Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAP kinase activation*


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Running title: Inhibition of the PI3K/Akt pathway by the Rap GTPases.

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SUMMARY

Signaling by the B cell antigen receptor (BCR) activates the Rap1 and Rap2 GTPases, putative antagonists of Ras-mediated signaling. Since Ras can activate the Raf-1/ERK pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, we asked whether Rap activation limits the ability of the BCR to signal via these pathways. To do this, we blocked the activation of endogenous Rap1 and Rap2 by expressing the Rap-specific GTPase-activating protein RapGAPII. Preventing Rap activation had no effect on BCR-induced activation of ERK. In contrast, BCR-induced phosphorylation of Akt on critical activating sites was increased two- to three-fold when Rap activation was blocked. Preventing Rap activation also increased the ability of the BCR to stimulate Akt-dependent phosphorylation of the FKHR transcription factor on negative regulatory sites and decreased the levels of p27^Kip1, a pro-apoptotic factor whose transcription is enhanced by FKHR. Moreover, preventing Rap activation reduced BCR-induced cell death in the WEHI-231 B cell line. Thus activation of endogenous Rap by the BCR limits BCR-induced activation of the PI3K/Akt pathway, opposes the subsequent inhibition of the FKHR/p27^Kip1 pro-apoptotic module, and enhances BCR-induced cell death. Consistent with the idea that Rap-GTP is a negative regulator of the PI3K/Akt pathway, expressing constitutively active Rap2 (Rap2V12) reduced BCR-induced phosphorylation of Akt and FKHR. Finally, our finding that Rap2V12 can bind PI3K and inhibit its activity in a manner that depends upon BCR engagement provides a potential mechanism by which Rap-GTP limits activation of the PI3K/Akt pathway, a central regulator of B cell growth and survival.
INTRODUCTION

Signaling by the B cell antigen receptor (BCR)\(^1\) is required for B cell development and survival, for the elimination or silencing of self-reactive B cells, and for the activation of B cells that recognize foreign antigens (1). The BCR activates multiple signaling pathways including the phospholipase C pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the kinase cascades that lead to activation of the mitogen-activated protein kinases (MAPKs) (1-3). Downstream targets of the phospholipase C pathway include protein kinase C (PKC) enzymes as well as the NF-AT and NF-kB transcription factors (4). PI3K produces lipid second messengers that regulate a network of protein kinases including 3-phosphoinositide-dependent kinase-1 (PDK1), PKC-\(\xi\), PKC-\(\epsilon\), p70 S6 kinase, the Btk tyrosine kinase (1,5), and Akt/protein kinase B, a kinase that plays a key role in B cell survival (6). The three major classes of MAPKs, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, phosphorylate a variety of transcription factors, increasing their ability to promote transcription. The activation of distinct combinations of these signaling pathways may account for the ability of the BCR to promote survival, apoptosis, proliferation, or differentiation depending on the maturation state of the B cell and the nature of the antigen (2).

A key feature of signaling by the BCR and many other receptors is the use of monomeric GTPases as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. Activated GTPases bind downstream effector proteins and thereby regulate their subcellular localization and activity. Previous work has shown that BCR engagement results in the activation of the Ras and Rac1 GTPases (7). Ras controls the kinase cascade leading to the activation of the ERK1 and ERK2 MAPKs while Rac1 regulates the kinase cascades leading to the activation of the JNK and p38 MAPKs. We have shown that the BCR
also activates the Rap1 GTPase (8) and in this report we show that the BCR activates the Rap2 GTPase. Recent work has shown that the Rap GTPases play a key role in receptor-induced integrin activation and, hence cell adhesion, in many cell types (9) including B cells2. However, the Rap GTPases were first described as potential antagonists of Ras-mediated signaling.

The idea that Rap is an antagonist of Ras-mediated signaling originated with experiments showing that overexpressing Rap1 caused Ras-transformed 3T3 cells to assume a more normal fibroblast-like morphology (10). Similarly, activated Rap1 can oppose Ras-dependent Xenopus oocyte differentiation (11). Since Ras and Rap1 have identical core effector binding domains (residues 32-40) and activated Rap1 can bind \textit{in vitro} to Ras effectors including Raf-1 (12,13), it was proposed that activated Rap1 limits Ras-mediated signaling by sequestering Ras effectors in inactive complexes. Although Rap2 has a phenylalanine at position 39 instead of the serine present in Rap1, this substitution does not appear to have any functional consequences (14,15), suggesting that activated Rap2 can also bind Ras effectors and act as a Ras antagonist. Indeed, several groups have shown that both activated Rap1 and activated Rap2 can inhibit Ras-dependent activation of ERK (15-18). Other studies however did not find that Rap activation impaired Ras-dependent ERK activation (19-21), suggesting that this could a cell type-specific effect.

Signaling pathways regulated by Ras play an essential role in BCR signaling. Loss-of-function studies have shown that activation of the Ras/Raf-1/MEK/ERK signaling pathway is essential for B cell development (22) and for BCR-induced proliferation of mature B cells (23). In addition to activating the Raf-1/MEK/ERK pathway, Ras can also promote signaling via PI3K. Activated Ras can bind directly to the p110 catalytic subunit of PI3K (24,25), thereby recruiting PI3K to the plasma membrane where it can produce the lipid second messengers
phosphatidylinositol 3,4,5-trisphosphate (PIP₃) and phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂) which activate downstream targets such as Akt. Akt plays a critical role in regulating survival versus apoptosis in B cells (6). Consistent with the idea that Ras regulates the PI3K/Akt pathway in B cells, recent work has shown that expressing constitutively active Ras leads to Akt activation in the A20 B cell line (26). The BCR can also initiate PI3K-dependent signaling by inducing the SH2 domain-dependent binding of PI3K to membrane-associated docking proteins such as CD19, Gab1, BCAP, and Cbl that are tyrosine phosphorylated after BCR engagement (27-30). Thus there may be two distinct mechanisms by which the BCR initiates PI3K signaling. Nevertheless, if activated Rap were to act as an antagonist of Ras-mediated signaling in B cells, it could limit the activation of two critical BCR signaling pathways, the Ras/Raf-1/MEK/ERK pathway and the Ras/PI3K/Akt pathway.

In contrast to the ability of activated Rap to inhibit Ras-dependent ERK activation in some cell types, Rap-GTP can in fact act as a positive regulator of both the ERK and p38 MAPKs in some situations. In neuronal cells such as the PC12 cell line, Stork and colleagues have shown that Rap1-GTP activates B-Raf, leading to sustained activation of ERK (31,32). Brummer et al. have recently shown that B-Raf plays a significant role in the activation of ERK by the BCR (33), suggesting that a Rap/B-Raf pathway could contribute to BCR-induced ERK activation. Thus, depending on the cell type, the Rap GTPases may act as either positive or negative regulators of ERK activation. There is also some evidence that Rap activation is important for activation of the p38 MAPK (34).

