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B Cell Receptor Signaling Down-Regulates Forkhead Box Transcription Factor Class O 1 mRNA Expression via Phosphatidylinositol 3-Kinase and Bruton's Tyrosine Kinase[1]

Rochelle M. Hinman, Jessica N. Bushanam, Whitney A. Nichols, and Anne B. Satterthwaite[2]

BCR cross-linking promotes mature B cell proliferation and survival. PI3K-mediated down-regulation of proapoptotic and antimitogenic genes such as forkhead box transcription factor class O 1 (FOXO1) is an important component of this process. Previously, BCR-induced phosphorylation of FOXO1 was shown to lead to a block in nuclear localization and subsequent protein degradation. We demonstrate that the BCR also signals through PI3K to down-regulate FOXO1 mRNA expression. Bruton's tyrosine kinase (Btk), a downstream effector of PI3K, signals through B cell linker protein (BLNK) and phospholipase C (PLC)γ2 to mediate B cell proliferation and survival in response to BCR cross-linking. BCR-induced down-regulation of FOXO1 mRNA was impaired in murine knockouts of Btk, BLNK, and PLCγ2. Because B cells in these models are predominantly immature, experiments were also performed using mature B cells expressing low levels of Btk and BLNK. Similar results were obtained. Inhibitors of downstream components of the Btk/BLNK/PLCγ2 pathway were used to define the mechanism by which Btk signaling inhibits FOXO1 expression. The protein kinase Cβ inhibitor Gö6850 had minimal effects on BCR-mediated FOXO1 mRNA down-regulation. However, cyclosporin A, an inhibitor of the Ca^{2+}-dependent phosphatase calcineurin, had similar effects on FOXO1 mRNA expression as the PI3K inhibitor LY294002. Neither Btk deficiency nor cyclosporin A prevented FOXO1 protein phosphorylation, indicating that PI3K down-regulates FOXO1 via two independent pathways. We show that the Btk/BLNK/PLCγ2 pathway mediates BCR-induced changes in expression of the FOXO1 target gene cyclin G2. These observations support the hypothesis that Btk mediates BCR-induced proliferation and survival in part via inhibition of FOXO expression. ***The Journal of Immunology,*** **2007, 178: 740–747.**

The forkhead box family of transcription factors, including forkhead box transcription factor class O 1 (FOXO1),[3] FOXO3a, and FOXO4, has been identified in species ranging from yeast to humans (1). FOXO transcription factors participate in a diverse range of physiological processes, including the inhibition of cellular proliferation and the promotion of apoptosis and metabolism (2–4). Although a basic understanding of the mechanisms regulating the subcellular localization and transcriptional activity of these proteins has been achieved, much remains to be learned about the cell-specific effects of FOXOs, as well as their control at the mRNA level.

A role for FOXO transcription factors in the maintenance of cellular quiescence was first suggested by studies of the *Caenorhabditis elegans* homologue DAF-16. Mutations in this gene disrupt the worm's ability to enter a so-called Daur or nonaging larval stage upon adverse environmental conditions. The worm's resistance to both oxidative stress and UV is also noticeably reduced (5, 6). Additional studies in mammals emphasize the role of FOXO transcription factors in cell cycle control. In humans, these factors were initially isolated at chromosomal breakpoints present in tumors such as rhabdomyosarcomas (7) and leukemias (8). The expression of constitutively active forms of FOXOs in a variety of cell types has been shown to lead to either cell cycle arrest or apoptosis (9–11).

In order for cells to undergo the cell cycle and survive, they must eliminate FOXO transcription factors present in the nucleus (12). It has been established that both DAF-16 and the FOXO proteins are posttranslationally controlled via Akt (also known as protein kinase B) in the PI3K signaling pathway. Phosphorylation of FOXO proteins by Akt in response to cytokines and growth factors such as insulin and insulin-like growth factor-1 results in their exclusion from the nucleus and their subsequent degradation (13–18). Although this means of posttranslational control for the FOXO family has been well defined, other levels of regulation, such as mRNA expression, remain largely unexplored.

Understanding the mechanisms controlling FOXO expression and function is particularly important in the case of the immune system, in which the maintenance of homeostasis is highly regulated. The number of T and B cells that enter the periphery is only a fraction of the total that is initially generated. Cells that do reach the periphery must be kept in a quiescent state, only to divide and

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[3] Abbreviations used in this paper: FOXO1, forkhead box transcription factor class O 1; BLNK, B cell linker protein; Btk, Bruton's tyrosine kinase; CsA, cyclosporin A; Ct, comparative threshold cycle; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PKC, protein kinase C; PLC, phospholipase C; Q-PCR, quantitative real-time PCR.

differentiate if/when they encounter their specific Ag. Programmed cell death is important both in eliminating B and T lymphocytes with nonfunctional or autoreactive Ag receptors and in terminating immune responses. Imbalances in cellular homeostasis within the immune system have been linked to immunodeficiency, cancer, and autoimmunity.

