PI 3-kinase and the Ras pathway in BCR signal transduction. Our results indicate that this is indeed the case. We used different methods to inhibit PI 3-kinase signaling and observed that ERK activation is correspondingly blocked. This influence of PI 3-kinase on ERK is independent of intracellular calcium mobilization. Additional experiments show that PI 3-kinase influences the Ras cascade both upstream and downstream of Ras itself. Finally, we show that Ras is required for the activation of PI 3-kinase, as it is in fibroblasts (21–23). Taken together, our results demonstrate that the PI 3-kinase and Ras pathways in B cells are intimately connected at several levels.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Lines**—Splenic B cells were prepared from the spleens of 5–8-week-old male BALB/cByJ mice (Jackson Laboratories) as described earlier (35). In brief, erythrocytes were removed by osmotic lysis, and T cells were removed by incubation with anti-Thy 1.2 followed by guinea pig complement serum. The resulting population is 85–95% spleen FcyRII+ (FcγRII). The murine B cell lymphoma A20 and its FcγRII-deficient derivative IIA1.6 were maintained at 37 °C in RPMI 1640 (ICN) containing 10% fetal bovine serum (Invitrogen), 50 μg/mL 2-mercaptoethanol, and antibiotics.

**Antibodies and Reagents**—Anti-ERK2-C(14), anti-Grb2, and anti-MEK1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-HA (high affinity) was from Roche Molecular Biochemicals; F(ab′)2 rabbit anti-mouse IgG (H+L) was from Pierce; anti-IgH was from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Sos was a subcloned into pEFneo with an internal EcoRI and orientation of the insert confirmed by sequencing. All other reagents were obtained from Dr. Ben Margolis (University of Michigan Medical School, Ann Arbor, MI) (41). GST-ERK2 in pEBG and Grb2R6EK in pEBB were from Dr. Bruce Mayer (Howard Hughes Medical Institute, Children’s Hospital, Boston, MA) (42); and pSsAΔinter-H2Sh2 in pSG5 was obtained from Dr. Julian Dowward (Imperial Cancer Research Fund, London, UK) and subcloned into pEF1a/myc-HisA(Invitrogen) with EcoRI and orientation of the insert confirmed by sequencing. All the constructs in these plasmids are driven by the EF-1α promoter with the exception of Shc Y239F/Y240F/Y317F, which is in the retroviral expression vector pBabe-Myc (41).

**Stimulations**—For immunoprecipitations, 10 × 106 cells were washed and resuspended in 100 μl of Hanks’ balanced salt solution and stimulated at 37 °C with 10 μg/ml PMA (Calbiochem), 1 μM all-trans retinoic acid (Sigma), 10 μM EdTA, 10 mM Na3VO4, 10 mM NaF, and 1% Triton X-100 containing 3 mM activated Na3VO4, 10 μg/ml aprotinin, 25 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and, for anti-HA-Akt immunoprecipitations, 1 μg/ml microcystin-LR. For spectrofluorometric measurements, 1–2 × 106 loaded cells were resuspended in 1 ml of HBSS (Hanks buffered salt solution, pH 7.4). Stimulation reagents were added directly to the cuvette. PMA was added to 20 ng/ml for 10 min at 37 °C. Wortmannin and LY294002 were added to equilibrated cells for 10 min at 37 °C, 30% wortmannin and LY294002 were added to equilibrated cells for 10 min at 37 °C, 4°C. Cells were stimulated and treated as described earlier and lysed, and the lysates were boiled with 5× SDS sample buffer (1× SDS sample buffer: 65 mM Tris-HCl, 10% glycerol, and 2.3% SDS). Affinity precipitation of Shc was performed as described earlier (35). Boiled samples were analyzed by SDS-PAGE and transferred to nitrocellulose, and the membranes were blocked for 1 h at room temperature in 5% nonfat milk or 1% gelatin in Tris-buffered saline-Tween. The membranes were incubated at 0.5–1 μg/ml primary antibody overnight at 4 °C, washed, and incubated with the appropriate horseradish peroxidase-linked secondary reagent for 40 min at room temperature. The Pierce SuperSignal ECL kit was used to develop the membranes, and images were captured on a Roche Lumilager. Where necessary, the bands were quantitated using ImageQuant software.

