The B cell antigen receptor (BCR) activates Ras, a GTPase that promotes cell proliferation by activating the Raf-1/MEK/ERK signaling module and other signaling enzymes. In its active GTP-bound form, the Rap1 GTPase may act as a negative regulator of Ras-mediated signaling by sequestering Ras effectors (e.g., Raf-1) and preventing their activation. In this report, we show that BCR engagement activates Rap1 and that this is dependent on production of diacylglycerol (DAG) by phospholipase C-γ. Activation of Rap1 by the BCR was greatly reduced in phospholipase C-γ-deficient B cells, whereas both a synthetic DAG and phorbol dibutyrate could activate Rap1 in B cells. We had previously shown that C3G, an activator of Rap1, associates with the Crk adapter proteins in B cells and that BCR engagement causes Crk to bind to the Cas and Cbl docking proteins. However, the DAG-dependent pathway by which the BCR activates Rap1 apparently does not involve Crk signaling complexes since phorbol dibutyrate could activate Rap1 without inducing the formation of these complexes. Thus, the BCR activates Rap1 via a novel DAG-dependent pathway.

The binding of antigens to the B cell antigen receptor (BCR) is essential for the generation of an antibody (Ab) response. BCR signaling activates resting B lymphocytes and, in the presence of T cell-derived costimulatory factors, drives B cells to proliferate and differentiate into antibody-secreting plasma cells (1).

The BCR activates multiple signaling pathways, including those regulated by Ras, phospholipase C-γ (PLC-γ), phosphatidylinositol (PtdIns) 3-kinase, Vav, and HS-1 (2). Ras is a low molecular weight GTPase that acts as a molecular switch, cycling between an inactive GDP-bound form and an active GTP-bound form. GTP-bound Ras binds to and activates the Raf-1 kinase (3). Raf-1 then initiates a kinase cascade that leads to activation of the ERK family of mitogen-activated protein kinases. Activated ERKs migrate to the nucleus where they phosphorylate and activate transcription factors that belong to the TCF and ETS families (4). The Ras/Raf-1/ERK pathway is also directly involved in cell cycle progression and has been shown to up-regulate the expression of cyclins D and E1 while down-regulating the expression of the p27Kip1 cell cycle inhibitor (5). Other downstream effectors of activated Ras include PtdIns 3-kinase, RalA, and p120 RasGAP (6–8). Lipid second messengers produced by PtdIns 3-kinase activate Akt, a kinase involved in preventing apoptosis (9). The RalA GTPase may also be an important target of Ras since dominant-negative forms of RalA can suppress the ability of activated Ras to transform cells (7). Thus, Ras controls multiple pathways that promote cell growth and survival. Consistent with this idea, mutated forms of Ras that are constitutively active are potent transforming agents. These activated forms of Ras are found in a large fraction of human tumors (10) and may be responsible for the uncontrolled proliferation of these cells.

Given the importance of Ras as a mediator of proliferation, differentiation, and oncogenesis, it is crucial to understand how Ras-mediated signaling is regulated. The Rap1 GTPases may be negative regulators of Ras-mediated signaling. The two mammalian Rap1 proteins, Rap1A and Rap1B, are 97% identical at the level of their amino acid sequence and are closely related to Ras. Rap1A (also known as Krev-1) was first identified by its ability to reverse the transformation of NIH 3T3 cells by an activated form of Ki-Ras (11). Expression of activated Rap1 proteins also blocks the actions of Ras in Drosophila eye development and in the maturation of Xenopus oocytes (12, 13). These results suggest that activation of the endogenous Rap1 proteins may be a way in which the magnitude or duration of Ras-mediated signaling is limited. However, little is known about the regulation of the endogenous Rap1 proteins. It is also not clear whether Rap1A and Rap1B have different functions; therefore, we will refer to them collectively as Rap1.