When mature resting B cells do encounter their cognate Ag, the BCR is engaged. As a result, the cells enter and progress through the cell cycle, receiving a variety of survival signals. Each of these processes depends on PI3K (19). A major downstream target of PI3K signaling in BCR-stimulated B cells is Akt (20). Yusuf et al. (21) have shown recently that BCR cross-linking leads to PI3K-dependent phosphorylation of FOXO1, a substrate of Akt (13–18), and subsequent nuclear exclusion of the FOXO1 protein. Overexpression of either constitutively active FOXO1 or FOXO3a protein in activated primary B cells causes cell cycle arrest and apoptosis (21). Taken together, these results suggest that PI3K-mediated down-regulation of FOXO family members is important for the activation of resting B cells by Ag.

A second major pathway emanating from PI3K is mediated by Bruton's tyrosine kinase (Btk) (20, 22, 23). Btk is a Tec family kinase that signals through the adaptor protein B cell linker protein (BLNK) and phospholipase C (PLC)γ2 to mediate BCR-induced Ca^{2+} flux and activation of protein kinase C (PKC)β (23–25). Btk is required for up-regulation of cell cycle regulators and antiapoptotic genes in response to BCR cross-linking (26–29). In this study, we demonstrate that BCR stimulation down-regulates FOXO1 mRNA via the PI3K/Btk/BLNK/PLCγ2 pathway. These observations reveal a novel mechanism for FOXO1 mRNA regulation in B cells.

Materials and Methods

Mice

Animals were housed and studied in accordance with protocols approved by the institutional animal care and use committee. *Btk*^{low} mice (mice expressing 25% of endogenous Btk levels in B cells) are *Btk*$^{-/-}$ mice (26) carrying a wild-type *Btk* transgene driven by the Ig H chain promoter and enhancer (30). *BLNK*$^{+/-}$ (31) mice were mated to *Btk*^{low} mice to generate progeny of the appropriate genotypes, as described in Whyburn et al. (27). Because all mice, including *BLNK*$^{-/-}$ (31) and *PLC*γ*2*$^{-/-}$ (32), were of mixed genetic background (C57BL/6 $\times$ 129), littermates were compared directly when possible and experiments were repeated with multiple litters. Mice were genotyped by PCR.

B cell purification

Splenocytes were depleted of RBC by 5-min incubation in 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA. Splenic B cells were then purified by negative selection with anti-CD43 magnetic beads using the Miltenyi Biotec MidiMACS system, according to the manufacturer's instructions. The purified cells were typically >90% B220$^+$ as measured by flow cytometry.

B cell stimulation

Purified B cells were harvested at time 0, incubated in medium alone, or stimulated with 10 μg/ml goat anti-mouse IgM F(ab′)$_2$ (Jackson ImmunoResearch Laboratories) for either 1, 6, 12, or 16 h. For inhibitor studies, cells were pretreated for 15 min with LY294002 (10 μM), U0126 (10 μM), Gö6850 (50 μM), or cyclosporin A (CsA; 100 ng/ml) (Calbiochem) before stimulation.

Quantitative real-time PCR (Q-PCR)/semiquantitative PCR

Total RNA was prepared using the RNeasy kit (Qiagen). cDNA was generated with a cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed in an Applied Biosystems 7300 Real Time PCR System using TaqMan reagents specific for mouse FOXO1, FOXO3a, FOXO4, cyclin G2, cyclin D2, and the internal control GAPDH (Applied Biosystems). Data were normalized to GAPDH using the delta comparative threshold cycle (Ct) method. Semiquantitative PCR was performed for 26–30 cycles (22 s at 95°C, 22 s at 59°C, 45 s at 72°C) using the following primers: FOXO1 forward, 5′-AAG AGC GTG CCC TAC TTC AAG GAT-3′; FOXO1 reverse, 5′-ATT TCA GAC AGA CTG GGC AGC GTA-3′; actin forward, 5′-GAG GCC CAG AGC AAG AGA G-3′; actin reverse, 5′-GTC ATC TTT TCA CGG TTG G-3′.

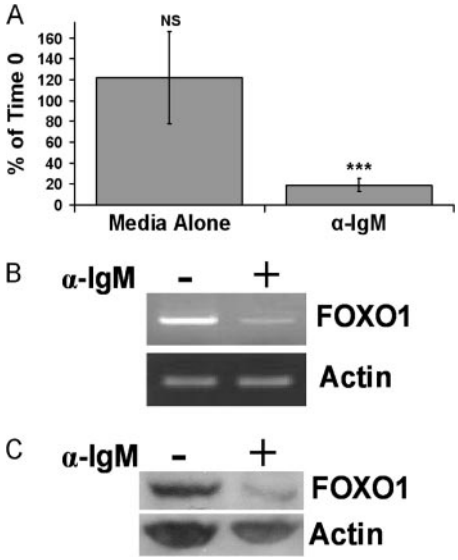

FIGURE 1. BCR signaling down-regulates FOXO1 mRNA expression through PI3K. *A*, Purified wild-type B cells were either harvested immediately, incubated for 6 h in medium alone, or stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$. Independent RNA samples derived from at least three separate B cell preparations were analyzed by Q-PCR for expression of FOXO1. FOXO1 expression levels were normalized to GAPDH using the delta Ct method and plotted as an average ($\pm$SD) percentage of the expression level in fresh unstimulated B cells (time 0). To determine significance, p values in relation to time 0 were calculated using a Student's t test and set to the following scale: NS $>$ 0.05, 0.05 $>$ * $>$ 0.005, 0.005 $>$ ** $>$ 0.0005, *** $<$ 0.0005. *B*, RNA samples derived from unstimulated B cells or B cells stimulated for 12 h with 10 μg/ml anti-IgM F(ab′)$_2$ were analyzed by semiquantitative PCR for expression of FOXO1. Actin was used to determine equal loading. The gel is representative of three independent experiments, each performed at multiple cycles to ensure linearity. *C*, Whole cell lysates from unstimulated B cells or B cells stimulated for 16 h with 10 μg/ml anti-IgM F(ab′)$_2$ were resolved by SDS-PAGE and immunoblotted for total FOXO1 protein. The blot was probed with actin to determine equal loading and is representative of three separate trials.