**ERK and Akt in Vitro Kinase Assays**—In vitro kinase assays for ERK-2 and HA-Akt were performed essentially as described earlier (35, 43). In brief, cells were lysed in TN-1 lysis buffer, and cleared supernatants were incubated with 2–4 μg of anti-HA or anti-ERK for 2 h followed by incubation with protein A/G-Sepharose for 1 h. Immune complexes were washed 4–6 times in lysis buffer and twice in assay buffer (20 mM MOPS, pH 7.2, 25 mM sodium glycophosphate, 1 mM activated Na3VO4, 1 mM dithiothreitol), and resuspended in the latter. To this suspension 10–20 μCi of [γ-32P]ATP was added along with 10–20 μg of histone H2B for Akt and myelin basic protein for ERK, and the mixture was incubated at 30 °C for 10 min. The 5× kinase reactions were terminated by the addition of 5× sample buffer. The supernatants were analyzed by SDS-PAGE and transfer to nitrocellulose, and the substrates quantitated using a Molecular Dynamics Storm system or by Coomassie staining to visualize the substrate followed by liquid scintillation counting of the excised bands.

**MEK1 in Vitro Kinase Assay**—A modification of the assay described in for MEK1 was used. Cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM NaF, 10 mM Na3VO4, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS), and MEK1 was immunoprecipitated overnight. Immune complexes were washed three times in radioimmune precipitation buffer and twice in kinase buffer (100 mM β-glycerophosphate, 160 mM HEPES, pH 7.2, 200 mM sodium vanadate, 40 mM MgCl2). The beads were incubated in 30 μl of kinase buffer at 30 °C for 30 min with 10 μg of kinase-inactive ERK2 and 4000 dpm/pmol of [γ-32P]ATP (166 μCi) with gentle agitation. Substrate was analyzed as detailed above. The specificity of phosphorylation was confirmed by blocking the reaction with the MEK inhibitor PD98059.

**Ras Assay**—The assay was performed essentially as described in for the assay was used. GST-Raf-RBD beads and GST control beads were prepared. The freshness of the fusion protein was found to be critical to the success of the experiment, so the beads were not stored for more than 48 h at 4 °C. Cells were stimulated and treated as described earlier and lysed, and Ras-GTP was precipitated from the lysates using the GST-Raf-RBD beads. The precipitated Ras was detected by immunoblot with the anti-ras (Ab-3) monoclonal antibody. The immunoblots were quantitated using a Roche Lumilager. Samples were run in triplicate or quadruplicate. The experiment shown in Fig. 5 is a “blinded” trial in which the tubes were relabeled by a colleague after the postnuclear spin. The identity of the samples was revealed at the end of the experiment.

**Transient Transfections**—A20 and its derivatives were transfected as described in for 45. 1 × 106 cells were electroporated with plasmid

Cross-talk between PI 3-Kinase and Ras in B Cells

2343
DNA in a 4-mm gap cuvette containing warm RPMI, using a Bio-Rad GenePulser at 310 V and 960 microfarads. 5 μg of GST-ERK or HA-Akt DNA, 25–50 μg of Shc Y239F/Y240F/Y317F or Grb2R86K DNA, 25 μg of V12Ras or N17Ras DNA, and 5–50 μg of p85lnter-SH2 DNA was added to each cuvette, depending on the experiment. The cells were resuspended in 8–10 ml of warm growth medium. Stimulations and immunoprecipitations were performed as above after incubation overnight at 37 °C in an incubator. To normalize for protein expression, whole lysates of the transfected cells were run in parallel and subjected to Western blot with anti-HA (in the case of HA-Akt) or anti-GST (in the case of GST-ERK). In some cases, the nitrocellulose used for the in vitro kinase assay itself was cut, and the top half was subjected to Western blot.

