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Reciprocal Control of Forkhead box O 3a and c-Myc via the Phosphatidylinositol 3-Kinase Pathway Coordinately Regulates p27Kip1 Levels

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B cell receptor (BCR) engagement of murine WEHI 231 immature B lymphoma cells leads sequentially to a drop in NF-κB and c-Myc, and induction of the p27Kip1 cyclin-dependent kinase inhibitor, which promotes growth arrest and apoptosis. BCR engagement was recently shown to induce a drop in phosphatidylinositol 3-kinase (PI3K)/Akt signaling, preceding the increase in p27. As induction of p27 is due to an increase in gene transcription, we investigated the role of the Forkhead box O (FOXO) transcription factor family, which has been shown to potently induce p27 promoter activity. We demonstrate that pharmacologic inhibitors of PI3K or BCR engagement lead to decreased inactive cytoplasmic levels and increased active functional nuclear FOXO3a. In contrast, inhibition of PI3K/Akt signaling decreased the levels of NF-κB and c-Myc, which has been shown to repress p27 promoter activity. To test the effects of ectopic c-Myc on endogenous p27 levels, WEHI 231 cells stably expressing c-Myc or empty vector DNA were prepared. Ectopic c-Myc blocked the induction of p27 expression upon either inhibition of PI3K or BCR engagement. Thus, p27Kip1 is coordinately regulated via two arms of a signaling pathway that are inversely controlled upon inhibition of PI3K: induction of the activator FOXO3a and down-regulation of the repressor c-Myc. The Journal of Immunology, 2004, 172: 5522–5527.

The regulation of p27 is quite complex. Post-translational regulation of protein stability is a major site of control (8). Translational efficiency of p27 mRNA can be controlled via a sequence within the 5′-untranslated region (9). Transcriptional regulation of the p27 gene has also been seen (10–12). The induction of p27 protein after BCR engagement of WEHI 231 cells is due to elevated levels of p27 mRNA and promoter activity and increased gene transcription (2, 5, 12). The three Forkhead box O (FOXO) transcription factors, mammalian orthologues of Caenorhabditis elegans daf-16, FOXO1a, FOXO3a, and FOXO4, which were previously known as FKHR, FKHR-L1, and AFX, respectively, have been shown to trans-activate the p27 promoter via binding to upstream elements. FOXO4 controlled cell cycle progression by activating p27 gene expression (13). FOXO3a induced p27 transcription and apoptosis of Ba/F3 cells (14). The activity of the FOXO proteins is controlled by phosphorylation mediated by Akt/protein kinase B activity, i.e., upon phosphorylation of Thr32, Ser253, and Ser315 by Akt, FOXO3a is inactivated by translocation to the cytoplasm via interaction with 14-3-3 (15). Inhibition of Akt leads to activation of FOXO protein, as judged by dephosphorylation and nuclear localization. Recent studies have shown that anti-IgM treatment of WEHI 231 results in a transient activation of phosphatidylinositol 3-kinase (PI3K) and Akt, followed by a drop, such that PI3K and Akt activities are well below basal values by 4–6 h (16). These observations led us to hypothesize a role for a FOXO factor in activation of the p27 promoter in WEHI 231 cells, and we report that inhibition of PI3K or anti-IgM treatment activates FOXO3a. The decline in PI3K activity also promotes a decrease in c-Myc levels via inhibition of NF-κB, and ectopic c-Myc blocks the induction of p27 levels upon anti-IgM treatment. Overall, these findings demonstrate that p27 gene expression is reciprocally regulated by FOXO3a and c-Myc, which are themselves inversely controlled by PI3K.

Materials and Methods

Cell culture conditions

WEHI 231 cells were maintained and treated with anti-μ H chain Ab at a concentration of 4 μg/ml as previously described (17). Where indicated, cells were treated with either LY294002 or wortmannin (Sigma-Aldrich, St. Louis, MO). For trypan blue exclusion analysis of viability, cells were incubated in 0.2% trypan blue (Life Technologies, Gaithersburg, MD) for 10–20 min, and the percentage of cells excluding dye (viable cells) was determined.