Immunoblots

Cells were lysed by boiling in SDS sample buffer for 10 min. Total cell extracts were electrophoresed by 8 or 10% SDS-PAGE gels and blotted to nitrocellose. Blots were blocked in 5% BSA in 10 mM Tris (pH 7.5) and 150 mM NaCl, and subsequently probed with anti-phospho-FOXO1 (Ser^{256}), anti-FOXO1, anti-phospho-Akt (Ser^{473}), anti-Akt (Cell Signaling Technology), or anti-actin (Sigma-Aldrich) diluted in 10 mM Tris (pH 7.5), 250 mM NaCl, 0.05% Tween 20, and 0.2% sodium azide. Blots were washed in 10 mM Tris (pH 7.5), 500 mM NaCl, and 0.05% Tween 20, and then incubated with HRP-conjugated goat anti-rabbit Ig (anti-phospho-FOXO1 (Ser^{256}), anti-FOXO1, anti-phospho-Akt (Ser^{473}), and anti-Akt) (Bio-Rad) or goat anti-mouse Ig (actin) (Bio-Rad) diluted in 10 mM Tris (pH 7.5), 250 mM NaCl, and 0.05% Tween 20. Blots were washed in 10 mM Tris (pH 7.5), 500 mM NaCl, and 0.05% Tween 20, and HRP was subsequently visualized using an ECL kit (Amersham). The program ImageJ was used to analyze relative band intensities.

Results

It has been demonstrated recently that stimulation of B lymphocytes through the BCR triggers PI3K-dependent phosphorylation and nuclear exclusion of the FOXO1 protein (21). We have extended these findings to show that BCR signaling also regulates FOXO1 at the level of mRNA expression. Q-PCR showed a ~80%