Measurement of Intracellular Calcium—Cells were washed and resuspended in growth medium (1 × 10^6/ml) and loaded with 5 μM Fluo-4AM for 30 min at 37 °C. The cells were washed, resuspended in growth medium without phenol red, and excited at 495 nm in a PerkinElmer Life Sciences LS55B luminescence spectrometer using a stirred cuvette maintained at 37 °C. The emission was measured at 516 nm. The intracellular calcium concentration was calculated using the formula [Ca^{2+})_o = K_d (F - F_{min})/F_{max} - F_{min}], F represents the instantaneous fluorescence reading of the loaded cells. F_{max}, the fluorescence of the indicator when saturated with calcium, was obtained by lysing the cells with Triton X-100, and F_{min}, the fluorescence in the absence of calcium, was obtained by the subsequent addition of EGTA. The K_d of Fluo-4 as given by the manufacturer is 345 nm.

RESULTS

Activation of ERK by the BCR Requires PI 3-Kinase Activity—We treated murine splenic B cells with F(ab')_2 anti-Ig to stimulate the BCR and examined the resulting phosphorylation of ERK in the presence of the PI 3-kinase inhibitor LY294002 or carrier alone. As seen in Fig. 1A, ERK phosphorylation in carrier-treated B cells peaks within 1 min of stimulation and is still above baseline at 30 min. In cells pretreated with LY294002, ERK phosphorylation is greatly reduced and peaks much later, around 5 min. Fig. 1B shows that the use of Wortmannin, another PI 3-kinase inhibitor, has the same effect on ERK phosphorylation. The inhibition of PI 3-kinase is demonstrated by the lack of Akt phosphorylation in the presence of these compounds (Fig. 1B, bottom panel). At these concentrations, both compounds are reported to be highly specific for PI 3-kinase (46, 47). Note that in Fig. 1, A and B, the different secondary reagents used in the immunoblot detect the endogenous B cell immunoglobulin heavy and light chains that are expressed on the cells used in the experiments. The equal intensity of these bands across all lanes demonstrates equivalent loading of the cell lysates.

We also directly measured ERK activity by in vitro kinase assay (Fig. 1C). For these experiments, splenic B cells from male BALB/cByJ and C57/BL6 mice were isolated and preincubated with the PI 3-kinase inhibitors LY294002 or carrier and stimulated with F(ab')_2 anti-mouse Ig. The activity of immunoprecipitated ERK2 was assayed in vitro. Fig. 1C represents the average values from three independent experiments done with B cells from both BALB/cByJ and C57/BL6 mice. Preincubation of the B cells with 10 μM LY294002 completely inhibits the activation of ERK. The PI 3-kinase inhibitor had no direct effect on ERK in the in vitro kinase reaction, ruling out the possibility that it directly inactivates ERK.

The data shown in Fig. 1 suggest that PI 3-kinase is required for the phosphorylation and activation of ERK in response to BCR ligands. To confirm this observation, we transfected the murine B cell line A20 with a GST-tagged ERK2 reporter in combination with P85Sh2 or SH2. The latter is a mutant construct of the PI 3-kinase regulatory subunit p85c lacking the minimal binding site for the catalytic subunit p110. This mutant has been used to block PI 3-kinase signaling in a dominant-negative fashion (31). Fig. 2 shows that P85Sh2 inhibits GST-ERK activation by BCR in a dose-dependent manner. Together with the inhibitor studies shown in Fig. 1, the data indicate that the induction of ERK by the B cell antigen receptor requires PI 3-kinase activity.