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Abbreviations used in this paper: CDK, cyclin-dependent kinase; BCR, B cell receptor; CKI, cyclin-dependent kinase inhibitor; FOXO, Forkhead box O; IRS, insulin-responsive sequence; Oct-1, octomer-1; PI3K, phosphatidylinositol-3-kinase.

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Stable WEHI 231 transfectants

To prepare stable WEHI 231 transfectants, cells were resuspended in culture medium supplemented with 20% FBS at a concentration of 20 × 10⁶ cells/ml. Cells (250 μl) were preincubated on ice for 10 min with pRC-e-Myc vector DNA, which expresses mouse WT e-Myc protein under control of the CMV promoter (12) or parental pCDNA3. DNA was transfected by electroporation at 240 mV and 960 μF using a GenePulsar (Bio-Rad, Hercules, CA). After incubation on ice for 5 min, the cell suspension was mixed with 1.75 ml of culture medium and incubated for 10 min at room temperature, then at 37°C. After 24 h, 1 mg/ml G418 (Life Technologies) was added to the culture medium, and selective growth conditions were maintained for ~3 wk.

Immunoblot analysis

Cell extracts were prepared, and samples (30 μg) were subjected to immunoblotting as previously described (18). FOXO3a (06-931), p27 (sc-776), and β-actin (AC-15) Abs were purchased from Upstate Biotechnology (Lake Placid, NY) Santa Cruz Biotechnology (Santa Cruz, CA), and Sigma-Aldrich, respectively. The rabbit polyclonal c-Myc Ab was a gift from S. Hann.

EMSA

Nuclear extracts were prepared as described previously (19). The sequence of the FOXO3a binding site-containing oligonucleotide from the insulin-like growth factor-binding protein IGFBP-1 gene is 5'-ATTGCTAG CAAGCAGAAAACACCGCGATGCTTA-3' (termed insulin-responsive sequence (IRS)) (15), where the core Forkhead element is underlined. The sequence of the upstream regulatory element NF-κB-containing oligonucleotide from the c-myc gene is as follows: 5'-CAAGTCCGGGTTTC CCAAACC-3', where the underlined region indicates the core binding element. The octomer-1 (Oct-1) oligonucleotide has the following sequence: 5' TGTGAGAAGATCAAATCACTAGTA-3'. Double-stranded oligonucleotides were labeled and subjected to EMSA as described previously (19).

Results

Inhibition of PI3K or anti-IgM treatment induces FOXO3a in WEHI 231 cells

We first sought to determine whether inhibition of PI3K activity leads to activation of a member of the FOXO family. WEHI 231 cells in exponential growth were treated with increasing doses of either LY294002 or wortmannin, two pharmacologic inhibitors of PI3K. FOXO3a was detected in the nuclear as well as cytoplasmic extracts of the asynchronously dividing population of WEHI 231 cells (Fig. 1A). Both inhibitors caused a dose-dependent decline in the level of FOXO3a in the cytoplasm and a commensurate increase in the level of the faster migrating, hypophosphorylated form of FOXO3a in the nucleus (Fig. 1A). No nuclear activation of the other members of the family, FOXO1a or FOXO4, could be detected (data not shown). Thus, WEHI 231 cells express the Forkhead box O family member FOXO3a, and addition of inhibitors of PI3K activity leads to its activation. To test whether inhibition of PI3K is sufficient to elevate the levels of p27 protein, WEHI 231 cells were treated with either 50 μM LY294002 or 200 nM wortmannin for 4 or 8 h. In both cases, an increase in p27 protein levels was observed at 4 h, which further increased by 8 h of treatment (Fig. 1B). Thus, p27 levels increase in WEHI 231 cells upon inhibition of PI3K with pharmacologic inhibitors.