Although the regulation of Rap1 is not well understood, there is considerable structural information indicating how Rap1 opposes the actions of Ras (14). The effector binding domain of Rap1 is identical to that of Ras, suggesting that Rap1-GTP competes with Ras-GTP for downstream effectors. In vitro experiments have shown that Rap1-GTP binds to Raf-1 and other targets of Ras but does not activate them (7, 14–16). In vivo, Rap1 may also keep Ras effectors physically separated from Ras since Rap1 is located primarily on the cytoplasmic face of the Golgi apparatus (17), whereas Ras is at the inner face of the plasma membrane. Consistent with the idea that Rap1-GTP sequesters Ras effectors in inactive complexes, expressing constitutively active Rap1A in fibroblasts inhibits Ras-dependent processes such as anchorage-independent growth.
activation of the ERK kinases (18).

In addition to opposing the ability of Ras to stimulate proliferation, Rap1 may also activate a distinct signaling pathway that inhibits proliferation. Genetic analysis in Drosophila has shown that the *dacapo* gene product is a target of Rap1 (19). *Dacapo* is a cyclin-dependent kinase (cdk) inhibitor similar to p27Rip1 and blocks cell cycle progression during Drosophila eye development.

Previous work by our group and others suggested that the Rap1 proteins may be a target of BCR signaling. C3G, an exchange factor that activates Rap1A and Rap1B (20, 21), is constitutively associated with the Crk adaptor proteins in B cells (22). In response to BCR engagement, the membrane-associated docking proteins p130Cas and p120Cbl are tyrosine phosphorylated, and the Crk proteins then use their SH2 domains to bind to them (22–24). Thus, BCR signaling may recruit Crk-C3G complexes to the cellular membranes where Rap1 is located, allowing C3G to activate Rap1. In this report, we show that the BCR does indeed activate Rap1 but that this occurs via a novel PLC-γ-dependent pathway, which may be independent of Crk and C3G.

**EXPERIMENTAL PROCEDURES**

**Materials**—Goat anti-mouse immunoglobulin M (IgM) Abs, goat anti-mouse IgG Abs, and goat anti-human IgM Abs were from Bio-Can (Mississauga, Ontario). Goat Abs against murine κ light chain were from Southern Biotechnology Associates (Birmingham, AL). The 2–9B1 monoclonal Ab to chicken IgM was a gift of M. Ratcliffe (McGill University, Montreal, Quebec). Phorbol dibutyrate (PdBu) was from Sigma, and ionomycin was from Calbiochem. Diisopropylfluorophosphate (diop) was purchased from Avanti Polar Lipids (Alabaster, AL), dissolved in chloroform, and stored at −20 °C. Immediately before use, it was dried under nitrogen gas and resuspended in MeSO. Abs to Rap1/Krev-1, Crk, and p120Cbl were from Santa Cruz Biotechnology, the Ab to p130Cas was from Transduction Laboratories (Lexington, KY), and the 4G10 anti-phosphotyrosine (anti-P-Tyr) monoclonal Ab was from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Cells**—The WEHI-231, BAL17, and A20 murine B cell lines as well as the RAMOS human B cell line were grown as described (22). The wild-type DT40 chicken B cell line, as well as the PLC-γ-deficient (25), and integrin receptor-deficient (26) variants of this cell line, were grown in RPMI 1640 supplemented with 10% fetal calf serum (Intergen, Purchase, NY), 1% chicken serum (Life Technologies, Inc.), 50 μM 2-mercaptoethanol, and 4 mM glutamine. Small resting B cells were isolated from the spleens of C57BL/6 mice by Percoll density centrifugation after incubation at 37 °C for 45 min with a 1:4 dilution of guinea pig complement (Life Technologies, Inc.) and 1:4 dilutions of culture supernatants from the HO13.4 anti-Thy1 hybridoma (ATCC), the 3.155 anti-CD8 hybridoma (from D. Hanson, Washington University, St. Louis, MO). The small resting B cells were recovered from the interface of 60 and 75% isotonic Percoll gradients.

**Cell Stimulation and Preparation of Cell Lysates**—Cells were resuspended to 2.5 × 10^6/ml in 37 °C modified Hepes-buffered saline (27) and stimulated with anti-Ig Abs or other stimuli. Reactions were terminated by adding an equal volume of cold Nonidet P-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin). After 30 min on ice, detergent-insoluble material was removed by centrifugation. Cell lysates were used immediately for Rap1 activation assays. Human platelets were isolated from freshly drawn venous blood as described (28) and stimulated with 0.25 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The lysate was centrifuged at 30,000 rpm for 45 min in the cold. The supernatant was stored at −80 °C in small aliquots.