The assembly of the Shc-Grb2-Sos Complex Is Unaffected by PI 3-Kinase Inhibition—To examine which of the steps in the cascade from antigen receptor to ERK is dependent on PI 3-kinase, we studied the assembly of the Shc-Grb2-Sos complex. The need for Shc and Grb2 in Ras induction is based on experiments with fibroblasts stimulated with growth factors and has not been studied in the context of BCR signaling. We investigated this issue using dominant-negative mutants of Shc and Grb2. Shc Y239F/Y240F/Y317F is unable to bind Grb2 (41), and Grb2R86K has an inactivated SH2 domain and is

FIG. 1. ERK activation by the B cell receptor is sensitive to inhibition of PI 3-kinase. A, B cells were isolated from the spleens of male BALB/cByJ mice, preincubated with the carrier Me2SO (lanes 2–6) or the PI 3-kinase inhibitor LY294002 at 10 μM (lanes 7–11), and stimulated with F(ab')_2 rabbit anti-mouse Ig for varying lengths of time. The presence of phosphorylated ERK in whole lysates was detected by immunoblot with anti-phospho-ERK antibody. The position of phosphorylated ERK (p-ERK) and p-ERK2) and the endogenous immunoglobulin heavy chain (lg H) is indicated. The first lane contains unstimulated cells, and the last lane contains cells stimulated for 10 min with 20 ng/ml PMA; they represent background and maximal ERK phosphorylation, respectively. B, B cells were isolated as in A and pretreated with the carrier or the indicated concentrations of the PI 3-kinase inhibitors LY294002 (LY) or Wortmannin (Wm) and then stimulated with F(ab')_2 rabbit anti-mouse Ig for 2 min. Whole lysates were prepared, and aliquots run in parallel were immunoblotted for phospho-ERK (top panel) or phospho-Akt (p-Akt; bottom panel). The positions of these proteins and of endogenous immunoglobulin light chain (lg L chain) are indicated. The first and last lanes are the controls described for A. C, B cells were isolated from the spleens of male BALB/cByJ or male C57/BL6 mice, stimulated as in B in the presence or absence of 10 μM LY294002, and the activity of immunoprecipitated ERK2 was determined by in vitro kinase assay. The average of three trials is plotted as a percentage of activity in the stimulated samples. Black bars, C57/BL6; gray bars, BALB/cByJ mice. Error bars represent S.D.
therefore unable to bind Shc (42). A20 B cells were co-transfected with the reporter GST-ERK in combination with cDNA encoding these mutant proteins, and the kinase activity of immunoprecipitated GST-ERK was measured in vitro. As seen in Fig. 3, both Shc and Grb2 mutants reduced the activation of GST-ERK by about 50% (compare gray bars). This finding is consistent with a need for the Shc-Grb2 complex in Ras activation by BCR.

We next investigated whether the assembly of Shc-Grb2-Sos is dependent on PI 3-kinase. Cell lysates were incubated with a GST-Grb2 fusion protein as described earlier (35), and co-precipitating Shc was detected by immunoblot. Fig. 4 shows that inhibition of PI 3-kinase with LY294002 had no impact on the anti-Ig-induced phosphorylation of Shc, and phosphorylated Shc was competent to bind the SH2 domain of Grb2 (compare lanes 2 and 3). We also examined the association of Grb2 and Sos by co-immunoprecipitation using anti-Grb2 antibodies, as previously described (48). As shown in Fig. 4B, the anti-Ig-induced association of Sos and Grb2 was also unaffected by the presence of LY294002. These results suggest that the assembly of the Shc-Grb2-Sos complex does not require PI 3-kinase activity.

**PI 3-Kinase Activity Affects the Activation of Ras and MEK**—We directly measured Ras activation by BCR using the affinity precipitation method (38). For these experiments, B cell lysates were incubated with a GST fusion protein containing the Ras binding domain of Raf. Active Ras was detected by immunoblotting the precipitated proteins with a pan-anti-Ras antibody. The samples were measured in triplicate, and the identity of the samples was “blinded” before the assay. Fig. 5 shows a representative experiment; in this case, a 32% decrease in Ras-GTP loading was observed in B cells treated with LY294002. Although the amount of active Ras detected by this method varied from experiment to experiment, in every case we observed a decrease in Ras activation in the presence of LY294002. However, in contrast to ERK activation, in no case was the GTP-loading of Ras completely blocked by inhibition of PI 3-kinase.