We next asked whether BCR engagement affects FOXO3a expression (Fig. 1, C and D). Anti-IgM treatment caused an initial decrease in mobility of FOXO3a in the cytoplasm at 1–2 h, followed by a subsequent increase in mobility and a substantial decline in its level by 4–8 h (Fig. 1C), consistent with the time course of changes in PI3K/Akt activity observed previously (16). Importantly, a low level of FOXO3a was detected in the nucleus at 0 h (better seen on a darker exposure), and this level remained low until an increase was detected by 4–8 h (Fig. 1D), preceding the induction of p27 mRNA and protein levels observed previously (12). Next, to verify that the FOXO3a protein in the nucleus is functional, a time course was performed of the effects of anti-IgM treatment on binding to a Forkhead site. As probe, we selected the FOXO3a binding site from the insulin-like growth factor-binding protein IGFBP-1 gene, termed IRS (Fig. 2). Consistent with the presence of FOXO3a in the nucleus, anti-IgM treatment first decreased the binding of FOXO3a to the IRS after 1–2 h, followed by a substantial increase after 4 and 8 h of treatment (Fig. 2). (Oct-1 oligonucleotide as a probe. Alternatively, Oct-1 oligonucleotide was used as a probe to test for equal loading.

FIGURE 1. Inhibition of PI3K or BCR engagement leads to activation of FOXO3a and induction of p27 levels in WEHI 231 cells. A, WEHI 231 cells were treated with the indicated dose of LY294002 (LY) for 2 h or wortmannin (Wort) for 3 h, and cytoplasmic and nuclear extracts were subjected to immunoblot analysis for FOXO3a protein or β-actin as a loading control. *, Position of nonspecific band. B, WEHI 231 cells were treated with LY294002 or wortmannin for 0, 4, or 8 h, and nuclear extracts were subjected to immunoblot analysis for p27 or β-actin. C and D, WEHI 231 cells were treated with anti-IgM for 0, 1, 2, 4, or 8 h. Samples of cytoplasmic (C) or nuclear (D) extracts, isolated at the indicated times, were subjected to immunoblot analysis for FOXO3a protein or β-actin as a loading control. *, Position of nonspecific band.
binding indicated that the 4 h point is slightly overloaded.) The higher relative level of binding in the untreated WEHI 231 cells probably reflects differential modifications or protein-protein interactions that affect FOXO3a binding activity (see Discussion). Thus, anti-IgM treatment as well as inhibition of PI3K activate FOXO3a in WEHI 231 cells.

Inhibition of PI3K prevents NF-κB-mediated activation of c-Myc

BCR engagement of WEHI 231 cells leads to a transient induction of c-Rel/p50 NF-κB trans-activating complexes, followed by a decline, such that levels are reduced below baseline at 4 h (20). As we found that the PI3K/Akt kinases mediate induction of p65/p50 NF-κB complexes in breast cancer cells (19), we next assessed the effects of inhibition of PI3K on NF-κB binding in WEHI 231 cells. Treatment with LY294002 caused a dose-dependent drop in formation of c-Rel/p50 and p50 homodimer binding complexes (Fig. 3A, left panel). WEHI 231 cells, preincubated with LY294002, were then treated with anti-IgM. Inhibition of PI3K with a low dose of LY294002 (10 μM) prevented the normal increase in binding of NF-κB trans-activating complexes (c-Rel/p50), whereas the higher dose of 50 μM reduced NF-κB levels below baseline, comparable to levels seen after 4 h of anti-IgM treatment (Fig. 3A, right panel). Thus, inhibition of PI3K in WEHI 231 cells reduces basal NF-κB activity and blocks the early induction normally seen upon BCR engagement.

As NF-κB regulates c-Myc gene transcription in WEHI 231 cells (20), we tested the effects of PI3K inhibition on the early changes in c-Myc levels after anti-IgM treatment. WEHI 231 cells were left untreated or were pretreated with LY294002 or wortmannin, then either harvested or incubated with anti-IgM for 1 h. In the absence of PI3K inhibitor, anti-IgM treatment caused a substantial induction of c-Myc protein (Fig. 3B), as observed previously (21, 22), consistent with the increase in trans-activating NF-κB subunits. Inhibition of PI3K prevented the increase, causing a dose-dependent decrease in c-Myc even after anti-IgM treatment (Fig. 3B). Thus, the PI3K signaling pathway controls activation of c-Myc mediated by NF-κB c-Rel/p50 complexes in WEHI 231 cells.