**Rap1 Activation Assay**—Bacterial lysate (20 μl per sample) containing the GST-RalGDS(RBD) fusion protein was mixed with glutathione-Sepharose 4B beads (Pharmacia, Baie d’Urfé, Quebec) for 1–2 h in the cold. After washing the beads twice with Nonidet P-40 lysis buffer, cell lysates (1.25 × 10^7 cell equivalents in 0.5 ml) were added and mixed with the beads for 1 h. The beads were then washed three times with Nonidet P-40 lysis buffer, and bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer containing 100 mM dithiothreitol. Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The filters were blocked with TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), containing 5% dry milk powder for 1 h at room temperature, and then incubated overnight in the cold with TBS containing 1 mg/ml bovine serum albumin and 1 μg/ml anti-Rap1 Ab. After washing with TBS/0.05% Tween 20, the filters were incubated with protein A-horseradish peroxidase (1:3000 in TBS; Bio-Rad) for 1 h at room temperature. The filters were washed extensively with TBS/0.1% Tween 20, and immunoreactive bands were visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

**RESULTS**

Rap1 opposes the actions of Ras and may therefore be a key regulator of cell growth. To determine whether the BCR activates Rap1, we used a novel assay for Rap1 activation developed by Franke et al. (29). This assay is based on the observation that the RapGDS protein has high affinity for active Rap1-GTP but does not bind the inactive GDP-bound form of Rap1 (30). Thus, we used a GST fusion protein containing the RBD of RapGDS to selectively precipitate activated Rap1. The recovery of activated Rap1 was monitored by immunoblotting with an anti-Rap1 Ab, which recognizes both Rap1A and Rap1B.

Using this assay, we found that initiating BCR signaling with anti-IgM Abs increased the amount of activated Rap1 in WEHI-231 B lymphoma cells (Fig. 1A). An increase in the amount of activated Rap1 was seen at 15 s after adding anti-IgM to the cells. This BCR-induced Rap1 activation was maximal at 2–15 min and persisted for at least 60 min. Several controls were done to confirm that the 23-kDa band detected by anti-Rap1 immunoblotting was activated Rap1 and not an artifact (Fig. 1B). First, the band detected in WEHI-231 cell lysates had the same electrophoretic mobility as activated Rap1 precipitated from thrombin-stimulated platelets. Rap1 is highly expressed in platelets, and thrombin causes strong activation of Rap1 in these cells (29). Second, the protein detected by the anti-Rap1 Ab was not precipitated by an irrelevant GST fusion protein containing the Crk SH2 domain. Finally, adding anti-IgM to WEHI-231 cells after they had been solubilized with Nonidet P-40 did not increase the intensity of the band detected in this assay. This shows that intact cells were necessary for this response and that the band detected by anti-Rap1 immunoblotting was not the 25-kDa Ig light chain of either the cells’ BCR or the stimulating Ab. Together, these controls support the conclusion that BCR engagement activates Rap1.

BCR signaling regulates multiple steps in B cell development and differentiation, influencing the survival and activation of pre-B cells, immature B cells, mature B cells, and memory B cells (1). Therefore, we asked whether BCR ligation activates Rap1 in cell lines corresponding to different stages of B cell development (Fig. 1C). BCR ligation increased the amount of activated Rap1 in the immature IgM⁺ WEHI-231 B cell line, the mature IgM⁺ IgD⁺ B cell line, and the IgM⁺ IgD⁺ B cell line and the IgG⁺ A20 murine cell line. A20 is thought to resemble a memory B cell. BCR-induced Rap1 activation was also observed in mature resting B cells isolated from mouse spleen (Fig. 2A), indicating that this response is not restricted to transformed B cell lines. Finally, we showed that BCR ligation-activated Rap1 not only in mouse cells but also in the DT40...
FIG. 1. Activation of Rap1 following BCR ligation. A, WEHI-231 cells were incubated with 100 μg/ml anti-IgM Abs for the indicated lengths of time. A Rap1 activation assay was performed on the cell lysate. Cell lysates were precipitated with GST-RalGDS(RBD), and precipitated proteins were analyzed by anti-Rap1 immunoblotting. B, WEHI-231 cells were incubated with (+) or without (−) 100 μg/ml anti-IgM for 2 min. Asterisk indicates that anti-IgM Abs were added to WEHI-231 cells following solubilization with Nonidet P-40 lysis buffer. Platelets were incubated with (+) or without (−) thrombin (0.25 units/ml) for 1 min. Cell lysates were precipitated with the indicated fusion protein. Precipitated proteins were analyzed by immunoblotting with the anti-Rap1 Ab. C, WEHI-231, BAL17, and A20 cells were incubated for 2 min with (+) or without (−) 100 μg/ml anti-IgM. A Rap1 activation assay was performed on the cell lysates. Molecular mass standards (in kDa) are indicated to the left of each panel. The experiments shown in each panel were performed three times, and similar results were obtained each time.