The activity of MEK1, which is immediately upstream of ERK in the Ras cascade, was determined by in vitro kinase assay using recombinant inactivated ERK2 as a substrate. The results are presented in Fig. 6A. We found that the activation of MEK1 kinase activity by BCR is very sensitive to the presence of LY294002, since MEK activity was completely blocked with 10 μM LY294002. The induction of MEK kinase activity requires phosphorylation by Raf (49) and can be used as an indicator of Raf activity in cells (50, 51). We examined MEK phosphorylation by immunoblotting with anti-phospho-MEK antibody (Fig. 6B) and found it to be similarly affected by inhibition of PI 3-kinase. These data suggest that PI 3-kinase is required upstream of Raf and MEK activation.

**ERK Activation Requires PLC-γ Activity**—The activation of ERK has been reported to require intracellular calcium influx (18). PLC-γ activation and consequently the mobilization of calcium are at least partially dependent on PI 3-kinase (27, 28). Therefore, the requirement for PI 3-kinase activity in BCR-stimulated ERK activity might reveal an effect on PLC-γ and calcium flux. We confirmed that BCR-induced ERK activation requires calcium mobilization (data not shown) using the intracellular calcium chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid. To establish a corresponding requirement for PLC-γ activity, we used the PLC inhibitor U73122 to block BCR-induced calcium flux. The efficacy of U73122 was first tested in a dose-response experiment, shown in Fig. 7A. The emission of cells loaded with the fluorescent calcium-binding dye Fluo-4 was monitored spectrofluorometrically before and after BCR stimulation. We found that a 30-s preincubation with U73122 at 1 μM is sufficient to inhibit greater than 80% of BCR-induced calcium flux. 1 μM U73122 was not toxic to B cells,
as assessed by trypan blue dye exclusion (data not shown). We then assayed the phosphorylation of ERK after treatment with U73122 using the same conditions. As seen in Fig. 7B, ERK phosphorylation by BCR is completely inhibited, indicating a requirement for PLC-γ activity. This observation is consistent with earlier findings that calcium mobilization lies distal to PI 3-kinase and proximal to ERK activation.

These results raised the possibility that deficient calcium mobilization might account for the PI 3-kinase requirement in the induction of ERK. To test this, we asked whether calcium flux induced by the calcium ionophore ionomycin could reconstitute the necessary signal in the absence of PI 3-kinase activity. High concentrations of ionomycin (>1 μM) alone activate ERK (not shown); therefore, we first established an appropriate ionomycin concentration that mimics the level of calcium flux similar to that induced by BCR ligation. The titration shown in Fig. 8A established that 50 nM ionomycin very closely mimics BCR-induced calcium influx. To see whether increased cytoplasmic calcium caused by ionomycin could restore ERK activity when PI 3-kinase is inhibited, we assessed B cell lysates for phospho-ERK in the presence and absence of LY294002 and 50 nM ionomycin. The results (Fig. 8B) show that 50 nM ionomycin does not by itself induce ERK phosphorylation (lane 4 versus lane 1); nor does it enhance ERK phosphorylation induced by BCR stimulation (lane 6 versus lane 2). Furthermore, the addition of ionomycin to B cells incubated in the presence of LY294002 did not restore BCR-induced ERK phosphorylation (lane 7). These findings demonstrate that although PI 3-kinase affects BCR-induced calcium influx (27, 28), PI 3-kinase influences ERK by a calcium-independent pathway.