Since the Rap GTPases can act as negative regulators of Ras-dependent signaling and, depending on the cell type, as positive regulators of MAPK signaling pathways, we investigated whether Rap activation in B cells modulates the activation of the PI3K/Akt pathway or the
MAPK pathways, critical mediators of BCR signaling. We show that Rap is neither a positive nor negative regulator of BCR-induced ERK activation and that Rap activation has no effect on the ability of the BCR to activate the JNK and p38 MAPKs. In contrast, we found that activation of the endogenous Rap GTPases limits the activation of Akt by the BCR. Preventing the activation of endogenous Rap by the BCR enhanced BCR-induced Akt phosphorylation while expressing Rap2V12, a constitutively active form of Rap2, inhibited BCR-induced Akt phosphorylation. Consistent with the idea that Rap-GTP opposes Akt activation, we found that Rap activation inhibits Akt-dependent signaling events, in particular phosphorylation of the FKHR transcription factor. We also found that the ability of Rap-GTP to limit BCR-induced signaling via Akt correlated with effects on B cell survival. Finally, we show that Rap2V12-GTP can bind to PI3K and inhibit its enzymatic activity. Thus, the Rap GTPases act as negative regulators of the PI3K/Akt pro-survival signaling pathway in B cells and may do so via a mechanism that involves the binding of Rap-GTP to PI3K, the upstream activator of Akt.
EXPERIMENTAL PROCEDURES

Antibodies. Goat antibodies against mouse immunoglobulin (Ig) G (\(\gamma\) chain-specific) or mouse IgM (\(\mu\) chain-specific) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit antibodies specific for Akt, Akt phosphorylated on serine 473 (anti-P-Ser473 Akt), Akt phosphorylated on threonine 308 (anti-P-Thr308 Akt), and p38 were purchased from Cell Signaling Technologies (Beverly, MA) as were the antibodies specific for the phosphorylated forms of ERK, JNK, p38 and the Forkhead-related transcription factor FKHR/FOXO1. The antibodies specific for ERK, JNK, Rap1 and p85\(\alpha\) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibodies to Rap2, PDK1, and p27\(^{kip1}\) were obtained from BD Transduction Laboratories (Mississauga, Ontario, Canada). The M2 anti-FLAG monoclonal antibody and the monoclonal antibody to actin were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

B cell lines. The A20 and WEHI-231 murine B cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 50 \(\mu\)M 2-mercaptoethanol, 1 mM pyruvate, 2 mM glutamine, 15 U/ml penicillin and 50 \(\mu\)g/ml streptomycin (complete medium). A20 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector (BD Biosciences Clontech, Palo Alto, CA) were generated by electroporation (400 V, 975 \(\mu\)F) followed by selection in medium containing 4 \(\mu\)g/ml puromycin (Calbiochem, La Jolla, CA). The cDNAs encoding FLAG-tagged RapGAPII (35) or Rap2V12 in pMSCVpuro were gifts from Dr. M. Matsuda (Osaka University, Osaka, Japan). A20 clones expressing RapGAPII were obtained by single-cell cloning. Expression of FLAG-RapGAPII or FLAG-Rap2V12 was detected by immunoblotting with the M2 anti-FLAG
monoclonal antibody. All experiments were performed using clone 16 and results confirmed using clone 3. The Rap2V12-expressing A20 cells were an oligoclonal population that was not subjected to single-cell cloning. Bulk populations of WEHI-231 cells expressing RapGAPII, Rap2V12 or the empty pMSCVpuro vector were generated by retrovirus-mediated gene transfer (36) followed by selection in medium containing 0.25 µg/ml puromycin. A20 cells expressing a myristoylated Akt-estrogen receptor fusion protein (mER-Akt) or a non-myristoylated version with a glycine to alanine substitution at position 2 (A2-ER-Akt) were generated by electroporation and single cell cloning in the presence of puromycin. The cDNAs encoding the mER-Akt and A2-ER-Akt proteins (37) (gifts from Dr. R. Roth, Stanford University, Stanford, CA) were subcloned into the pMXpuro-IRES-EGFP vector, a derivative of pMXpuro (DNAX, Palo Alto, CA) (38).

B cell stimulation and preparation of cell lysates. Cells were washed once with modified HEPES-buffered saline (39), resuspended in this buffer at 1 x 10^7 or 2 x 10^7 per ml, and warmed to 37°C for 10 to 30 min. The cells were then stimulated with anti-Ig antibodies for the indicated times. Activation of the mER-Akt fusion protein was achieved by treating the cells with 2 µM 4-hydroxytamoxifen (4-HT; Sigma-Aldrich). For stimulation reactions lasting longer than 1 h, the cells were resuspended at 5 x 10^5 per ml in complete medium and treated with anti-Ig antibodies at 10 µg/ml for the indicated times. The reactions were terminated by adding ice-cold phosphate-buffered saline containing 1 mM Na3VO4 and then centrifuging the cells for 1 min at 1100 x g in a microfuge at 4°C. For analyzing the phosphorylation of Akt, ERK, JNK, and p38, the cell pellets were solubilized in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 8.0], 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride,
1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na$_3$VO$_4$, 25 mM β-glycerophosphate, 1 µg/ml microcystin-LR). For Rap activation assays, immunoprecipitation assays, PI3K enzyme assays, and detection of p27$^{kip1}$ protein levels, the cell pellets were solubilized in Rap lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Igepal [ICN, Costa Mesa, CA], 200 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na$_3$VO$_4$, 25 mM β-glycerophosphate). For ERK in vitro kinase assays, the cell pellets were solubilized in ERK assay buffer (50 mM Tris-HCl [pH 7.5], 1% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM MoO$_4$, 0.2 mM Na$_3$VO$_4$, 1 mM dithiothreitol, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 0.7 µg/ml pepstatin, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor). In all cases, the samples were left on ice for 10 min and then insoluble material was removed by centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

**Immunoblotting.** Cell lysates (20 µg protein) or immunoprecipitated proteins were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked for 1-2 h with 5% (w/v) skim milk powder in TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween-20) and then incubated overnight at 4°C or 1 h at room temperature with the primary antibody. All antibodies were diluted in TBST/1% bovine serum albumin. The membranes were then washed with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). To re-probe membranes, bound antibodies were eluted by incubating the membrane in 10 mM Tris-HCl [pH 2], 150 mM NaCl for 30 min.
The membranes were then re-blocked and probed with antibodies as described above. To quantitate results, scans of ECL exposures were saved as TIFF files and analyzed using ImageQuant 1.2 software (Molecular Dynamics, Sunnyvale, CA).

*Rap activation assays.* Cells were stimulated as above, solubilized in Rap lysis buffer, and then assayed for Rap activation as described previously (8). Briefly, a glutathione S-transferase (GST) fusion protein containing the Rap1/2-binding domain of the RalGDS protein (GST-RalGDS (RBD)) was used to selectively precipitate the active GTP-bound forms of Rap1 and Rap2, which were then detected by immunoblotting with antibodies specific for either Rap1 or Rap2. In some experiments the filters were probed with anti-Rap2 antibodies, stripped, and then re-probed with anti-Rap1 antibodies.

*Immunoprecipitation.* Cell lysates were pre-cleared with Sepharose-CL-4B beads (Sigma-Aldrich) for 30 min at 4°C. For anti-FLAG immunoprecipitation, cell lysates were transferred to tubes containing 10 µl of agarose beads covalently coupled with the M2 anti-FLAG-monoconal antibody (Sigma-Aldrich) plus 10 µl of Sepharose-CL-4B beads as filler. After mixing for 1 h at 4°C, the beads were pelleted, washed three times with Rap lysis buffer, and bound proteins were eluted using SDS-PAGE sample buffer.

*ERK and PI3K in vitro kinase assays.* ERK2 was immunoprecipitated using monoclonal anti-ERK2 antibodies conjugated to agarose beads (Santa Cruz Biotechnology). ERK in vitro kinase assays were performed as described previously (40) using either 30 µg myelin basic protein (Sigma-Aldrich) or 10 µg GST-ELK-1 (Cell Signaling Technologies) as the substrate. PI3K
enzyme assays were performed on anti-FLAG immunoprecipitates as described by Gold et al. (41). The immunoprecipitates were incubated with phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and $^{32}$P-$\gamma$-ATP for 20 min at room temperature. The resulting $^{32}$P-labeled phosphatidylinositol 3-phosphate was separated from the $^{32}$P-$\gamma$-ATP by thin layer chromatography and quantified using a phosphorimager (Molecular Dynamics).