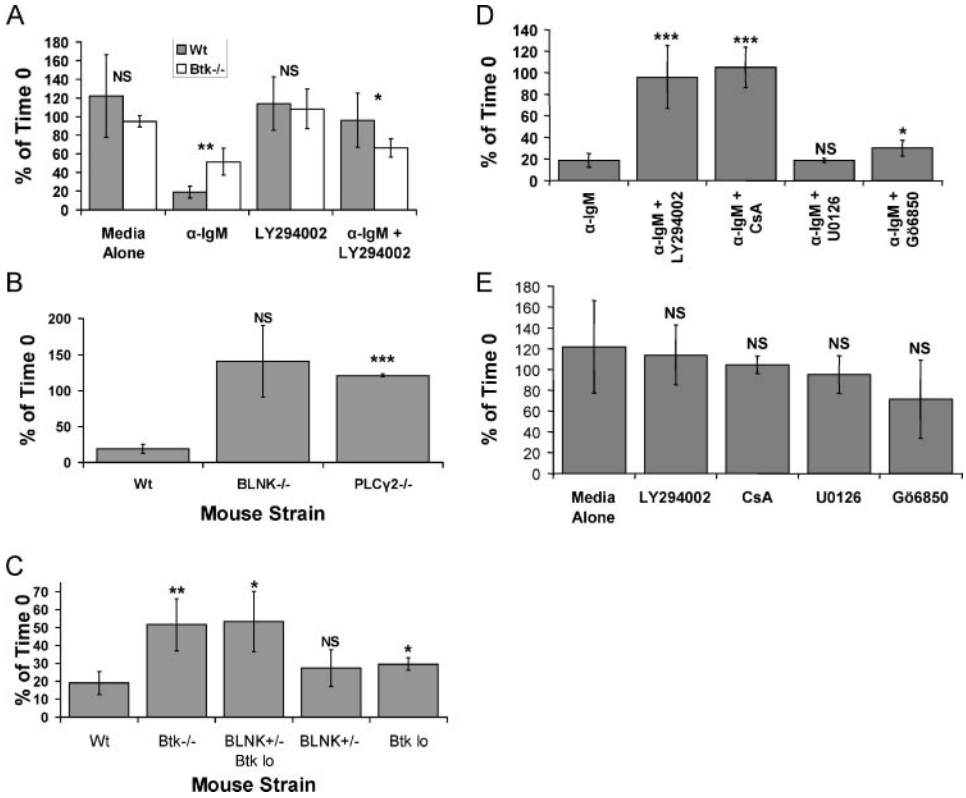

FIGURE 2. BCR-induced down-regulation of FOXO1 mRNA is mediated by Btk. *A*, Purified B cells of the indicated mouse strain were either harvested immediately (time 0) or incubated for 6 h in the presence and/or absence of 10 μg/ml anti-IgM F(ab′)$_2$ and the PI3K inhibitor LY294002 (10 μM). *B* and *C*, Purified B cells of the indicated mouse strain were either harvested immediately or stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$. *D*, Purified wild-type B cells were stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$ in the presence or absence of LY294002 (10 μM), the calcineurin inhibitor CsA (100 ng/ml), the PKCβ inhibitor Gö6850 (50 μM), or the Erk inhibitor U0126 (10 μM). *E*, Purified wild-type B cells were incubated for 6 h with each inhibitor alone. *A–E*, Independent RNA samples derived from at least two to three separate B cell preparations were analyzed by Q-PCR for expression of FOXO1. FOXO1 expression levels were normalized to GAPDH using the δ Ct method and plotted as an average ($\pm$SD) percentage of the expression level in fresh unstimulated B cells of the corresponding genotype (time 0). To determine significance, *p* values in relation to either the corresponding wild-type sample (*A*), anti-IgM-stimulated wild-type cells (*B–D*), or wild-type cells incubated in medium alone (*E*) were calculated using a Student's *t* test and set to the following scale: NS $>$ 0.05, 0.05 $>$ * $>$ 0.005, 0.005 $>$ ** $>$ 0.0005, *** $<$ 0.0005.

decrease in the levels of FOXO1 mRNA transcript upon stimulation of purified wild-type splenic B cells with anti-IgM for 6 h (Fig. 1*A*). This effect was maintained for at least 12 h poststimulation (Fig. 1*B*). This decrease was not observed in cells pretreated with the PI3K inhibitor LY294002 (Fig. 2, *A* and *D*), indicating a previously unidentified role for PI3K in the control of FOXO1 at the transcriptional level in addition to its known posttranslational effects. Our results were confirmed by microarray data generated through the Alliance for Cellular Signaling showing that stimulation of primary murine B cells with anti-IgM for 4 h induces down-regulation of FOXO1 mRNA (33). Consistent with the change in mRNA levels, total FOXO1 protein was observed to decrease 16 h poststimulation with anti-IgM (Fig. 1*C*). Culture in medium alone also resulted in some decrease in FOXO1 protein levels (data not shown), most likely due to the ability of growth factors present in serum to induce the phosphorylation and degradation of FOXO1 protein (34). However, the down-regulation of FOXO1 mRNA was specific for the BCR-induced signals and was not observed in the presence of medium alone (Fig. 1*A*).

The Tec family kinase Btk acts downstream of PI3K (20, 22, 23) and is required for proliferation and survival of B cells in response to BCR cross-linking (26–29). Previous research has suggested a connection between Btk and FOXO1. In quiescent cells, cyclin D2 expression is blocked by FOXO1 (35), whereas Btk is known to promote cyclin D2 expression in response to BCR cross-linking (27, 28, 36). We therefore asked whether BCR-mediated down-regulation of FOXO1 may be mediated by Btk. Although wild-type B cells exhibited a 5-fold average decrease in FOXO1 RNA levels upon BCR stimulation, $<$2-fold reduction was displayed in $Btk^{-/-}$ B cells, as measured by Q-PCR (Fig. 2*A*).

Btk has been shown to activate PLCγ2 both directly, by phosphorylating it (37–39), and indirectly, by increasing the local concentration of its substrate via an interaction with phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (40). Direct activation of PLCγ2 by Btk is facilitated by the adaptor protein BLNK (24). We therefore asked whether BCR-induced down-regulation of FOXO1 was mediated by BLNK and/or PLCγ2 by examining B cells from $BLNK^{-/-}$ and $PLC\gamma2^{-/-}$ mice. Both failed to down-regulate FOXO1 mRNA in response to anti-IgM treatment (Fig. 2*B*).

$Btk^{-/-}$, $BLNK^{-/-}$, and $PLC\gamma2^{-/-}$ B cells are immature relative to wild-type B cells (26), and immature B cells are known to respond differently than mature B cells to BCR cross-linking (41, 42). As such, we expanded our studies to include a mouse model in which signaling through the Btk/BLNK pathway is prevented, but B cells remain phenotypically mature. $BLNK^{+/-}Btk^{low}$ mice express low levels of both Btk and BLNK (27). It has been established that a transgene expressing 25% of endogenous Btk levels restores the development of normal numbers of mature B cells when crossed to $Btk^{-/-}$ mice. These cells, however, remain functionally impaired and have reduced, although measurable, response to BCR cross-linking

(30). Haploinsufficiency of *BLNK* further exacerbates this defect without affecting B cell development (27). BCR-stimulated down-regulation of FOXO1 mRNA was similarly impaired in $BLNK^{+/-}Btk^{low}$ and $Btk^{-/-}$ B cells (Fig. 2*C*). Further examination of B cells expressing reduced dosage of BLNK ($BLNK^{+/-}$) or Btk (Btk^{low}) alone suggests that BCR-mediated inhibition of FOXO1 mRNA expression could result from an additive effect of Btk and BLNK (Fig. 2*C*). Thus, the observed effect in $Btk^{-/-}$, $BLNK^{-/-}$, and $PLC\gamma2^{-/-}$ B cells is due to impaired signaling through the Btk/BLNK/PLCγ2 pathway rather than the relative immaturity of the cells.

