G₁ cell cycle progression and the expression of G₁ cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells

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¹Mary Babb Randolph Cancer Center, Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown 26506-9300; ²Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and ³College of Pharmacy, University of New Mexico, Albuquerque, New Mexico 87131

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G₁ cell cycle progression and the expression of G₁ cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. Am J Physiol Cell Physiol 287: C281–C291, 2004. First published March 17, 2004; 10.1152/ajpcell.00422.2003.—Ovarian cancer is one of the most common cancers among women. Recent studies demonstrated that the gene encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K) is frequently amplified in ovarian cancer cells. PI3K is involved in multiple cellular functions, including proliferation, differentiation, antiapoptosis, tumorigenesis, and angiogenesis. In this study, we demonstrate that the inhibition of PI3K activity by LY-294002 inhibited ovarian cancer cell proliferation and induced G₁ cell cycle arrest. This effect was accompanied by the decreased expression of G₁-associated proteins, including cyclin D1, cyclin-dependent kinase (CDK) 4, CDC25A, and retinoblastoma phosphorylation at Ser795, Ser780, and Ser207/208. Expression of CDK6 and β-actin was not affected by LY-294002. Expression of the cyclin kinase inhibitor p16INK4a was induced by the PI3K inhibitor, whereas steady-state levels of phosphatidylinositol 3-kinase; cyclin-dependent kinases; retinoblastoma protein

Address for reprint requests and other correspondence: B.-H. Jiang, 1820 MBR Cancer Center and Dept. of Microbiology, Immunology and Cell Biology, West Virginia Univ., Morgantown, WV 26506-9300 (E-mail: bbjiang@hsu.wvu.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the cell cycle (38, 41, 51). p14<sup>INK4a</sup> is capable of binding to CDK4, thereby inhibiting the catalytic activity of the CDK4/cyclin D1 enzymes. p21<sup>CIP1/WAF1</sup> also interferes with CDK/cyclin complexes and thereby blocks DNA replication (55).

PI3K signaling has been observed to play an important role in human ovarian cancer cells (17, 48). However, the role of PI3K in cell cycle progression in ovarian cancer cells is not well studied. Here we investigated the effect of PI3K inhibitor LY-294002 on cell proliferation and cell cycle progression in ovarian cancer cells. We examined the expression of proteins associated with the cell cycle and analyzed the downstream molecules involved in the PI3K-mediated cell cycle progression.

**MATERIALS AND METHODS**

**Reagents and cell culture.** The PI3K inhibitor, LY-294002, and mTOR inhibitor, rapamycin, were purchased from Calbiochem (San Diego, CA). Propidium iodide (PI) was from Molecular Probes (Eugene, OR). The antibodies against p16, p21, CDC25A, CDK4, CDK6, cycline D1, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibodies against Rb, phospho-Rb (Ser<sup>795</sup>), phospho-Rb (Ser<sup>780</sup>), phospho-Rb (Ser<sup>779</sup>), phospho-phospho (Ser<sup>780</sup>/795), phospho-p70S6K1 (Thr<sup>389</sup>), phospho-p70S6K1 (Thr<sup>421/Ser<sup>424</sup></sup>) were from Cell Signaling Technology (Beverly, MA). The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were from Perkin Elmer Life Sciences (Boston, MA). The human ovarian cancer cell lines OVCAR-3 and A2780/CP70 (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (GIBCO BRL, Grand Island, NY), supplemented with 10% FBS, 2 mM L-glutamine, and 0.2% insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cultured at 37°C in 5% CO<sub>2</sub> incubator. Trypsin-0.25%/EDTA solution was used to detach the cells from the culture flask for passing the cells.

**Cell proliferation assays.** Cells were seeded in a 60-mm dish at a density of 1 × 10<sup>5</sup> cells/dish in RPMI 1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO<sub>2</sub> incubator, followed by the treatment of the cells with or without LY-294002 (10 and 20 μM). After 24, 48, and 72 h of the treatment, cells were scraped and washed twice with PBS and centrifuged at 1,000 rpm for 5 min. Cells were resuspended in 1 ml of Hanks’ balanced salt solution and counted. The total cell number was normalized to that in the control at 24 h. All samples were assayed in duplicate. The proliferation assays were performed three times.

**Cell cycle analysis.** Cells were seeded in a 100-mm dish at a density of 5 × 10<sup>5</sup> cells/dish in RPMI 1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO<sub>2</sub> incubator. Cells were then treated with or without LY-294002 (10 and 20 μM) for 6–48 h. For cell cycle analysis, cells were scraped and washed twice with PBS. Then cells were fixed with 70% ice-cold ethanol, followed by the incubation of the freshly prepared nuclei staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml RNase, and 20 g/ml PI) for 15 min at 37°C. Cell-cycle histograms were generated after analysis of PI-stained cells by fluorescence-activated cell sorting (FACS) with a Becton Dickinson FACScan. For each sample, at least 1 × 10<sup>4</sup> events were recorded. Histograms generated by FACS were analyzed by ModFit Cell Cycle Analysis software (Verity, Topsham, ME) to determine the percentage of cells in each phase (G<sub>1</sub>, S, and G<sub>2</sub>M).

**Plasmid constructs.** The active form of AKT, Myr-AKT, was inserted into pBSFI adaptor vector and then inserted into pEGFP-N vector (Clontech, San Francisco, CA) to make the fusion protein of Myr-AKT upstream of enhanced green fluorescent protein. A constitutive active rapamycin-resistant p70S6K1 with the mutation of P54A, T389E, S411D, S418D, T421E, and S424D was inserted into pRK7 vector (kindly provided by Dr. John Blenis).