PI3K inhibition promotes the induction of p27 and WEHI 231 cell death

Previously, we showed that induction of p27 promotes WEHI 231 cell death (5). To determine whether inhibition of PI3K would have a similar effect on WEHI 231 cell survival, cultures were treated with either 50 μM LY294002 or anti-IgM, alone or in combination, and trypan blue-positive cells were counted as a function of time (Fig. 4A). Addition of LY294002 alone effectively induced cell death, and the time course appeared somewhat more rapid than with anti-IgM treatment alone (Fig. 4A). The combination of the anti-IgM plus LY294002 resulted in the earliest and

![Figure 3](http://www.jimmunol.org/)

![Figure 4](http://www.jimmunol.org/)
most extensive induction of cell death. Consistent with the ability of p27 to induce apoptosis of these cells, the extent and time course of death correlated with the relative increases in p27 (Fig. 4B). The additive effects of LY294002 and anti-IgM probably reflect the use of a suboptimal dose of LY294002 (see Figs. 1A and 3B) or, alternatively, the ability of anti-IgM to induce a second signaling pathway.

Ectopic c-Myc ablates PI3K-mediated induction of p27 and death of WEHI 231 cells

Ectopic c-Myc represses p27 promoter activity in multiple cell types (12). To determine whether c-Myc expression can counteract the effects of PI3K inhibition on the induction of p27 and cell death, WEHI 231 cells were stably transfected with either pRC-c-Myc or empty pCDNA3 vector (WEHI 231-c-Myc and WEHI 231-pCDNA3, respectively). Cultures were treated as described above, and trypan blue-positive cells were plotted as a function of time (Fig. 5A). Ectopic c-Myc protected cells from death induced by LY294002 and anti-IgM added alone or in combination. In contrast, WEHI 231-pCDNA3 cell death profiles were identical with those obtained with parental WEHI 231 cells shown above in Fig. 4A (data not shown). We next assessed the effects of ectopic c-Myc on levels of p27 and c-Myc expression (Fig. 5B). The basal level of c-Myc was only slightly overexpressed in WEHI 231-c-Myc cells, as found previously (21), consistent with the known autoregulation of unrearranged c-Myc genes, as present in WEHI 231 cells (23; reviewed in Ref. 24). Of note, sustained c-Myc expression was found after treatment for 8 h with either anti-IgM or LY294002 alone or in combination compared with WEHI 231-pCDNA3 cells (Fig. 5B). Thus, the drop in c-Myc levels normally seen upon anti-IgM treatment is completely overridden by ectopic c-myc expression, and basal c-Myc levels are retained. Importantly, the increase in p27 level seen at the 8 h point in WEHI 231-pCDNA3 cells was almost completely blocked in WEHI 231-c-Myc cells. Thus, ectopic c-Myc expression reduces the extent of induction of p27 and resulting cell death mediated upon inhibition of PI3K or BCR engagement.

Discussion

In this study we show that a decrease in the PI3K signaling pathway upon BCR engagement of WEHI 231 cells leads reciprocally to the induction of FOXO3a and the inhibition of c-Myc. Thus, the PI3K signaling pathway coordinately and inversely controls simultaneously both the activator and the repressor of p27 Kip1 gene transcription. Overall, these findings identify PI3K as the upstream mediator of the NF-kB to c-myc pathway that controls the survival and proliferation of WEHI 231 B cells (20) (see model in Fig. 6). This signaling is presumably mediated via the PI3K downstream target Akt, which has been shown to lead to degradation of IκBα and release of NF-κB as well as to mediate phosphorylation and thereby inactivation of FOXO3a (15). Importantly, BCR engagement of mature B cells leads to sustained PI3K/Akt activity and proliferation (25) (Fig. 6A), whereas similar treatment of immature B cells (such as WEHI 231 or CH 33) leads to cell death (16, 26) (Fig. 6B). The findings presented in this study identify FOXO3a/p27 and NF-κB/c-Myc as downstream molecular targets of the

FIGURE 5. Ectopic c-Myc protects from death upon induction of p27. A, WEHI 231-c-Myc cells were treated with 50 μM LY294002 or anti-IgM alone or in combination or with DMSO as a control for 18, 24, 36, 48, or 72 h. Trypan blue-positive (dead) cells were determined, and the average value ± SD are presented. B, WEHI 231-pCDNA3 or WEHI 231-c-Myc cells were treated with either 0 or 50 μM LY294002 for 8 h or with anti-IgM alone for 1 and 8 h or were pretreated with LY294002 for 8 h, then with anti-IgM for 1 h. Nuclear extracts were subjected to analysis for c-Myc, p27, and β-actin from both cell populations on the same immunoblot.