Once activated via the Btk/BLNK complex, PLCγ2 cleaves phosphatidylinositol-4,5-bis-phosphate to produce 1,2-diacylglycerol and inositol 1,4,5-triphosphate. The 1,2-diacylglycerol and inositol 1,4,5-triphosphate activate PKCβ and initiate Ca^{2+} influx, respectively (23–25). To better define the mechanisms by which Btk signaling down-regulates FOXO1 mRNA expression, we stimulated wild-type cells through the BCR alone or in the presence of inhibitors of downstream components of the Btk/BLNK/PLCγ2 pathway. The PKCβ inhibitor Gö6850 had minimal effects on BCR-regulated FOXO1 down-regulation. These effects alone could not account for observations made using either the $Btk^{-/-}$ or the $BLNK^{+/-}Btk^{low}$ model. However, the effects of CsA, an inhibitor of the Ca^{2+}-dependent phosphatase calcineurin, were roughly equivalent to that seen with the PI3K inhibitor, LY294002 (Fig. 2*D*), suggesting that this branch of the BCR signaling cascade may have a more prominent role in the BCR-induced down-regulation of FOXO1.

PI3K has also been shown to mediate BCR-induced activation of Erk (43). To determine whether this pathway contributes to the down-regulation of FOXO1 mRNA expression, B cells were treated with the Erk inhibitor U0126 before incubation with anti-IgM. These cells showed normal levels of BCR-stimulated FOXO1 down-regulation (Fig. 2*D*). Q-PCR of the cyclin D2 gene, whose expression depends on Erk activity (44), confirmed that the inhibitor was effective (data not shown). Thus, the control of FOXO1 expression by BCR engagement is independent of Erk, but dependent on PI3K, Btk, BLNK, PLCγ2, and calcineurin. This observation is consistent with previous work from our lab and others demonstrating that BCR-induced Erk activation is unaffected in $Btk^{-/-}$, $BLNK^{-/-}$, and $BLNK^{+/-}Btk^{low}$ B cells (27, 45, 46).

It has been established that BCR stimulation induces PI3K-dependent phosphorylation and nuclear exclusion of the FOXO1 protein (21). To determine the contribution of Btk to this aspect of FOXO1 regulation, we first assessed Akt activation in $Btk^{-/-}$ B cells. Consistent with previous studies in $Btk^{-/-}$ (45, 47) and $BLNK^{-/-}$ (46) B cells, BCR-induced phosphorylation of Akt was unimpaired in the absence of Btk in our hands (Fig. 3*A*). Phosphorylation of FOXO1 at Ser^{256}, an Akt consensus site, was then examined. As expected based on normal Akt activation, a similar 2- to 3-fold increase in FOXO1 phosphorylation was observed in wild-type and $Btk^{-/-}$ B cells (Fig. 3, *B–D*). The PI3K inhibitor Ly294002 blocked phosphorylation of both Akt and FOXO1 regardless of the presence of Btk (Fig. 3, *A–C*). Taken together, these observations indicate that FOXO1 protein phosphorylation and mRNA expression are most likely controlled by distinct signaling pathways stemming from PI3K. This finding was further supported through the use of the calcineurin inhibitor, CsA. Although CsA was shown to block the down-regulation of FOXO1 mRNA (Fig. 2*D*), it had no appreciable effect on protein phosphorylation (Fig. 3*B*).

To determine the importance of the Btk-dependent pathway for FOXO down-regulation, we asked whether this pathway also controls expression of cyclin G2, an antimitogenic gene that inhibits cell cycle progression and contributes to the maintenance of the quiescent state of differentiated cells (48). Cyclin G2 has been shown to be a direct target of FOXO proteins (49), and recent studies have indicated that overexpression of FOXO1 promotes cyclin G2 expression in B lineage cells (50). It has also been shown to be down-regulated by BCR cross-linking via PI3K (21). Our research indicates that cyclin G2 and FOXO1 show similar responses to inhibition of the Btk pathway (Fig. 4). This observation, taken in concert with data that the forced expression of a PI3K-independent variant of FOXO1 in activated B cells induces cell cycle arrest and increased apoptosis (21), suggests that this newly described means of BCR-PI3K-FOXO1 regulation at the level of mRNA expression is functionally relevant.