Anti-IgM-induced cell death. WEHI-231 cells were plated at $2 \times 10^5$ per ml in complete medium and cultured for 48 to 72 h at 37°C. The cells were then pelleted and resuspended in FACS buffer (phosphate buffered saline supplemented with 2% fetal calf serum) containing 4 µg/ml 7-amino-actinomycin D (7-AAD; Calbiochem), a membrane-impermeable DNA-binding compound that is excluded from live cells with intact membranes. After staining the cells in the dark for 10 min, the cells were pelleted and resuspended in FACS buffer. Flow cytometry was used to determine the percent of cells that had taken up significant amounts of 7-AAD.
RESULTS

Expressing RapGAPII inhibits BCR-induced activation of Rap1 and Rap2

We have previously shown that BCR engagement results in an increase in the amount of active GTP-bound Rap1 (8). Since Rap2 has a nearly identical effector region to that of Rap1 (14), can bind Ras effectors, and can inhibit Ras-dependent ERK activation (15), we investigated whether the BCR also activates Rap2. We found that inducing BCR signaling with anti-Ig antibodies resulted in the activation of both Rap1 and Rap2 in the WEHI-231 and A20 murine B cell lines (Fig. 1A). WEHI-231 is an IgM+ B cell line that resembles an immature/transitional B cell that can undergo antigen-induced clonal deletion while A20 is an IgG+ B cell line that resembles a mature B cell that has undergone Ig class switching. The activation of Rap1 and Rap2 occurred with similar kinetics, consistent with the idea that they are regulated by the same exchange factors and GTPase-activating proteins (GAPs).

To investigate whether activated Rap GTPases modulate BCR signaling pathways we employed a loss-of-function approach in which we blocked the activation of endogenous Rap1 and Rap2. To do this, we expressed in both WEHI-231 and A20 cells a Rap-specific GAP called RapGAPII (Fig. 1B) that converts Rap1 and Rap2 to their inactive GDP-bound forms. RapGAPII is normally expressed in brain (35) but not in B cells (42) and has been shown to specifically inhibit the activation of Rap1 and Rap2 (15,35) while having no effect on the activation of other closely related GTPases such as Ha-Ras, R-Ras (35), RhoA (43), and Rac1 (42). We found that expressing RapGAPII in B cell lines completely blocked BCR-induced activation of Rap1 and inhibited BCR-induced activation of Rap2 by at least 80% (Fig. 1C).

{Insert Figure 1}
Inhibiting Rap activation has no effect on BCR-induced activation of the ERK, JNK, or p38 MAPKs

The BCR activates ERK (40,44) in a Ras-dependent manner (7,45) and this is important for both B cell development (22) and BCR-induced proliferation of mature B cells (23). Depending on the cell type, activated Rap may be either a positive or a negative regulator of ERK activation. In fibroblasts, expression of the constitutively active forms of Rap1 or Rap2 can inhibit ERK activation, perhaps by competing with Ras-GTP for Raf-1, the first kinase in the Raf-1/MEK/ERK cascade (15,16). In contrast, Rap-GTP can activate ERK in the PC-12 neuronal cell line by activating B-Raf (31,32). Thus we were interested in determining whether the Rap GTPases act as either positive or negative regulators of BCR-induced ERK activation.

To assess whether Rap activation modulated the ability of the BCR to activate ERK, we compared BCR-induced ERK activation in vector control cells versus RapGAPII-expressing cells where activation of Rap1 and Rap2 was inhibited (see Fig. 1C). We first assessed ERK activation by using phospho-specific antibodies to detect the phosphorylation of ERK1 and ERK2 on sites that are required for their activation. The BCR causes strong activation of ERK2 and weaker activation of ERK1 (Fig. 2A,B), consistent with our previous findings (40). When we blocked the activation of Rap1 and Rap2 by expressing RapGAPII, we found that this had no effect on either the magnitude or duration of anti-Ig-induced ERK phosphorylation in A20 cells (Fig. 2A) or WEHI-231 cells3. To rule out the possibility that the effects of Rap-GTP might only be evident when there are low levels of ERK activation, we stimulated the cells with different concentrations of anti-Ig antibodies. Figure 2B shows that the dose response for anti-Ig-induced ERK phosphorylation was identical in vector control and RapGAPII-expressing WEHI-231 cells. We went on to perform ERK2 in vitro kinase assays to directly show that Rap does not modulate
ERK activation. We found that the magnitude and duration of BCR-induced ERK2 activation was virtually identical in the vector control and RapGAPII-expressing A20 cells and WEHI-231 cells (Fig. 2C,D). Thus, Rap-GTP is neither a positive nor a negative regulator of BCR-induced ERK activation. {Insert Figure 2}

We also investigated whether Rap activation modulated BCR-induced activation of the JNK and p38 MAPKs. Sawada et al. showed that preventing Rap activation via the expression of RapGAPII inhibits cell stretching-induced activation of p38, but not JNK, in the L-929 fibroblast cell line (34). Using phospho-specific antibodies to detect the activated forms of JNK and p38, we found that preventing Rap activation by expressing RapGAPII did not have a significant effect on BCR-induced activation of JNK or p38 in either A20 cells (Fig. 2E,F) or WEHI-231 cells. Thus, activation of endogenous Rap1 and Rap2 does not modulate the ability of the BCR to activate any of the MAPKs.

**Rap activation negatively regulates BCR-induced Akt phosphorylation**

PI3K is also a downstream effector of Ras (24,25,46,47), and recent work has shown that expressing constitutively active Ras in the A20 B cell line leads to increased activation of the PI3K/Akt pathway (26). Since the effector-binding domains of Rap1 and Rap2 are nearly identical to that of Ras (14), Rap1 and Rap2 may also bind PI3K and modulate the activation of PI3K-dependent signaling pathways. An important downstream target of PI3K is the serine/threonine kinase Akt/protein kinase B. The BCR activates Akt in a PI3K-dependent manner (39) and Akt activity opposes BCR-induced apoptosis and promotes B cell survival (6). Therefore, we investigated whether Rap activation modulates BCR-induced activation of Akt. The activation of Akt involves two steps, recruitment of Akt to the plasma membrane via the
binding of the Akt PH domain to PIP3 and PI(3,4)P2 and subsequent phosphorylation of Akt on threonine 308 (Thr308) by PDK1 (48) and on serine 473 (Ser473) by one of several candidate PDK2 enzymes (49-51). Phosphorylation of Akt on these two sites induces a structural reorganization of the kinase domain that is required for kinase activity (52). We therefore assessed Akt activation by using phospho-specific antibodies to analyze the phosphorylation of Akt on Thr308 and Ser473.

To determine whether activation of endogenous Rap1 and Rap2 by the BCR modulates its ability to activate Akt, we compared BCR-induced Akt phosphorylation in RapGAPII-expressing cells to that in vector control cells. We found that inhibiting Rap activation resulted in increased BCR-induced phosphorylation of Akt on both Ser473 (Fig. 3A) and Thr308 (Fig. 3B). Similar results were obtained in both the A20 and WEHI-231 B cell lines. BCR-induced phosphorylation of Akt on Ser473 and Thr308 was at least two-fold higher in the RapGAPII-expressing cells than in the vector control cells, and in some experiments was as much as 5-fold higher. Since inhibiting Rap activation resulted in increased phosphorylation of Akt, it suggests that BCR-induced activation of endogenous Rap1 or Rap2 normally limits BCR-induced phosphorylation of Akt. Thus, the Rap GTPases act as negative regulators of Akt activation in B cells. {Insert Figure 3}

To support the conclusion that Rap-GTP is a negative regulator of the PI3K/Akt pathway, we wished to perform the complementary gain-of-function experiment and show that increasing the amount of activated Rap results in a decrease in Akt activation. To do this, we expressed a constitutively active form of Rap2, Rap2V12, in both the A20 and WEHI 231 B cell lines. Figure 4A shows that the FLAG-tagged Rap2V12 is indeed constitutively active and that the total amount of activated Rap2 in these cells after BCR engagement was greater than in the
vector control cells. Rap2V12 expression did not have a significant effect on BCR-induced ERK phosphorylation, indicating that Rap2V12 did not impair BCR signaling in general and supporting the idea that Rap does not regulate ERK activation in B cells. In contrast, Rap2V12 expression inhibited BCR-induced phosphorylation of Akt on Ser473 by approximately 50% (Fig 4B). BCR-induced phosphorylation of Akt on Thr308 phosphorylation was also decreased by approximately 50% in the Rap2V12-expressing cells (Fig. 4C). These data show that activated Rap can inhibit BCR-induced phosphorylation of Akt and is consistent with the idea that BCR-induced activation of endogenous Rap normally limits the ability of the BCR to activate Akt. {Insert Figure 4}