chicken B cell line (Fig. 2C) and the RAMOS human B cell line (Fig. 2, D and E). Thus, BCR-induced activation of Rap1 is a consistent feature of B cells.

Having shown that Rap1 is activated by the BCR, we were interested in determining the mechanism of BCR-induced Rap1 activation. Our previous work had suggested that Crk-C3G complexes might be involved in this process. In B cells, we showed that the Crk adaptor proteins constitutively bind C3G (22), a nucleotide exchange factor that can activate Rap1 (20). BCR ligation causes Crk to bind via its SH2 domain to two membrane-associated docking proteins, p130Ca and p120Cbl, both of which are tyrosine phosphorylated in response to BCR engagement (22). Thus, BCR ligation may recruit Crk-C3G complexes to cellular membranes where Rap1 is located, allowing C3G to activate Rap1. Recently, Franke et al. (29) showed that there is another mechanism for receptor-induced activation of Rap1. They found that increases in cytoplasmic Ca2+ concentrations could activate Rap1 in platelets and that this was required for activation of Rap1 by thrombin in these cells. Since the BCR activates PLC-γ, which results in the production of IP3 and subsequent increases in intracellular Ca2+ concentrations, the BCR could activate Rap1 either via a Ca2+-dependent pathway or via the binding of Crk-C3G complexes to tyrosine-phosphorylated docking proteins.

To distinguish between these two possibilities, we first asked whether the Ca2+-dependent pathway for Rap1 activation is present in B cells. We used the Ca2+-selective ionophore ionomycin to increase intracellular Ca2+ levels in murine splenic B cells and in B cell lines. We found that ionomycin concentrations (250–500 nM) that cause similar increases in intracellular Ca2+ levels as do BCR stimulation (31) did not activate Rap1 to a significant extent (Fig. 2, A–D). Summed over eight experiments, the amount of activated Rap1 in unstimulated cells was 19.9 ± 13.9% of that in anti-IgM-stimulated cells, whereas the amount of activated Rap1 in cells stimulated with 500 nM ionomycin was 14.4 ± 8.1% of that in anti-IgM-stimulated cells. Thus, in most experiments the amount of activated Rap1 in ionomycin-treated cells was not significantly different than that in untreated cells. Higher concentrations of ionomycin (1 μM), which cause larger increases in intracellular Ca2+ concentrations, also failed to activate Rap1 in B cells.2

Although the Ca2+-dependent pathway for Rap1 activation seems not to be significant in B cells, we found that the other PLC-γ-derived second messenger, diacylglycerol (DAG), could activate Rap1 in B cells. PdBu, a phorbol ester that mimics the action of DAG, caused strong activation of Rap1 in murine splenic B cells, the WEHI-231 murine B cell line, the DT40 chicken B cell line, and the RAMOS human B cell line (Fig. 2, A–F). The kinetics of PdBu-induced Rap1 activation (Fig. 2G) were similar to that for anti-IgM-induced Rap1 activation (Fig. 1A). A synthetic DAG, diC8, also caused strong activation of Rap1 in WEHI-231 cells,2 RAMOS cells (Fig. 2E), and DT40 cells (Fig. 2F). Thus, a DAG-dependent pathway for Rap1 activation is present in B cells. This, together with the rapid kinetics of PdBu-induced Rap1 activation, suggested that the BCR may activate Rap1 via this DAG-dependent pathway.