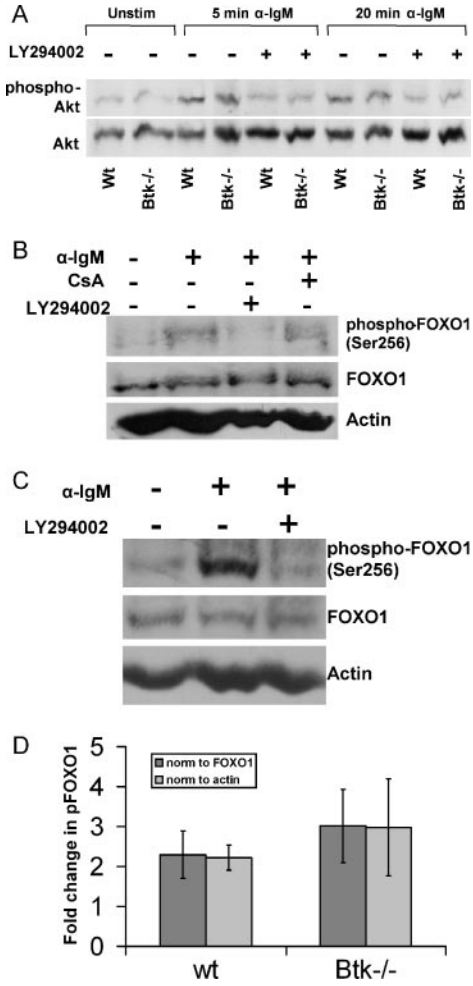

FIGURE 3. BCR stimulation induces PI3K-dependent phosphorylation of the FOXO1 protein independent of Btk. *A*, Purified B cells of the indicated mouse strains were stimulated with 10 μg/ml anti-IgM $F(ab')_2$ in the presence or absence of the PI3K inhibitor LY294002 (10 μM) for the indicated times. Whole cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-Akt (Ser^{473}) and total Akt. Blots were also probed for actin to determine equal loading. *B*, Purified wild-type B cells were stimulated for 1 h with 10 μg/ml anti-IgM $F(ab')_2$ in the presence or absence of LY294002 (10 μM) or the calcineurin inhibitor CsA (100 ng/ml). *C*, Purified $Btk^{-/-}$ cells were stimulated for 1 h with 10 μg/ml anti-IgM $F(ab')_2$ in the presence or absence of the PI3K inhibitor LY294002 (10 μM). Whole cell lysates from *B* and *C* were resolved by SDS-PAGE and immunoblotted for phospho-FOXO1 (Ser^{256}) and total FOXO1. Blots were also probed for actin to determine equal loading. Blots shown are representative of three separate trials. *D*, ImageJ was used to determine relative band intensities. The fold change in pFOXO1 upon anti-IgM stimulation was calculated for each mouse strain after normalizing to either total FOXO1 or actin as loading controls. Data are presented as mean ± SD, $n = 3$ (wild type) or 4 ($Btk^{-/-}$).

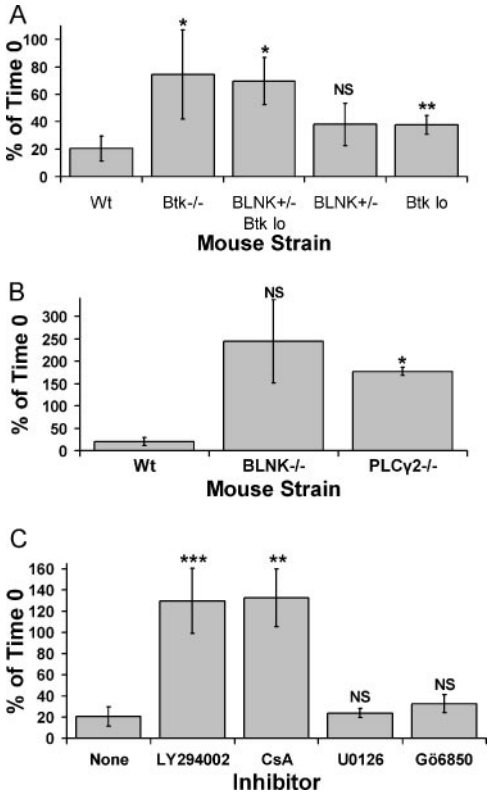

FIGURE 4. Cyclin G2 and FOXO1 mRNA levels show similar responses to inhibition of the Btk pathway. *A* and *B*, Purified B cells of the indicated mouse strains were harvested immediately (time 0) or stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$. *C*, Purified wild-type B cells were harvested immediately (time 0) or stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$ alone or in the presence of the PI3K inhibitor Ly294002 (10 μM), the calcineurin inhibitor CsA (100 ng/ml), the PKCβ inhibitor Gö6850 (50 μM), or the Erk inhibitor U0126 (10 μM). *A–C*, Independent RNA samples derived from at least three separate B cell preparations were analyzed by Q-PCR for expression of cyclin G2. Cyclin G$_2$ expression levels were normalized to GAPDH using the delta Ct method and plotted as an average ($\pm$SD) percentage of the expression level in fresh unstimulated B cells of the corresponding genotype (time 0). To determine significance, *p* values in relation to anti-IgM-stimulated wild-type cells were calculated using a Student's *t* test and set to the following scale: NS $>$ 0.05, 0.05 $>$ $*$ $>$ 0.005, 0.005 $>$ $**$ $>$ 0.0005, $***$ $<$ 0.0005.

Although the murine homozygous knockout of *FOXO1* is embryonic lethal (51, 52), the knockout of *FOXO3a*, a related FOXO family member, is viable (52, 53). These mice display, among other characteristics, increased lymphoproliferation (53). One might assume that the disparity between the *FOXO1* and *FOXO3a* knockouts indicates that these proteins, in general, have unique functions (52). However, the observation that cyclin G2 transcription in B cells is also enhanced by FOXO3a (50) prompted us to ask whether FOXO3a mRNA expression was down-regulated by BCR cross-linking in a manner similar to FOXO1. Q-PCR indicated a ~3-fold reduction in FOXO3a mRNA levels following 6 h of anti-IgM stimulation (Fig. 5*A*). This was completely inhibited by the PI3K inhibitor LY294002. Likewise, FOXO4 mRNA levels were also observed to be down-regulated via PI3K in anti-IgM-stimulated B cells, as measured by Q-PCR (Fig. 5*B*). Thus, one might hypothesize that the coincident decrease in expression of multiple FOXO family members in response to BCR cross-linking promotes the proliferation and survival of activated B cells.