**Rap2V12 can associate with PI3K**

We were interested in determining the mechanism by which Rap-GTP inhibits BCR-induced activation of the PI3K/Akt pathway. We hypothesized that activated Rap may bind to one of the components in this pathway and inhibit its activity. Therefore, we immunoprecipitated the FLAG-tagged Rap2V12 and performed immunoblots to determine whether it associated with PI3K, PDK1, or Akt. Figure 5A shows that neither Akt nor PDK1 associated with Rap2V12 to a significant extent. The bands observed in the anti-PDK1 blots have a different mobility than the PDK1 in the cell lysates and are most likely the Ig heavy chains of the anti-FLAG antibody used for immunoprecipitation. In contrast, Rap2V12 bound very efficiently to PI3K, as judged by immunoblotting with antibodies to the p85α subunit of PI3K (Fig 5A, bottom panel). Moreover, similar amounts of the PI3K p85 subunit co-precipitated with Rap2V12 from unstimulated cells and anti-Ig-treated cells, indicating that this
was a constitutive association. These data suggest that activated Rap might exert its inhibitory effect on the PI3K/Akt pathway by binding to PI3K. {Insert Figure 5}

We went on to perform \textit{in vitro} kinase assays to assess the amount of PI3K enzyme activity that was associated with Rap2V12 precipitated from unstimulated and anti-Ig-stimulated cells. A substantial amount of PI3K enzyme activity associated with the Rap2V12 precipitated from unstimulated cells (Fig. 5B). However, considerably less PI3K enzyme activity was associated with Rap2V12 precipitated from anti-Ig-stimulated cells (Fig. 5B). The amount of PI3K activity associated with Rap2V12 isolated from anti-Ig-treated cells was only $36 \pm 6\%$ (n = 4 independent experiments) of the PI3K activity associated with Rap2V12 isolated from unstimulated cells (Fig. 5C). Thus, even though similar amounts of the PI3K p85 subunit associated with Rap2V12 in unstimulated and anti-Ig-treated cells, considerably less PI3K enzyme activity was associated with the Rap2V12 isolated from anti-Ig-treated cells. The simplest interpretation of this result is that some BCR-induced signaling event allows Rap2V12 to inhibit the enzymatic activity of PI3K. In any case, our results indicate that activated Rap can inhibit the activation of the PI3K/Akt pathway by negatively regulating PI3K activity in some manner.

\textbf{Rap activation modulates phosphorylation of the FKHR transcription factor, a downstream target of Akt.}

Akt-dependent phosphorylation regulates the activity of a number of proteins that are involved in cell survival, cell growth and cell cycle progression (53). Substrates of Akt include Bad (54,55), GSK-3 (56), mTOR (57), tuberin (58), and p21$^{\text{Cip1/Waf}}$ (59,60). In addition, Akt-mediated regulation of the NF-\kappaB and Forkhead transcription factors leads to changes in gene
expression that promote cell survival (61-64). Akt enhances the activation of NF-κB, a transcription factor that regulates the expression of multiple pro-survival factors. In contrast, Akt inhibits the activity of Forkhead transcription factors which normally induce the expression of pro-apoptotic proteins. Akt-mediated phosphorylation of Forkhead transcription factors causes them to translocate from the nucleus to the cytoplasm and in this way inhibits their transcriptional activity (65). Gene targets of Forkhead include the cell cycle inhibitor p27^Kip1 (66), a pro-apoptotic member of the Bcl-2 family called Bim1 (67) and the Fas ligand (63). Phosphorylation of Forkhead transcription factors by Akt results in decreased transcription of these pro-apoptotic genes and represents one mechanism by which Akt promotes cell survival. Since we found that Rap-GTP limits BCR-induced activation of Akt, an important question was whether Rap-GTP negatively regulates Akt-dependent anti-apoptotic/pro-survival signaling pathways in B cells.

To test whether Rap-GTP regulates Akt-dependent pro-survival signaling pathways, we analyzed the effect of RapGAPII expression and Rap2V12 expression on BCR-induced phosphorylation of the Forkhead-related transcription factor FKHR/FOXO1, a known target of Akt in B cells (68). Akt-mediated phosphorylation of FKHR on serine 256 and threonine 24 induces the cytoplasmic translocation of FKHR and inhibits FKHR-dependent transcription (65). Consistent with our finding that inhibition of endogenous Rap1 and Rap2 enhanced BCR-induced activation of Akt, we found that preventing the activation of endogenous Rap by expressing RapGAPII enhanced BCR-induced phosphorylation of FKHR on serine 256 by approximately 2-fold (Fig. 6A). This indicates that activation of endogenous Rap limits the ability of the BCR to induce FKHR phosphorylation, an Akt-dependent pro-survival pathway. Supporting the idea that Rap-GTP negatively regulates Akt-dependent phosphorylation of
FKHR, we found that expressing the constitutively active Rap2V12 in A20 cells reduced BCR-induced phosphorylation of FKHR by approximately 60% (Fig. 6B). {Insert Figure 6}

**Endogenous Rap modulates expression of the FKHR target p27^Kip1**

Since FKHR regulates the expression of proteins that promote cell cycle arrest and apoptosis, we wished to determine if modulation of the Akt/FKHR signaling module by activated Rap altered the expression of these proteins. In particular we focused on expression of the p27^Kip1 cell cycle inhibitor, an FKHR target that can promote apoptosis when ectopically expressed (69-73). p27^Kip1 plays a key role in BCR-induced growth arrest and apoptosis (70,73,74) in B cell lines such as WEHI-231 and CH31 which correspond to immature/transitional B cells that undergo clonal deletion in response to antigen engagement. Prolonged BCR signaling in these immature B cell lines leads to increases in the expression of p27^Kip1 (73,74). Moreover, ectopic expression of p27^Kip1 can induce apoptosis in these immature B cell lines (70,73) while antisense-induced decreases in p27^Kip1 levels prevent apoptosis (70). This suggests that p27^Kip1 expression is a key determinant of survival versus apoptosis in B cells.

Consistent with the idea that Akt is an important pro-survival factor for B cells, expression of constitutively active Akt in immature B cell lines delays BCR-induced upregulation of p27^Kip1 and apoptosis (73). Suppressing the expression of p27^Kip1 may therefore be an important mechanism by which Akt promotes B cell survival. Our finding that activation of endogenous Rap by the BCR limits the activation of Akt, as well as the ability of Akt to phosphorylate FKHR on negative regulatory sites, suggested that Rap activation would enhance the expression of p27^Kip1. If this were the case, then preventing BCR-induced activation of
endogenous Rap via the expression of RapGAPII should inhibit the expression of $p27^{kip1}$ by increasing BCR-induced Akt activation (as shown in Fig. 3) and increasing the phosphorylation of FKHR on negative regulatory sites (as shown in Fig. 6A) such that FKHR would have a decreased ability to promote the transcription of $p27^{kip1}$ (see pathway model in Fig. 8).

To test this hypothesis, we first developed an approach to show that enhancing Akt activation leads to increased BCR-induced phosphorylation of FKHR and decreased expression of $p27^{kip1}$. We expressed in A20 cells a conditionally active form of Akt (mER-Akt) that consists of the kinase domain of Akt fused to a mutant form of the estrogen receptor that is responsive to the estrogen analog 4-HT. Although the mER-Akt protein lacks the PH domain of Akt, it contains a myristoylation sequence at the N-terminus that localizes it to the inner leaflet of the plasma membrane. In response to 4-HT, the mER-Akt estrogen receptor undergoes a conformational change that exposes the Akt activation sites, allowing it to be phosphorylated and activated by PDK1 and PDK2 (37). As a negative control for any effects of 4-HT, we also expressed a variant of the mER-Akt protein called A2-ER-Akt that has an alanine to glycine mutation at position 2 and therefore cannot be myristoylated. This A2-ER-Akt fusion protein is restricted to the cytosol and cannot be activated by PDK1 and PDK2 (37).