To test whether the BCR activated Rap1 via this DAG-dependent pathway, we made use of several variants of the DT40 chicken B cell line. To determine whether activation of Rap1 by the BCR is dependent on the generation of PLC-γ-derived second messengers, we asked whether the BCR could activate Rap1 in a variant of the DT40 chicken B cell line in which the genes encoding PLC-γ have been disrupted (25). Since these cells do not express PLC-γ, they are unable to produce IP3, or DAG in response to BCR ligation (25). We found that BCR-induced activation of Rap1 was dramatically reduced in the PLC-γ-deficient DT40 cells as compared with wild-type DT40 cells.

2 S. J. McLeod and M. R. Gold, unpublished observations.
In contrast, PdBu caused strong activation of Rap1 in both the wild-type and PLC-γ-deficient DT40 cells, indicating that Rap1 could still be activated in the PLC-γ-deficient DT40 cells. Thus, PLC-γ expression, and presumably activation, is required for the BCR to activate Rap1.

Since activation of PLC-γ is necessary for the majority of BCR-induced Rap1 activation (Fig. 3A) and both PdBu and diC₈ can activate Rap1 (Fig. 2), it strongly suggests that the BCR activates Rap1 via a DAG-dependent pathway. To confirm that activation of Rap1 by the BCR does not require increases in intracellular Ca²⁺ concentrations in addition to DAG production, we asked whether the BCR could activate Rap1 in a variant of the DT40 cell line in which the genes encoding all three IP₃ receptors have been disrupted (26). The IP₃ receptors are responsible for the release of intracellular Ca²⁺ stores in response to IP₃, and in these IP₃ receptor-deficient DT40 cells, there is no increase in intracellular Ca²⁺ following BCR engagement (26). We found that BCR ligation caused strong activation of Rap1 in the IP₃ receptor-deficient DT40 cells (Fig. 3B) and that the extent of Rap1 activation in these cells was similar to that caused by engaging the BCR on wild-type DT40 cells. This suggests that DAG production is sufficient to induce maximal activation of Rap1. Consistent with this idea, we found that increasing intracellular Ca²⁺ concentrations with ionomycin did not potentiate the ability of PdBu to activate Rap1 (Fig. 2B). In sum, these data argue that increases in intracellular Ca²⁺ are neither necessary nor sufficient for activation of Rap1 by the BCR.

The ability of PdBu and diC₈ to activate Rap1 indicates that a novel DAG-dependent pathway for Rap1 activation exists in B cells. Our previous work had suggested that the BCR might activate Rap1 by recruiting CrkC3G complexes to cellular membranes where Rap1 is located. In this model, translocation of CrkC3G complexes to cellular membranes was mediated by the binding of the Crk SH2 domain to membrane-associated docking proteins (e.g., p120Cbl, p130Cas) that are tyrosine phosphorylated in response to BCR ligation. To determine whether the formation of these Crk complexes is involved in the DAG-dependent activation of Rap1, we investigated whether PdBu induced CrkL, the most abundant form of Crk in B cells, to associate with Cbl or Cas. Fig. 4A shows that BCR ligation caused CrkL to associate with 120–130-kDa tyrosine-phosphorylated proteins in RAMOS cells but that PdBu treatment did not. Immunoblotting with specific Abs confirmed that PdBu treatment did not cause CrkL to associate with Cas (Fig. 4B) or Cbl (Fig. 4C). Thus, the binding of Crk-C3G complexes to tyrosine-phosphorylated docking proteins is not necessary for PdBu-induced activation of Rap1. Moreover, the formation of these Cbl/Cas/Crk-C3G complexes is not sufficient for BCR-induced Rap1 activation. There was very little BCR-induced Rap1 activation in PLC-γ-deficient DT40 cells (Fig. 3A), even though BCR ligation caused CrkL to associate with 120–130-
kDa tyrosine-phosphorylated proteins in these cells to the same degree as in wild-type DT40 cells (Fig. 4D). Thus, the BCR seems to activate Rap1 primarily via a DAG-dependent pathway that does not require the interaction of Crk proteins with membrane-associated docking proteins.