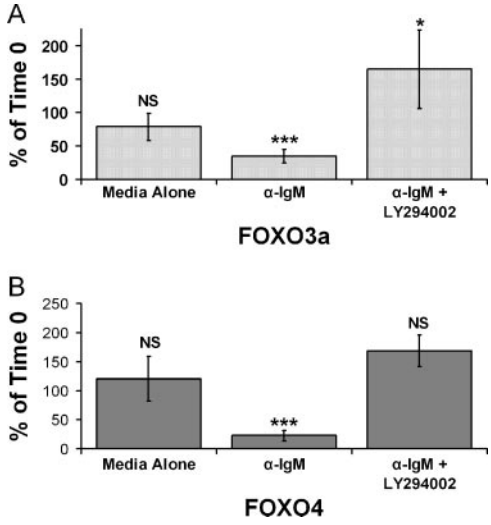

FIGURE 5. BCR signaling also down-regulates FOXO3a and FOXO4 mRNA expression through PI3K. Purified wild-type B cells were either harvested immediately, incubated for 6 h in medium alone, or stimulated for 6 h with 10 μg/ml anti-IgM F(ab′)$_2$ in the presence or absence of the PI3K inhibitor LY294002 (10 μM). Independent RNA samples derived from at least three separate B cell preparations were analyzed by Q-PCR for expression of FOXO3a (*A*) or FOXO4 (*B*). Expression levels were normalized to GAPDH using the delta Ct method and plotted as an average ($\pm$SD) percentage of the expression level in fresh unstimulated B cells (time 0). To determine significance, *p* values in relation to time 0 were calculated using a Student's *t* test and set to the following scale: NS $>$ 0.05, 0.05 $>$ $*$ $>$ 0.005, 0.005 $>$ $**$ $>$ 0.0005, $***$ $<$ 0.0005.

Discussion

It has been shown in numerous cell types, including B cells, that activation of PI3K leads to phosphorylation and nuclear exclusion/degradation of the FOXO1 protein (13–18, 21). In this study, we describe another level of FOXO1 regulation. BCR signaling down-regulates FOXO1 mRNA via the PI3K/Btk/BLNK/PLCγ2 pathway. The discovery of this additional means of control emphasizes the importance of FOXO transcription factors in the maintenance of immune cell homeostasis.

Our studies suggest that control of FOXO1 at the mRNA level may take place via a separate signaling pathway from that established for the protein (Fig. 6). Both pathways share PI3K, as demonstrated by Western and Q-PCR experiments using the PI3K inhibitor, LY294002. Akt appears largely responsible for the posttranslational control of FOXO1 (13–18, 21), whereas the Btk/BLNK/PLCγ2 pathway down-regulates mRNA expression. Our observations are consistent with previous studies of primary B cells showing BCR-stimulated activation of Akt to be independent of Btk and BLNK (45–47). Westerns comparing *Btk*$^{-/-}$ and wild-type cells showed no significant difference in FOXO1 phosphorylation following BCR stimulation. Inhibition of calcineurin with CsA also blocked BCR-induced down-regulation of FOXO1 mRNA, but did not affect protein phosphorylation. Whether calcineurin exerts its effects on FOXO1 mRNA downstream of Btk via PLCγ2 and Ca^{2+}, downstream of PI3K independent of Btk (see below), or both, these results strongly support the idea that FOXO1 mRNA expression and protein phosphorylation are regulated differently.

It is important to note that our work does not rule out other roles for Btk in the posttranslational control of FOXO1. Multiple sites for both phosphorylation and acetylation have been identified within the FOXO1 protein (54). The Ab used in this study is specific for Ser^{256}, a site phosphorylated by Akt (55). Btk may

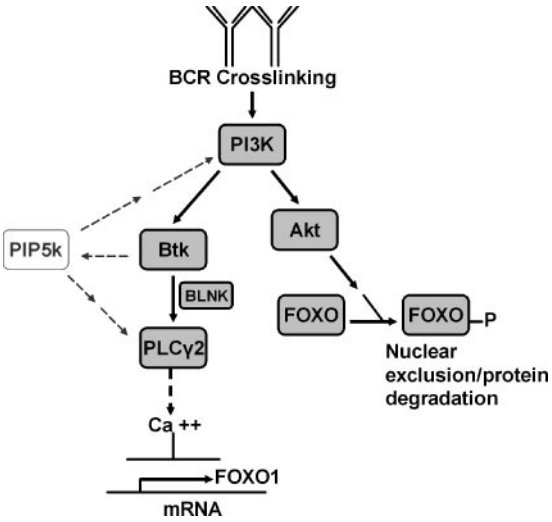

FIGURE 6. Model for the regulation of FOXO1 by PI3K in BCR-stimulated B cells. Following BCR stimulation, PI3K/Akt controls initial FOXO1 protein degradation, whereas PI3K/Btk/BLNK/PLCγ2 later down-regulates mRNA expression to prevent generation of additional FOXO1.

regulate phosphorylation at other sites that would not be detected with this reagent. IκB kinase, which is downstream of Btk, is known to phosphorylate FOXO3a at Ser^{644} (56). Although this residue is not conserved in FOXO1, it is possible that there are additional IκB kinase-dependent phosphorylation sites in FOXO1. Possible interactions with secondary molecules such as the 14-3-3 proteins that bind to phosphorylated FOXO protein and sequester it to the cytosol also remain to be investigated (57).