Figure 7A shows that 4-HT stimulation of the mER-Akt protein could enhance Akt activation over a 24 h time course. Treating the control A2-ER-Akt-expressing A20 cells with the combination of anti-IgG plus 4-HT for 8-24 h resulted in sustained phosphorylation of only the endogenous Akt protein since the A2-ER-Akt protein cannot be activated by PDK1 and PDK2. In contrast, treating mER-Akt-expressing A20 cells with the combination of anti-IgG plus 4-HT induced sustained phosphorylation of the 90-kDa mER-Akt fusion protein and also enhanced the phosphorylation of the endogenous 60-kDa Akt protein compared to A2-ER-Akt-
expressing cells (Fig. 7A). The phosphorylation of the endogenous Akt protein was about 30% higher in the mER-Akt-expressing cells than in the A2-ER-Akt-expressing cells. The enhanced phosphorylation of endogenous Akt may be due to an interaction between the mER-Akt protein and the endogenous Akt protein. This is significant since the endogenous Akt protein, unlike the mER-Akt fusion protein, is not restricted to the plasma membrane and can phosphorylate nuclear substrates such as FKHR. {Insert Figure 7}

We then examined the consequence of enhanced Akt activation on FKHR phosphorylation in A20 cells. Although BCR signaling induces FKHR phosphorylation on the 5 to 15 min time scale (Fig. 6), in A2-ER-Akt-expressing cells stimulated with anti-IgG plus 4-HT, signaling via the BCR alone caused very little sustained phosphorylation of FKHR at 8 to 24 h (Fig. 7B). In contrast, in mER-Akt-expressing cells stimulated with anti-IgG plus 4-HT, the increased activation of Akt resulted in significant and sustained phosphorylation of FKHR at 16 h and 24 h (Fig. 7B). We then examined the effect of enhanced Akt activation on the levels of the FKHR target p27\textsuperscript{Kip1}. BCR signaling alone in the A2-ER-Akt-expressing cells resulted in a significant increase in p27\textsuperscript{Kip1} protein levels at 16 h and 24 h (Fig. 7C). In contrast, the enhanced Akt activation in the mER-Akt-expressing cells treated with anti-IgG plus 4-HT strongly suppressed the accumulation of p27\textsuperscript{Kip1} (Fig. 7C). This is consistent with previous findings showing that ectopic expression of constitutively active Akt suppresses BCR-induced accumulation of p27\textsuperscript{Kip1} in WEHI-231 cells (73).

Since inhibition of endogenous Rap via expression of RapGAPII increases the ability of the BCR to activate Akt and phosphorylate FKHR, we predicted that preventing Rap activation in this way would suppress the BCR-induced accumulation of p27\textsuperscript{Kip1}, similar to what we observed when we increased Akt activity using the mER-Akt fusion protein. Indeed, p27\textsuperscript{Kip1}
levels were lower, and the BCR-induced accumulation of p27Kip1 was smaller, in RapGAPII-expressing A20 cells than in the vector control cells (Fig. 7D). These findings indicate that activation of endogenous Rap limits Akt activation and in doing so prevents Akt from suppressing the expression of p27Kip1, a potent inducer of cell cycle arrest and apoptosis in B cells.

**Activation of endogenous Rap modulates anti-Ig induced cell death**

We have shown that preventing the activation of endogenous Rap, via RapGAPII expression, enhances the ability of the BCR to activate Akt. Since Akt is a potent pro-survival factor for B cells (6), we predicted that the enhanced Akt activation in RapGAPII-expressing cells would make the cells less sensitive to apoptotic stimuli. To test this hypothesis, we compared anti-Ig-induced cell death in WEHI-231 cells. WEHI-231 cells resemble immature/transitional B cells and undergo growth arrest followed by apoptosis upon prolonged treatment with anti-IgM antibodies (74). Moreover, expressing a constitutively active form of Akt in WEHI-231 cells has been shown to reduce BCR-induced cell death (73). Consistent with the enhanced BCR-induced Akt activation in RapGAPII-expressing WEHI-231 cells (see Fig. 3), we found that RapGAPII-expressing WEHI-231 cells underwent less anti-IgM-induced cell death than the vector control cells (Table 1). At both the 48 h and 72 h time points, anti-IgM induced cell death was consistently 15% lower in the RapGAPII-expressing WEHI-231 cells than in the vector control cells. The finding that preventing Rap activation via RapGAPII expression reduces anti-Ig-induced cell death suggests that activation of endogenous Rap by the BCR favors anti-Ig-induced cell death, presumably by limiting the activation of Akt and Akt-
regulated survival pathways such as the downregulation of the FKHR/p27Kip1 module. {Insert Table 1}
DISCUSSION

We have shown that activation of the Rap GTPases during BCR signaling selectively limits the ability of the BCR to activate the PI3K/Akt pathway while having no effect on other BCR-induced signaling events including phosphorylation of the ERK, JNK, and p38 MAPKs. We found that preventing the activation of endogenous Rap by the BCR enhanced BCR-induced phosphorylation of Akt and potentiated Akt-dependent effects on the phosphorylation of the FKHR transcription factor and the expression of the FKHR target p27^Kip1. These findings indicate that BCR-induced Rap activation normally limits the ability of the BCR to activate Akt, inhibit FKHR, and suppress the expression of p27^Kip1, a cell cycle inhibitor that can promote apoptosis (Fig. 8). Thus Rap activation functions as a negative regulator of a key Akt-regulated pro-survival pathway. Consistent with this idea, we found that blocking Rap activation enhanced the ability of WEHI-231 cells to survive prolonged treatment with anti-IgM antibodies. Finally, our finding that the constitutively active Rap2V12 can associate with PI3K and inhibit its activity in a manner that depends upon BCR engagement provides a potential mechanism by which Rap-GTP limits the activation of the PI3K/Akt pathway by the BCR. {insert Figure 8}

The Rap GTPases were originally described as negative regulators of Ras signaling. In vitro, the active GTP-bound forms of Rap1 and Rap2 can bind Ras effectors such as Raf-1 and RalGDS (12,13,15-17) but does not activate them. This suggested that activated Rap might limit Ras signaling by sequestering Ras effectors in inactive complexes. In support of this idea, expressing constitutively active forms of Rap1 or Rap2 inhibits Ras-dependent ERK activation in a variety of cell types (15,16) while preventing the activation of endogenous Rap, via expression of RapGAPII, enhances Ras-dependent ERK activation in 293T cells (35) and a CD45R0+ T cell line (75). However ERK activation is not inhibited when constitutively active Rap1 is expressed.
in the T cells of transgenic mice (76) or when endogenous Rap1 and Rap2 are selectively
activated in NIH 3T3 cells (21). One possible explanation for these divergent results is that the
ability of Rap-GTP to effectively compete with Ras for binding to Raf-1 is cell type-specific. In
B cells, we found that Rap-GTP did not act as a negative regulator of Ras-dependent ERK
activation. Preventing the activation of endogenous Rap1 and Rap2 did not augment BCR-
induced ERK activation, indicating that endogenous Rap does not limit the ability of the BCR to
activate ERK. Moreover, expressing the constitutively active Rap2V12 protein did not inhibit
BCR-induced ERK activation\(^3\). Thus Rap-GTP is unable to interfere with Ras-dependent ERK
activation in B cells. This may reflect the fact that the amount of Raf-1 is not limiting in B cells
or that Ras and Raf-1 are coupled very tightly by scaffolding proteins, such that Rap-GTP cannot
effectively interfere with their interaction.