**DISCUSSION**

By acting as an antagonist of Ras-mediated signaling, the Rap1 GTase may play a key role in regulating cell growth and survival. Using an assay that allows us to selectively precipitate the activated GTP-bound form of Rap1, we have shown that engaging the BCR activates Rap1 in normal B cells and in a variety of B cell lines. Moreover, our data suggest that BCR-induced Rap1 activation proceeds via a novel DAG-dependent pathway.

At present, the only other example of receptor-induced activation of Rap1 is in platelets, where various platelet agonists including thrombin can activate Rap1 (29). In this system, increases in intracellular free Ca\(^{2+}\) levels are both necessary and sufficient for activation of Rap1. Although BCR-induced Rap1 activation required PLC-\(\gamma\), experiments using IP\(_3\) receptor-deficient DT40 cells showed that increases in intracellular free Ca\(^{2+}\) were not required for the BCR to activate Rap1. Moreover, Ca\(^{2+}\) ionophores could not stimulate Rap1 activation to the same extent as anti-IgM Abs, indicating that increases in intracellular free Ca\(^{2+}\) concentrations cannot account for BCR-induced Rap1 activation. This suggested that the other PLC-\(\gamma\)-derived second messenger, DAG, might be involved in activation of Rap1 by the BCR. Indeed, the synthetic DAG diC\(_{2}\), as well as PdBu, which mimics the action of DAG, caused strong activation of Rap1 in murine, human, and chicken B cells. These results are consistent with the idea that the BCR activates Rap1 via a DAG-dependent pathway.

The components of this novel signaling pathway that link DAG to Rap1 activation in B cells are not known. We are currently addressing whether activation of protein kinase C (PKC) enzymes by DAG is involved in this process. In preliminary experiments, we found that BCR-induced Rap1 activation was significantly inhibited by one PKC inhibitor, chelerythrine chloride, whereas another PKC inhibitor Ro31–8220 had only marginal effects. Further work is needed to determine whether these results reflect nonspecific effects of chelerythrine chloride or the involvement of a PKC isoform that is inhibited to a greater degree by chelerythrine chloride than by Ro31–8220. Down-regulating PKC by treating B cells overnight with PdBu resulted in a higher basal level of activated Rap1 in the cells but abolished any further increase in Rap1 activation due to BCR ligation. Although this supports the idea that PKC enzymes are involved in BCR-induced activation of Rap1, additional studies are necessary.

We also do not know whether the ultimate target of this DAG-dependent Rap1 activation pathway is a Rap1 guanine nucleotide exchange factor or a Rap1 GTase-activating protein (GAP). An increase in the amount of activated Rap1 could be due to an increase in the rate at which exchange factors activate Rap1 or to a decrease in the rate at which Rap1 GAPs stimulate hydrolysis of the GTP bound to Rap1. There are likely to be multiple Rap1 exchange factors and Rap1 GAPs. In addition to C3G, Bos and colleagues have recently identified

**FIG. 3.** Activation of Rap1 by the BCR requires PLC-\(\gamma\) but does not involve IP\(_3\) receptor-mediated increases in intracellular Ca\(^{2+}\). Rap1 activation assays were performed on cell lysates. A, wild-type (wt) and PLC-\(\gamma\)-deficient DT40 B cells were left untreated (−) or incubated for 5 min with 50 \(\mu\)g/ml anti-IgM or 100 nM PdBu. B, Wild-type (wt) and IP\(_3\) receptor-deficient (IP\(_3\)R−/−) DT40 B cells were left untreated (−) or incubated for 5 min with 50 \(\mu\)g/ml anti-IgM or 100 nM PdBu. Molecular mass standards (in kDa) are indicated to the left of each panel. The experiments shown in each panel were performed three times, and similar results were obtained each time.