**PI 3-Kinase Is Downstream of Ras in B Cell Receptor Signaling**—Experiments in fibroblasts indicated that PI 3-kinase activation by growth factors is dependent on Ras (21–23). To examine whether the same is true in B cells, we used the activation of transiently transfected Akt as an indirect measurement for PI 3-kinase activity. A20 murine B cells were transfected with HA-tagged Akt cDNA in combination with an excess of vector or V12Ras, a GTPase-deficient mutant known to potently stimulate Ras-dependent events (52). After stimulation, cell lysates were immunoprecipitated with anti-HA monoclonal antibody, and the immunoprecipitates were subjected to an in vitro kinase assay, as described earlier (43). As can be seen in Fig. 9A, basal activity of HA-Akt (Vector; white

**Fig. 5.** Ras activation is decreased in the presence of PI 3-kinase inhibitor. Splenic B cells were isolated from BALB/c ByJ mice, pretreated with carrier (−) or LY294002, and stimulated as above. GTP-bound Ras was precipitated from the lysates using a GST-Raf-RBD fusion protein, visualized by immunoblot, and quantitated using a Roche LumiImager. Shown is a representative experiment; the values are the average of triplicate samples. Error bars represent S.D.

**Fig. 6.** The activation of MEK1 by the B cell receptor is dependent on PI 3-kinase activity. A, BALB/c ByJ splenic B cells were preincubated with carrier or the indicated concentrations of LY294002 and stimulated, and the activity of immunoprecipitated MEK1 was determined by in vitro kinase assay. The average of three independent trials is shown. B, whole lysates were made from B cells stimulated in the presence of carrier or 10 μM LY294002. The presence of phosphorylated MEK in these lysates was detected using an anti-phospho-MEK antibody. Half the amount of lysate was loaded in the PMA-stimulated sample as in the others. Phospho-MEK (p-MEK) is the upper band of the doublet, as confirmed by reprobe with anti-MEK1 antibody shown in the lower panel. A representative experiment is shown.

**Fig. 7.** The activation of ERK is dependent upon PLC activity. A, titration of U73122. Splenic B cells were loaded with Fluo-4/AM, and the fluorescence of the cells was monitored over time in a luminescence spectrometer. The concentration of the PLC inhibitor U73122 added is indicated as follows. Closed diamonds, carrier; open inverted triangles, 50 nM; open squares, 200 nM; closed triangles, 1 μM. The first arrow represents the addition of carrier (Me2SO) or inhibitor, and the second arrow represents the addition of F(ab’2) rabbit anti-mouse Ig. The concentration of intracellular calcium was calculated as described under “Experimental Procedures.” B, effect of U73122 on ERK phosphorylation. Splenic B cells were treated with carrier or 1 μM U73122 followed by F(ab’2) rabbit anti-mouse Ig as in A. Aliquots of whole lysates run in parallel were immunoblotted for p-ERK1/2 (top panel) or total ERK (lower panel). A representative experiment is shown.
Bar) is stimulated about 5-fold by the presence of active Ras. Furthermore, the induction of Akt by active Ras is completely blocked by inhibition of PI 3-kinase. These data are consistent with the notion that V12Ras induces Akt by activating PI 3-kinase.

To examine this issue in the context of BCR signaling, B cells were transiently transfected with HA-tagged Akt cDNA, in combination with an excess of vector or N17Ras, a dominant-negative mutant of Ras (53). As seen in Fig. 9A, stimulation of the BCR increases HA-Akt activity (Vector, white bar) by 3–5-fold (Vector, black bar), as earlier reported (43). However, in the presence of N17Ras, both basal (N17Ras, white bar) and BCR-stimulated (N17Ras, black bar) HA-Akt activity is completely inhibited. These findings establish that the activation of PI 3-kinase and its effector Akt by BCR is downstream of Ras, as demonstrated in other systems (21–23).

**DISCUSSION**

The results presented here demonstrate that PI 3-kinase is required for the activation of ERK by the BCR. In the presence of the PI 3-kinase inhibitor LY294002 or a dominant-negative mutant of Ras (53). As seen in Fig. 9B, stimulation of the BCR increases HA-Akt activity (Vector, white bar) by 3–5-fold (Vector, black bar), as earlier reported (43). However, in the presence of N17Ras, both basal (N17Ras, white bar) and BCR-stimulated (N17Ras, black bar) HA-Akt activity is completely inhibited. These findings establish that the activation of PI 3-kinase and its effector Akt by BCR is downstream of Ras, as demonstrated in other systems (21–23).