In some cell types, particularly neuronal cells, Rap-GTP acts a positive regulator of ERK
by activating B-Raf (31,32). B-Raf is an upstream activator of the ERK pathway that functions
similarly to Raf-1. Recent work by Brummer et al. showed that BCR-induced ERK activation
involves both B-Raf and Raf-1, with B-Raf making the major contribution (33). This suggested
that Rap-GTP could be a positive regulator of ERK activation in B cells. However, we found
that blocking Rap activation via the expression of RapGAPII did not reduce the ability of the
BCR to activate ERK. This indicates that Rap-GTP does not contribute to ERK activation in B
cells by activating the B-Raf/ERK pathway. Consistent with this idea, Brummer et al. (33) argue
that B-Raf-dependent ERK activation in B cells is controlled by Ras as opposed to Rap. It is not
clear why Rap-GTP activates the B-Raf/ERK signaling module in neuronal cells such as the PC-
12 cell line but not in B cells. There are multiple alternatively spliced isoforms of B-Raf, so it is
possible that there are neural-specific B-Raf isoforms that are regulated by Rap-GTP whereas the
B cell-specific isoforms are regulated by Ras. It is also possible that an intermediary protein or scaffolding protein that couples Rap to B-Raf is expressed in neurons but not in B cells.

We also investigated whether Rap activation was involved in BCR-induced activation of the p38 and JNK MAPKs. During BCR signaling, the p38 and JNK MAPKs are coordinately activated and both regulate the activity of transcription factors that contribute to B cell activation. Although stretching-induced p38 activation in L-929 cells is dependent on Rap activation (34), we found that preventing Rap activation had no effect on the ability of the BCR to activate either p38 or JNK.

Even though Rap-GTP did not inhibit the activation of the Ras/ERK pathway in B cells, it was possible that it selectively inhibited the activation of other Ras effectors. PI3K is a downstream effector of Ras in many cell types (24,25,46,47), including B cells (26) where it plays a key role in promoting B cell survival by activating the Akt kinase (6). Using a combination of loss-of-function and gain-of-function approaches, we found that Rap-GTP is a negative regulator of the PI3K/Akt pathway in B cells. Preventing Rap activation by expressing RapGAP-II enhanced the ability of the BCR to stimulate Akt phosphorylation. This indicates that activation of endogenous Rap limits the ability of the BCR to activate Akt. This may be important since Akt regulates multiple pro-survival pathways and the excessive activation of these pathways could lead to oncogenic transformation. Consistent with the idea that Rap-GTP inhibits the activation of the PI3K/Akt pathway, expressing the constitutively active Rap2V12 in B cell lines reduced the ability of the BCR to induce Akt phosphorylation. We have not been able to express the constitutively active Rap1V12 protein in B cell lines, perhaps because it is a stronger inhibitor of Akt activation than Rap2V12 and reduces Akt activity to the point where the cells are unable to survive.
The ability of Rap-GTP to regulate Akt phosphorylation and activation has been reported previously in the context of cAMP-mediated cell signaling. Depending on the cell type, cAMP can either activate or inhibit Rap activation and Rap-GTP can act as either a positive or a negative regulator of Akt activation. In 293 cells, cAMP analogues activate the Rap-specific nucleotide exchange factor EPAC, resulting in Rap activation and Rap-dependent Akt phosphorylation (77). Rap-GTP also appears to be a positive regulator of Akt in the C6 rat glioma cell line where cAMP inhibits Rap activation and this leads to decreased Akt phosphorylation (78). In contrast, Rap-GTP acts as a negative regulator of Akt in the PCCL3 thyroid cell line. In these cells, cAMP-induced activation and phosphorylation of Rap1B results in inhibition of Akt phosphorylation (79). The mechanisms by which Rap regulates the PI3K/Akt pathway in these systems are not known.

Our findings suggest that Rap-GTP inhibits BCR-induced Akt phosphorylation by binding to PI3K. The constitutively active Rap2V12 associated with PI3K in A20 cells but did not associate with PDK1 or Akt. This is the first report showing the association of Rap2 with PI3K. It had been proposed that Rap1 can bind PI3K (80,81) since Rap1 and Ras have identical effector-binding regions and Rap1 can bind most other Ras effectors but this has not been shown directly. Our preliminary data indicates that both Rap1V12 and Rap2V12 can associate with endogenous PI3K when expressed in 293 cells3. Thus, it is likely that both Rap1-GTP and Rap2-GTP can associate with PI3K in B cells and inhibit its ability to activate Akt. We have not been able to test this directly since we are unable to express the constitutively active Rap1V12 in B cell lines.

How the binding of Rap2-GTP to PI3K impairs the ability of PI3K to signal to Akt remains to be elucidated. We found that Rap2V12 associated with PI3K in both unstimulated
and anti-Ig-stimulated A20 B lymphoma cells, as judged by immunoblotting with antibodies against the p85 subunit of PI3K. However, PI3K enzyme assays revealed that the amount of PI3K enzyme activity associated with these Rap2V12/PI3K complexes was significantly lower when the complexes were isolated from anti-Ig-treated cells than when the complexes were isolated from unstimulated cells. Thus, Rap2V12 appears to inhibit the activity of PI3K in a manner that depends upon BCR engagement.

Although the nature of this BCR signaling contribution to Rap-mediated inhibition of PI3K is not known, a number of possibilities can be envisioned. One possibility is that Rap-GTP recruits an inhibitor of PI3K but can only do so in BCR-stimulated cells. The interaction between Rap-GTP and this putative inhibitor may depend on BCR signaling events, for example the phosphorylation of Rap-GTP or the putative inhibitor. Conversely, as opposed to recruiting an inhibitor of PI3K, the binding of Rap-GTP to PI3K in anti-Ig stimulated cells could cause an activator to dissociate from PI3K. Another possibility is that Rap-GTP recruits a lipid phosphatase such as PTEN that dephosphorylates the lipid second messengers produced by PI3K. Co-localization of PTEN with PI3K would prevent the accumulation of PIP3 and PI(3,4)P2 in vivo as well as the accumulation of phosphatidylinositol 3-phosphate in our in vitro PI3K assays in which phosphatidylinositol is used as the substrate. A final possibility is that Rap2V12, in conjunction with a BCR signaling event, induces the dissociation of the p110 catalytic subunit of PI3K from the p85 regulatory subunit. Further work is required to test these various models for how Rap-GTP, in the context of BCR signaling, inhibits PI3K activity.

The initiation of PI3K signaling requires the recruitment of PI3K to the plasma membrane. Since PI3K is a central regulator of B cells survival and activation, it is not surprising that the BCR uses multiple mechanisms to recruit PI3K to the plasma membrane. The
membrane-associated proteins that recruit PI3K to the plasma membrane after BCR engagement include a variety of tyrosine-phosphorylated docking proteins such as CD19, BCAP, Gab1, Gab2, and Cbl (27-30). Ras-GTP may also recruit PI3K to the plasma membrane after BCR engagement (26) and there may also be other non-phosphotyrosine-dependent mechanisms for initiating PI3K signaling. Interestingly, we found that Rap2V12 expression did not inhibit the activity of PI3K that associated with tyrosine-phosphorylated proteins and that Rap2V12 was not present in the same complexes with these tyrosine-phosphorylated proteins. This suggests that Rap-GTP inhibits the activity of a subset of PI3K molecules that the BCR mobilizes in a phosphotyrosine-independent manner that may involve Ras-GTP or a yet undescribed mechanism for PI3K recruitment to the plasma membrane.

An important question is whether Rap activation limits the activation of other PI3K targets besides Akt in B cells. Our preliminary work suggests that Rap-dependent inhibition of PI3K may only impair processes that require high levels of PI3K-derived lipids. We found that BCR-induced phosphorylation of Akt, which is strongly inhibited by low concentrations (5 µM) of the PI3K inhibitor LY294002, was inhibited by Rap activation whereas BCR-induced phosphorylation of PKC-ε, which is only inhibited by higher concentrations (25 µM) of LY294002, was not affected by expression of either RapGAPII or Rap2V12. The finding that Rap activation preferentially inhibits those PI3K-dependent signaling events that are most sensitive to PI3K inhibitors suggests that Rap activation selectively limits only those PI3K-dependent signaling events that require high levels of PI3K-derived lipids for their activation.