**FIG. 4.** Association of Crk complexes with Cas and Cbl is not necessary for activation of Rap1 by the BCR. A–C, RAMOS cells were left untreated (−) or incubated for 2 min with 100 \(\mu\)g/ml anti-IgM or 100 nM PdBu. Cell lysates were precipitated with an anti-CrkL Ab, and precipitated proteins were analyzed by immunoblotting with an anti-phosphotyrosine (anti-P-Tyr) monoclonal Ab (A), anti-Cas Abs (B), or anti-Cbl Abs (C). D, Wild-type (wt) and PLC-\(\gamma\)-deficient (PLC-\(\gamma\)−/−) DT40 cells were left untreated (−) or incubated for 2 min with 50 \(\mu\)g/ml anti-IgM. Cell lysates were precipitated with an anti-CrkL Ab, and precipitated proteins were analyzed by anti-P-Tyr immunoblotting. Molecular mass standards (in kDa) are indicated to the left of each panel. The experiments shown in each panel were performed twice, and similar results were obtained each time.
several novel Rap1 exchange proteins. Similarly, three Rap1-GAPs have been identified, SPA-1, RapGAP1, and tuberin (32–34). SPA-1 is expressed primarily in lymphoid cells (32), and it will be important to determine whether DAG regulates its activity or subcellular localization.

Our initial model for Rap1 activation by the BCR involved the binding of Crk-C3G complexes to tyrosine-phosphorylated Cbl and Cas (22). Since Cbl and Cas can associate with cellular membranes, this interaction could recruit cytosolic Crk-C3G complexes to membranes where Rap1 is located. However, we found that the association of Crk complexes with Cbl, Cas, or other tyrosine-phosphorylated proteins was neither necessary nor sufficient for the DAG-dependent activation of Rap1. PtdIns 4,5-P2 caused strong activation of Rap1 but did not cause Crk to associate with Cbl, Cas, or other tyrosine-phosphorylated proteins. Moreover, BCR-induced Rap1 activation was dramatically reduced in PLC-γ-deficient DT40 cells even though Crk associated with tyrosine-phosphorylated proteins in a normal manner. The small amount of residual Rap1 activation seen in the PLC-γ-deficient DT40 cells following BCR engagement may reflect the contribution of the Cbl/Cas/Crk/C3G pathway, whereas the majority of BCR-induced Rap1 activation may proceed via an independent DAG-regulated pathway. Our findings do not, however, rule out a role for Crk and C3G in activation of Rap1 by the BCR. It is possible that the DAG-dependent pathway regulates C3G or Crk/C3G complexes in some way that does not involve the binding of Crk to tyrosine-phosphorylated docking proteins such as Cbl or Cas. Alternatively, the target of the DAG-dependent pathway for Rap1 activation may be one of several other recently identified Rap1 exchange factors or one of the several known Rap1-GAPs.

The functions of the Rap1 proteins in B cells remain to be elucidated. As an antagonist of Ras, Rap1 may play a key role in regulating B cell activation. Depending on whether or not a B cell receives costimulatory signals from T cells, BCR ligation can result in activation and proliferation, functional inactivation (anergy), or apoptosis. Since activated Ras can transform B cells (35), activation of Ras by the BCR most likely promotes B cell proliferation. The concomitant activation of Rap1 may oppose this by limiting Ras-mediated signaling and perhaps by activating other signaling pathways that induce the expression of cell cycle inhibitors. Thus, the balance between the activation of Ras and Rap1 may be a key parameter that determines the biological outcome of BCR ligation. T cell-derived costimulatory signals in the form of CD40 ligand and interleukin-4 may shift the balance in favor of Ras and proliferation, whereas inhibitory signals delivered by Fc receptors may shift the balance in favor of Rap1, leading to cell cycle arrest and either anergy or apoptosis. We are currently investigating whether signaling by CD40, the interleukin-4 receptor, or Fc receptors modulates activation of Rap1 by the BCR.

Rap1A and Rap1B may also have other functions in B cells. Maly et al. have shown that the ability of the BCR to stimulate the oxidative burst is dependent on Rap1A (36). In neutrophils, Rap1A associates with cytochrome b556 and this may be important for assembly of the NADPH oxidase complex, which produces reactive oxygen species (37). The production of these reactive oxygen species may allow B cells to kill bacteria that they bind via their BCR.

In sum, we have provided the first direct evidence that the BCR activates Rap1, and our data suggest that this occurs primarily via a novel DAG-dependent pathway. We are currently investigating whether Rap1 activation is a general property of receptors that activate Ras and whether this DAG-dependent pathway for Rap1 activation is present in other cell types.

Acknowledgments—We thank Christian Stevens for assistance in obtaining and stimulating platelets and Linda Matsuuchi for critical reading of the manuscript.

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