By examining this issue in the context of BCR signaling, B cells were transiently transfected with HA-tagged Akt cDNA, in combination with an excess of vector or N17Ras, a dominant-negative mutant of Ras (53). As seen in Fig. 9B, stimulation of the BCR increases HA-Akt activity (Vector, white bar) by 3–5-fold (Vector, black bar), as earlier reported (43). However, in the presence of N17Ras, both basal (N17Ras, white bar) and BCR-stimulated (N17Ras, black bar) HA-Akt activity is completely inhibited. These findings establish that the activation of PI 3-kinase and its effector Akt by BCR is downstream of Ras, as demonstrated in other systems (21–23).

**FIG. 9.** Ras is upstream of PI 3-kinase in B cell receptor signaling. A, A20 cells were co-transfected with HA-Akt cDNA and an excess of vector or V12Ras cDNA as indicated, and the kinase activity of anti-HA immunoprecipitates was determined from resting cells. White bars, cells pretreated with carrier; black bars, cells pretreated with 25 μM LY294002. An average of three independent trials is shown; error bars represent S.D. B, IIA1.6 B cells were co-transfected with HA-Akt in combination with excess N17Ras cDNA as indicated, and the activity of HA-Akt was determined from resting samples (white bars) and samples stimulated with rabbit anti-mouse Ig (black bars). An average of two trials is shown; error bars represent S.D.

**FIG. 10.** Model of ERK activation by the B cell receptor. BCR induces Ras through the assembly of the Shc-Grb2-Sos complex as well as through other mechanisms. Ras causes the activation of the ERK and PI 3-kinase. PI 3-kinase contributes to the activation of PLC-γ2 as well as to that of ERK, feeding into the Ras pathway both upstream and downstream of Ras itself. PLC-γ2 causes the mobilization of calcium, which is also required for the activation of ERK. Thus, signals from Ras, PI 3-kinase, and PLC-γ2 converge on the activation of ERK. Closed arrowheads indicate links investigated in this study.

proteins with their endogenous counterparts. Alternatively, there may be other pathways that link the BCR to Ras activation that are independent of Shc and Grb2. Supporting this possibility, the results in Fig. 5 show that PI 3-kinase makes a small but...
significant contribution to Ras activation.

ERK activation is also dependent on PLC-γ activity (Fig. 7B), as suggested by earlier studies indicating a requirement for intracellular calcium mobilization (18). Since PI 3-kinase inhibitors cause a partial block in BCR-induced calcium flux (27, 28), we investigated whether this may contribute to the defect in ERK activity. We found that reconstitution of calcium with ionomycin failed to restore ERK activation (Fig. 8B), indicating that PI 3-kinase affects ERK through a calcium-independent mechanism.

This report provides evidence for a PI 3-kinase-dependent event downstream of Ras. Paradoxically, we also found that Ras acts upstream of PI 3-kinase in B cells, as it does fibroblasts (9). The effect of Ras on PI 3-kinase is presumably through a direct interaction of Ras-GTP with the p110 subunit of PI 3-kinase. This is in stark contrast to T cell receptor signaling, where this interaction does not take place (54).