Since Rap-GTP appears to selectively inhibit the PI3K/Akt pathway in B cells, we asked whether Rap activation limits the ability of the BCR to activate Akt-dependent pro-survival signaling pathways. An important Akt-dependent survival pathway involves the phosphorylation
of FKHR by Akt, a modification that prevents FKHR from promoting the transcription of pro-apoptotic genes such as p27^Kip1. Consistent with our finding that activation of endogenous Rap by the BCR limits BCR-induced activation of Akt, we found that Rap activation also opposes the Akt-dependent inhibition of the FKHR/p27^Kip1 module (Fig. 8). Preventing the activation of endogenous Rap by the BCR mimicked the effects of enhancing Akt activation by increasing BCR-induced phosphorylation of FKHR and suppressing the expression of p27^Kip1. Thus activation of the Rap GTPases by the BCR limits the ability of the BCR to activate Akt and also limits the ability of Akt to suppress a pro-apoptotic pathway that involves FKHR-dependent accumulation of p27^Kip1 (Fig. 8). Rap-dependent accumulation of p27^Kip1 is not limited to B cells. Although they did not implicate Akt as an intermediate, Katagiri et al. showed that expressing constitutively active Rap1 in T cells results in the accumulation of p27^Kip1 (82).

The ability of Rap-GTP to negatively regulate the activation of Akt and Akt-dependent survival pathways in B cells may be an important factor that limits B cell survival and activation. The BCR activates both pro-survival and pro-apoptotic pathways and Rap activation may tip the balance in favor of apoptosis in certain situations where it is appropriate, such as the deletion or silencing of self-reactive B cells. Consistent with this idea, Rap activation has been associated with T cell anergy and activation-induced cell death (82-84). Our experiments show that preventing BCR-induced Rap activation reduces the ability of anti-IgM antibodies to cause cell death in WEHI-231 cells, a model for antigen-induced clonal deletion. This suggests that Rap activation makes immature/transitional B cells more sensitive to antigen-induced clonal anergy or clonal deletion. Further in vivo experiments are required to support this idea that Rap activation affects the signaling threshold for the elimination or silencing of self-reactive B cells.
In summary, we have shown that activation of endogenous Rap limits the ability of the BCR to activate the PI3K/Akt pathway, limits the subsequent Akt-mediated inhibition of the FKHR/p27Kip1 pro-apoptotic module, and modulates the sensitivity of an immature B cell line to anti-Ig-induced cell death.
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FOOTNOTES

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1 The abbreviations used are: BCR, B cell antigen receptor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PDK1, 3-phosphoinositide-dependent kinase-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; Ig, immunoglobulin; Thr308, threonine 308; Ser473, serine 473; FKHR, Forkhead-related transcription factor; mER-Akt, myristoylated-estrogen receptor-Akt fusion protein; A2-ER-Akt, non-myristoylated estrogen receptor-Akt fusion protein with a glycine to alanine mutation at position 2; 4-HT, 4-hydroxytamoxifen; TBST, Tris-buffered saline with 0.1% Tween-20; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; 7-AAD, 7-amino-actinomycin D; GAP, GTPase-activating protein.


3 S.L.C. and M.R.G, unpublished observations
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FIGURE LEGENDS

FIG. 1. BCR-induced activation of Rap1 and Rap2 is inhibited by expression of RapGAPII.
A, A20 or WEHI-231 cells were stimulated with 40 µg/ml goat anti-mouse anti-Ig antibodies for the indicated times. Cell lysates were incubated with immobilized GST-RalGDS(RBD) in order to precipitate the active GTP-bound forms of Rap1 and Rap2 which were detected by immunoblotting with antibodies specific for Rap1 or Rap2. B, A20 and WEHI-231 cells were transfected with the empty pMSCVpuro vector or with pMSCVpuro containing the cDNA encoding FLAG-tagged RapGAPII. A20 clones and WEHI-231 bulk populations were analyzed for expression of FLAG- RapGAPII by immunoblotting with the anti-FLAG M2 antibody. C, A20 or WEHI-231 cells expressing either the empty vector or RapGAPII were stimulated with 40 µg/ml goat anti-mouse Ig antibodies for the indicated times. Rap activation assays were performed on the cell lysates as in A. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments.

FIG 2. Preventing Rap activation does not affect BCR-induced activation of ERK, JNK or p38.
A and B, Immunoblotting with antibodies that recognize the phosphorylated, active forms of ERK1 and ERK2 was performed on cell extracts from vector control and RapGAPII-expressing A20 cells (A) that were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times or WEHI-231 cells (B) that were stimulated with the indicated concentrations of goat anti-mouse IgM antibodies for 5 min. Equal loading was analyzed by re-probing the membranes with an anti-ERK antibody. C and D, ERK2 in vitro kinase assays were performed on cell extracts from vector control and RapGAPII-expressing A20 cells (C) that were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times or WEHI-231 (D) cells that
were stimulated with 40 µg/ml goat anti-mouse IgM antibodies for the indicated times. ERK2 activity was measured using GST-ELK-1 as a substrate (C) or myelin basic protein as a substrate (D). E and F, Immunoblotting with antibodies that recognize the phosphorylated, active forms of JNK (E) or p38 (F) was performed on cell extracts from vector control and RapGAPII-expressing A20 cells that were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times. Equal loading was analyzed by re-probing the membranes with antibodies to JNK or p38. A non-specific band recognized by the anti-phospho-JNK antibody is indicated with an asterisk (*). For each panel, similar results were obtained in two to five independent experiments. Molecular mass standards (in kDa) are indicated to the left of each panel.

FIG. 3. Preventing Rap activation increases BCR-induced phosphorylation of Akt. Vector control and RapGAPII-expressing A20 cells (left panels) or WEHI-231 cells (right panels) were stimulated with 40 µg/ml goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt that is phosphorylated on Ser473 (A) or on Thr308 (B). Equal loading was analyzed by re-probing the membranes with anti-Akt antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three to six independent experiments.

FIG. 4. The constitutively active Rap2V12 inhibits BCR-induced phosphorylation of Akt. Vector control and Rap2V12-expressing A20 cells (left panels) or WEHI-231 cells (right panels) were stimulated with 40 µg/ml goat anti-mouse Ig antibodies for the indicated times. A, Rap2 activation assays were performed on cell extracts. The active GTP-bound form of endogenous Rap2 or the transfected FLAG-tagged Rap2V12 was precipitated using a GST-
RalGDS(RBD) fusion protein and detected by immunoblotting with an antibody specific for Rap2.  

**B and C,** Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt that is phosphorylated on Ser473 (**B**) or on Thr308 (**C**). Equal loading was analyzed by re-probing the membranes with anti-Akt antibodies. Molecular mass standards (in kDa) are indicated to the left of each panel. To calculate the relative levels of Ser473 phosphorylation, the intensity of each band in the upper panel of (**B**) was determined by densitometry and divided by the intensity of the corresponding total Akt band in the lower panel. For each panel, similar results were obtained in three independent experiments.

**FIG. 5.** Rap2V12 associates with PI3K.  

**A,** A20 cells expressing FLAG-Rap2V12 were incubated with or without 40 µg/ml goat anti-mouse IgG antibodies for 5 min. Cell lysates from 1.25 x 10⁷ cells were incubated with the anti-FLAG M2 antibody conjugated to agarose beads or with an equivalent volume of Sepharose-CL-4B beads. Precipitated proteins, as well as total cell lysates (1.25 x 10⁶ cell equivalents) were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against the p85 subunit of PI3K. The upper portion of the membrane was subsequently re-probed with antibodies to PDK1 and Akt while the lower portion of the membrane was re-probed with the anti-FLAG antibody to show that equivalent amounts of FLAG-Rap2V12 had been precipitated from the unstimulated and anti-Ig-stimulated cells. Molecular mass standards (in kDa) are indicated to the left of each panel. The asterisks represent bands derived from the Ig heavy chains (IgH) or Ig light chains (IgL) of the anti-FLAG antibody used for immunoprecipitation. Similar results were obtained in two independent experiments.  