LY294002 had a more profound effect on FOXO1 mRNA down-regulation than did Btk deficiency. Although much less effective than in wild-type cells, LY294002 did cause a slight increase in FOXO1 mRNA levels in $Btk^{-/-}$ cells stimulated with anti-IgM (Fig. 2*A*). This indicates that PI3K most likely has some Btk-independent contribution to the down-regulation of FOXO1 mRNA. This is not via the Erk pathway, as the Erk inhibitor U0126 had no influence on BCR-induced FOXO1 mRNA down-regulation. The finding that inhibitors of PI3K and calcineurin have similar effects on FOXO1 mRNA levels suggests that this alternative pathway lies between these two molecules. This Btk-independent process could involve other Tec family kinases redundant with Btk for control of Ca^{2+} flux (Ca^{2+} mobilization is reduced, but not completely prevented, in B cells from $Btk^{-/-}$ or $BLNK^{+/-}Btk^{low}$ mice (27, 45)) or a unique means of calcineurin activation via PI3K.

Another explanation for the apparently greater requirement for PI3K than Btk in mediating FOXO1 mRNA down-regulation is that Btk is acting upstream, rather than downstream, of PI3K. In addition to phosphorylating and activating PLCγ2, Btk can act in a kinase-independent manner to recruit PIP5K to the membrane, thus increasing the local production of phosphatidylinositol-4,5-bis-phosphate, the substrate for both PI3K and PLCγ2 (40) (Fig. 6). In the absence of Btk, decreased substrate availability for PI3K would be predicted to reduce, but not completely inhibit, the ability of PI3K to down-regulate FOXO1 expression. However, in primary B cell cultures, neither we (Fig. 3*A*) nor others (45, 47) have observed a role for Btk in Akt phosphorylation or activation in response to BCR cross-linking. Our work also indicates that Btk does not regulate phosphorylation of FOXO1 protein at Akt consensus sites (Fig. 3, *C* and *D*). Thus, if Btk is acting upstream of PI3K to regulate FOXO mRNA expression, it is most likely selectively affecting an Akt-independent function of PI3K.

The Btk/PIP5K interaction may also contribute to FOXO1 mRNA down-regulation by providing substrate for PLCγ2 (40) (Fig. 6). This process is independent of the kinase activity of Btk, but dependent on the ability of the Btk pleckstrin homology domain to bind the product of PI3K. Such a model would place Btk downstream of PI3K and account for the observation made in one study that BCR-induced stimulation of Btk kinase activity was independent of PI3K (47).

Regardless of the relative order of PI3K and Btk in this pathway, the current findings suggest a model whereby PI3K-Akt controls the initial protein degradation, whereas PI3K, Btk, BLNK, PLCγ2, and calcineurin later down-regulate mRNA expression to prevent generation of additional FOXO1 (Fig. 6). Our work has shown that FOXO1 protein is phosphorylated 1 h poststimulation, whereas FOXO1 mRNA levels are unchanged at this early time point (data not shown). The work of Donahue and Fruman (19) supports this idea. This study demonstrated that sustained BCR signaling through PI3K is required for proliferation and survival of B cells. LY294002 and CsA were effective at blocking the activation of B cells at late time points in which Akt activation was weak and not PI3K dependent (19). This suggests that newly generated FOXO1 protein may be inefficiently phosphorylated by Akt and degraded at late times post-BCR stimulation, necessitating the newly described PI3K/Btk/calcineurin-dependent mechanism for inhibiting further expression of FOXO1 mRNA.

Our proposed model emphasizes the importance of FOXO1 in the control of cellular quiescence. The lack of B cell proliferation and survival in the $Btk^{-/-}$, $BLNK^{-/-}$, $PLC\gamma2^{-/-}$, and $BLNK^{+/-}$ Btk^{low} models aligns with that observed in activated B cells retrovirally infected with FOXO1 (21, 27, 31, 32). Given that FOXO1 knockouts are embryonic lethal (51, 52), this study lends credence to the need for development of a B cell-specific conditional knockout or inducible transgenic system. Such a system would allow for investigation of the importance of FOXO1 control for B cell development and function both in vivo and in vitro.

Initial examination of FOXO3a and FOXO4, two other FOXO family members, indicates similar control at the mRNA level through PI3K. However, whether all FOXO family members are affected by the same downstream components of the BCR/PI3K signaling cascade remains to be explored. Given the observation that cyclin G2 transcription in B cells is enhanced by both FOXO1 and FOXO3a (50), redundancy between these two molecules and FOXO4 could have important consequences in future research.

Our findings are also potentially intriguing in relation to other cell types. In the literature, control of FOXO1 at the mRNA level has been largely unexplored. Our model may be B cell specific, or this signaling pathway could control cell cycle progression in other cell types, particularly those of the immune system. Fabre et al. (58) have demonstrated recently that in T cells contacting APCs there is a sustained activation of PI3K, resulting in the sequestration of FOXO transcription factors outside the nucleus in a manner that permits cell growth. Is FOXO1 expression also controlled at the mRNA level in these cells, and, if so, what are the global consequences of the deregulation of this pathway? Continuing study of the factors controlling mRNA expression of the FOXO family will further our understanding of immune homeostasis and potentially illuminate novel therapeutic targets for immune cell malignancy, immunodeficiency, and/or autoimmune disease.

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Disclosures

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