The fact that Ras activation is not as sensitive to LY294002 as ERK activation suggests that PI 3-kinase provides essential pathway support at steps downstream of Ras activation. One mechanism might be the PI 3-kinase-dependent activation of PAK, which activates Raf-1 in other systems (15). In human tonsil B cells, ERK activation by BCR is partially dependent on PI 3-kinase, whereas the enhancement of ERK activation through the co-ligation of CD19 is completely dependent on PI 3-kinase (55). ERK activation by T cell receptor in Jurkat T cells (31) and by FcRI in human basophils (56) absolutely requires PI 3-kinase. In the former case, PI 3-kinase feeds into the Ras cascade downstream of MEK, whereas in the latter case PI 3-kinase is apparently required for the activation of Ras itself. Interestingly, the class IB PI 3-kinase p110γ links G protein-coupled receptors to ERK activation through its protein kinase activity rather than through lipid phosphorylation (33). Whether this is also true of class IA PI 3-kinases is currently unknown. In any case, these earlier reports together with our studies on B cells show that the requirement for PI 3-kinase in ERK activation is more universal than previously recognized. The findings suggest that signal transduction pathways are not isolated entities, but rather there is considerable cross-talk between them. Further investigation is necessary to identify the precise link between PI 3-kinase and ERK.

We have summarized our data in the model shown in Fig. 10. Ligation of the B cell receptor causes the induction of Ras through the translocation of the She-Grb2-Sos complex and possibly through other mechanisms as well. Active Ras induces the Raf cascade as well as PI 3-kinase, with the latter exerting a positive feedback on Ras activation. PI 3-kinase also contributes to the activation of ERK downstream of Ras and to the induction of PLC-γ. PLC-γ in turn induces calcium release, which supports the activation of ERK.

These results demonstrate the intricate relationship between the Ras, PLC-γ, and PI 3-kinase pathways in B cells. These proteins can no longer be thought of as initiating separate signal transduction cascades; rather, they are part of a complex network. We have documented connections between the pathways at several levels, from proximal events such as the induction of PI 3-kinase to distal ones such as that of ERK. Last, the induction of ERK by the B cell receptor appears to be under the simultaneous control of PI 3-kinase, Ras, and PLC-γ. Such multilayered regulation may be necessary to protect against inappropriate activation of ERK, given the established importance of this enzyme in cell cycle progression and differentiation.

REFERENCES

1. Gold, M. R. (2000) Curr. Top. Microbiol. Immunol. 245, 77–134
2. Beth, M., Wianends, J., and Schamel, W. W. (2000) Immunol. Rev. 176, 10–18
3. Hutchinson, J. E., Harrison, M. L., and Geahlen, R. L. (1992) J. Biol. Chem. 267, 8613–8619
4. Ma, H., Yankee, T. M., Hu, J., Asai, D. J., Harrison, M. L., and Geahlen, R. L. (2001) J. Immunol. 166, 1507–1516
5. Gold, M. R., and Aebersold, R. (1999) J. Immunol. 162, 42–50
6. Vanhaeckebroeck, B., and Waterfield, M. D. (1999) Exp. Cell Res. 253, 239–254
7. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) Annu. Rev. Biochem. 68, 965–1014
8. Suzuki, H., Terachy, U., Fujiwara, M., Aizawa, S., Yaaaki, Y., Kadowaki, T., and Koyasu, S. (1999) Science 283, 390–392
9. Fruman, D. A., Snapper, S. B., Ybaele, C. M., Davidson, L., Yu, J. Y., Alt, F. W., and Cantley, L. C. (1999) Science 283, 393–397
10. Aronheim, A., Engelberg, D., Li, N., al-Askawi, N., Schlessinger, J., and Karin, M. (1994) Cell 78, 949–961
11. Malumbres, M., and Pellicer, A. (1998) Front. Biosci. 3, 877–912
12. Lankester, A. C., van Schijndel, G. M., Rood, P. M., Verhoeven, A. J., and Broek, J. M. (1994) J. Immunol. 153, 6236–6246
13. Smitt, L., de Vries-Smits, A. M., Bos, J. L., and Borst, J. (1994) J. Biol. Chem. 269, 20290–20212
14. Sun, H., Reif, K., Beach, S., Kramer, I., and Cantrell, D. (1998) Oncogene 17, 1731–1738
15. Li, X., and Carter, R. H. (2000) Eur. J. Immunol. 30, 1576–1586
16. Miura, K., and MacGlashan, D. W. (2000) Blood 96, 2199–2205