**B and C,** A20 cells were stimulated as in **A.** Cell lysates were incubated with the anti-FLAG M2 antibody coupled to agarose beads and PI3K enzyme assays were performed on the
immunoprecipitates using phosphatidylinositol as a substrate. The phosphatidylinositol 3-phosphate (PI(3)P) produced by PI3K was separated by thin-layer chromatography (B). The amounts of $^{32}$P-PI(3)P produced were quantitated using a phosphorimager and are expressed as a % of the $^{32}$P-PI(3)P for the unstimulated (time 0) samples (C). The data are presented as the mean ± SEM for four independent experiments. P values were calculated using Student’s T-test.

**FIG. 6.** Rap activation modulates BCR-induced phosphorylation of FKHR. Cell extracts were analyzed by immunoblotting with antibodies that recognize FKHR that is phosphorylated on serine 256. *A*, Vector control and RapGAPII-expressing A20 cells were stimulated with 40 µg/ml goat anti-mouse IgG antibodies for the indicated times. *B*, Vector control and Rap2V12-expressing A20 cells were stimulated with 40 µg/ml goat anti-mouse IgG antibodies. A non-specific band in panel *A* is indicated with an asterisk (*). Equal loading was analyzed by re-probing the membranes with a monoclonal antibody to actin. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in three independent experiments.

**Fig. 7.** Both Akt activation and inhibition of endogenous Rap oppose BCR-induced accumulation of p27<sup>Kip1</sup>. *A-C*, A20 cells expressing either A2-ER-Akt or mER-Akt were stimulated with 10 µg/ml goat anti-mouse IgG antibodies plus 2 µM 4-HT for the indicated times. *A*, Cell extracts were analyzed by immunoblotting with antibodies that recognize Akt phosphorylated on serine 473. Equal loading was analyzed by re-probing the membranes with anti-Akt antibodies. *B*, Cell extracts were analyzed by immunoblotting with antibodies that recognize FKHR that is phosphorylated on serine 256. Equal loading was analyzed by re-
probing the membranes with a monoclonal antibody to actin.  C, Cell extracts were analyzed by immunoblotting with antibodies against p27\textsuperscript{Kip1}. Equal loading was analyzed by re-probing the membranes for actin.  D, Vector control and RapGAPII-expressing A20 cells were stimulated with 10 µg/ml goat anti-mouse Ig antibodies for the indicated times. Cell extracts were analyzed by immunoblotting with antibodies against p27\textsuperscript{Kip1}. As a loading control, the membrane was re-probed with antibodies to actin. To calculate the relative levels of p27\textsuperscript{Kip1}, the intensity of each band in the p27\textsuperscript{Kip1} blot (upper panel) was determined by densitometry and divided by the intensity of the corresponding actin band in the lower panel. Molecular mass standards (in kDa) are indicated to the left of each panel. For each panel, similar results were obtained in two to four independent experiments.

**FIG. 8.** Model depicting the effects of Rap activation and RapGAPII expression on the PI3K/Akt pathway. BCR-induced activation of endogenous Rap proteins limits the activation of the PI3K/Akt pathway by the BCR. In conjunction with other BCR signaling events (X), activated GTP-bound Rap can inhibit PI3K-dependent activation of Akt, apparently by binding to PI3K. Inhibition of Akt activation by activated Rap reduces BCR-induced phosphorylation of FKHR, resulting in increased levels of the FKHR target p27\textsuperscript{Kip1}. Thus, Rap activation limits the Akt-mediated downregulation of the FKHR/p27\textsuperscript{Kip1} pro-apoptotic module. The thick gray arrows represent the effect of preventing Rap activation via the expression of RapGAPII. Under these conditions, the negative effects of Rap-GTP on the PI3K/Akt pathway do not occur. The net result is that Akt activation is enhanced, FKHR phosphorylation is enhanced, and expression of the p27\textsuperscript{Kip1} cell cycle inhibitor is suppressed.
Table I

Preventing Rap activation via expression of RapGAPII reduces anti-IgM-induced cell death in WEHI-231 cells. Vector control and RapGAPII-expressing WEHI-231 cells were cultured with 10 μg/ml goat anti-mouse IgM antibodies for 48 h or 72 h. The cells were stained with 7-AAD, a probe for membrane integrity, and the percent of cells that were dead was determined by flow cytometry. For cells cultured in the absence of anti-IgM, cell death at 48 h and 72 h was less than 15%. P values were calculated using Student’s T test.

<table>
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<tr>
<th>% dead cells</th>
<th>48 h</th>
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<tr>
<td>Expt. I</td>
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<tr>
<td>MSCV</td>
<td>70.2%</td>
<td>Expt I MSCV: 87.2%</td>
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<tr>
<td>RapGAPII</td>
<td>55.0%</td>
<td>RapGAPII: 74.0%</td>
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</tr>
<tr>
<td>MSCV</td>
<td>66.2%</td>
<td>Expt V MSCV: 73.2%</td>
</tr>
<tr>
<td>RapGAPII</td>
<td>53.4%</td>
<td>RapGAPII: 58.8%</td>
</tr>
</tbody>
</table>

Difference in % dead cells (Mean ± SEM)

MSCV - RapGAPII: 15.0 ± 2.1% (n=3) MSCV - RapGAPII: 14.4 ± 1.1% (n=3)
P < 0.01 P < 0.005
Figure 2

A

<table>
<thead>
<tr>
<th>Anti-IgG (min)</th>
<th>Vector control A20 cells</th>
<th>RapGAPII-expressing A20 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Phospho-ERK1</td>
<td>Phospho-ERK2</td>
<td>ERK1</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Anti-IgM (μg/ml)</th>
<th>Vector control WEHI-231 cells</th>
<th>RapGAPII-expressing WEHI-231 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Phospho-ERK1</td>
<td>Phospho-ERK2</td>
<td>ERK1</td>
</tr>
</tbody>
</table>

C

Relative ERK2 activity


D

Relative ERK2 activity


E

<table>
<thead>
<tr>
<th>Anti-IgG (min)</th>
<th>Vector control A20 cells</th>
<th>RapGAPII-expressing A20 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Phospho-p54 JNK</td>
<td>Phospho-p46 JNK</td>
<td>p54 JNK</td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th>Anti-IgG (min)</th>
<th>Vector control A20 cells</th>
<th>RapGAPII-expressing A20 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Phospho-p38</td>
<td>p38</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4

A

A20 cells

Vector control A20 cells

Rap2V12-expressing A20 cells

Anti-IgG (min)

0 5 15 30

23

FLAG-Rap2V12-GTP

Rap2-GTP

WEHI-231 cells

Vector control WEHI-231 cells

Rap2V12-expressing WEHI-231 cells

Anti-IgG (min)

0 5 15 30

23

FLAG-Rap2V12-GTP

Rap2-GTP

B

Vector control A20 cells

Rap2V12-expressing A20 cells

Anti-IgG (min)

0 2 5 15

66

Phospho-Ser473Akt

Akt

Vector control WEHI-231 cells

Rap2V12-expressing WEHI-231 cells

Anti-IgG (min)

0 2 5 15

66

Phospho-Ser473Akt

Akt

C

Vector control A20 cells

Rap2V12-expressing A20 cells

Anti-IgG (min)

0 2 5 15

66

Phospho-Thr308Akt

Akt

Vector control WEHI-231 cells

Rap2V12-expressing WEHI-231 cells

Anti-IgG (min)

0 2 5 15

66

Phospho-Thr308Akt

Akt
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>anti-FLAG</th>
<th>beads only</th>
<th>total cell lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ippt Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-IgG (min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-FLAG blot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Akt blot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-PDK1 blot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-p85 blot</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

B

C

p < 0.05

[Rap2V12-associated PI3K activity (% of activity from unstimulated cells)]

unstimulated
anti-IgG
Figure 6

A

<table>
<thead>
<tr>
<th>Anti-IgG (min)</th>
<th>Vector control A20 cells</th>
<th>RapGAPII-expressing A20 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Phospho-FKHR

actin reprobe

B

<table>
<thead>
<tr>
<th>Anti-IgG (min)</th>
<th>Vector control A20 cells</th>
<th>Rap2V12-expressing A20 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Phospho-FKHR

actin reprobe
Figure 8
Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAP kinase activation

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