FIGURE 6. PI3K coordinately regulates two arms of a pathway that controls the induction of p27 gene transcription. The scheme indicates the signaling cascades under conditions of elevated vs inhibited levels of PI3K signaling, as would be seen after 1–2 h vs 4–8 h of BCR engagement of WEHI 231 cells.
PI3K/Akt kinase cascade, controlling the subsequent events leading to either cell proliferation or cell death.

The ability of PI3K to activate NF-κB has been demonstrated in several cell types, including rat liver epithelial cells and NF639 breast cancer cells (19, 27). This role of PI3K in activation of NF-κB in B cells was recently shown using PI3K−/− mice (28), although involvement of the Forkhead family and p27 were not explored. Previous work had linked p27 gene expression with PI3K activity in B cells. Induction of p27 mRNA levels was shown to follow IFN-induced growth arrest and inactivation of PI3K activity and decreased phosphorylation of FOXO1α in normal human B lymphocytes (29). CD40 receptor engagement induces the PI3K/Akt pathway (16), and we have shown that in WEHI 231 cells it results in an early and sustained induction of NF-κB, which prevents the drop in c-Myc (17), and the induction of p27 levels and apoptosis of these cells (5). In this study we implicate FOXO3α as the mediator between PI3K and p27 gene expression in WEHI 231 B cells upon BCR engagement.

Surprisingly, a higher relative level of binding was seen in the EMSA with nuclear extracts of untreated WEHI 231 cells using the IRS Forkhead binding site from the insulin-like growth factor-binding protein as probe compared with the level of FOXO3α protein detected by immunoblot. We believe that these differences most likely reflect the effects of either FOXO3α protein modifications (e.g., phosphorylation or acetylation) and/or its interactions with other proteins, rather than binding of other members of the Forkhead family, for the following reasons. The IRS has been shown to bind to FOXO3α (15). Nuclear extracts from NF639 breast cancer cells, which express a low level of FOXO3α, transfected with a FOXO3α expression vector showed an increase in a band that comigrated with the complex seen with WEHI 231 nuclear extracts (data not shown). Given that the other FOXO members are all of distinct size, changes in binding of these other members should yield an additional band(s) in the gel. Furthermore, as discussed above, we were unable to detect other members of the FOXO proteins within WEHI 231 cell nuclear extracts. Lastly, there is evidence that FOXO proteins can functionally interact with other transcription factors and modifier proteins (e.g., estrogen receptor α, CREB-binding protein, and 14-3-3σ).

Many cancers are typified by aberrant activation of NF-κB (30, 31). We and others have demonstrated the ability of NF-κB to control c-myc gene expression in culture and in vivo (18, 20, 32, 33). Recent analysis has indicated that c-Myc levels are elevated in as many as one-seventh of human cancer deaths in the United States (34). The elevated level of c-Myc has been identified as a poor prognostic marker (35). A growing body of literature has demonstrated decreased levels of p27 protein in various tumor types, which has been characterized as an independent factor of poor prognosis (6, 7). Our findings suggest that suppression of p27 in more malignant cancers may result from sustained elevated PI3K/Akt signaling mediating increased NF-κB/c-Myc and decreased FOXO activity, thereby promoting neoplastic transformation (Fig. 6A). Lastly, our previous studies demonstrated the ability of c-Myc to repress p27 promoter activity, whereas additional mechanisms has been seen by other groups (36, 37). In this study ectopic c-Myc was shown to block the induction of endogenous p27 levels upon either inhibition of PI3K activity or BCR engagement. This leads us to hypothesize that c-Myc can block the induction of p27 levels mediated by FOXO3α. Experiments are in progress to test this hypothesis and to elucidate the mechanisms of c-Myc-mediated repression.

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References