**Immunoblotting analysis.** The cells were plated in a 100-mm culture dish in RPMI 1640 media supplemented with 10% FBS for 24 h at 37°C, followed by serum starvation for 20 h. Cells were then treated with or without LY-294002 (10 and 20 μM) for 12 and 24 h, respectively. Cells were lysed on ice for 30 min in RIPA buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF], supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM pepstatin A. After centrifugation at 14,000 rpm for 15 min, the supernatant was harvested as the total cellular protein extract and stored at −70°C. The protein concentration was determined by using Bio-Rad protein assay reagent (Richmond, CA). The total cellular protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane in 20 mM Tris·HCl (pH 8.0), containing 150 mM glycine and 20% (vol/vol) methanol. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with antibodies against p16, p21, CDK4, CDK6, cycline D1, β-actin, Rb, phospho-PI3K (Ser<sup>780</sup>), phospho-p70S6K1 (Ser<sup>389</sup>), phospho-p70S6K1 (Ser<sup>421/Ser<sup>424</sup></sup>), phospho-p70S6K1 (Ser<sup>424</sup>), and phospho-CDC25 (Thr<sup>216/Ser<sup>217</sup></sup>) and then followed with appropriate HRP-linked antibodies. The specific proteins in the blots were visualized by using the enhanced chemiluminescence reagent (NEN, Boston, MA).

**RESULTS**

**PI3K activity is required for the cell proliferation and cell cycle progression.** To elucidate whether PI3K affects the proliferation of ovarian cancer cells, we selected human ovarian cancer OVCAR-3 cells, in which the p110α catalytic subunit of PI3K is amplified in high copy number (48), and A2780/CP70 cells, which contain a mutation in the p53 gene and an elevated level of PI3K activity. Thus OVCAR-3 cells are suitable for the investigation of PI3K activation and its downstream targets. A2780/CP70 cells permit an analysis of PI3K and its downstream molecules in cells in combination with p53 deficiency, a common mutation in many human cancers. OVCAR-3 and A2780/CP70 cells were seeded and cultured for 24 h, followed by incubation in the presence or absence of the PI3K inhibitor LY-294002. Total cell numbers were counted 24, 48, and 72 h after the incubation. As shown in Fig. 1A, the number of both OVCAR-3 and A2780/CP70 cells was greatly increased over the period of 72 h in culture.
in the absence of LY-294002. The proliferation of the cells was slightly decreased by LY-294002 24 h after the treatment. However, after 48 and 72 h of the treatment, the proliferation of the cells was significantly inhibited by LY-294002 in a dose-dependent manner. These results indicate that PI3K may play a role in the proliferation of the ovarian cancer cell lines OVCAR-3 and A2780/CPT0.

To determine whether the inhibition of PI3K activity by LY-294002 affects cell cycle progression, OVCAR-3 and A2780/CPT0 cells were treated as described above, and the cell cycle distribution was analyzed by flow cytometry. A typical histogram is shown in Fig. 1B. The treatment of the OVCAR-3 cells with 10 and 20 μM of LY-294002 for 24 h increased the percentage of the cell population to 53 and 58%, respectively, at the G1 phase compared with the control of 42% (Fig. 1B). The inhibition of PI3K activity decreased the percentage of OVCAR-3 cells at the S phase. Similarly, the inhibition of PI3K activity induced the G1 arrest and decreased the number of cells at the S phase in A2780/CPT0 cells (Fig. 1B). Moreover, the increased percentage of cells at G1 phase and the decreased percentage of cells at S phase were observed when we repeated the experiment three times with duplicate samples per experiment in both OVCAR-3 and A2780/CPT0 cells (Fig. 1C). These data indicate that PI3K is required for the cell cycle entry from the G1 to S phase, and the increases in the cell population at the G1 phase due to the inhibition of PI3K activity may account for the decrease in the cell population at S phase in the presence of the PI3K inhibitor LY-294002 (Fig. 1, B and C).

Inhibition of PI3K led to G1 arrest through p16INK4a/CDK4/cyclin D1/Rb pathway. Cell cycle progression through G1 is regulated principally by the sequential activation of the cyclin D/CDK4/CDK6, which induces the phosphorylation of Rb and the release of E2F. To explore the mechanism by which the inhibition of PI3K activity induced the cell cycle arrest at the G1 phase in OVCAR-3 and A2780/CPT0 cells, the cells were cultured and treated, as described above. The total cellular proteins were prepared and analyzed by immunoblot assays for the expression of cyclin D1, CDK4, CDK6, Rb, and phospho-Rb. The expression levels of cyclin D1, CDK4, CDC25A, Rb, and phospho-Rb (Ser780, Ser795, and Ser807/811) were inhibited by the treatment of cells with LY-294002 in a dose-dependent manner (Fig. 2A), whereas the levels of CDK6 and β-actin remained relatively unchanged in either the presence or absence of the PI3K inhibitor (Fig. 2A). CDK inhibitors are important negative regulators of cell cycle progression. After binding to cyclin/CDK complexes at the G1 phase of the cell cycle, these inhibitors block the CDK activity, preventing the phosphorylation of members of the Rb gene family and the transition from the G1 to S phase. To determine which CDK inhibitor was involved in the inhibition of CDK/cyclin complexes, we analyzed the expression of p16INK4a, p15, p27kip1, and p21CIP1/WAF1 in a dose-dependent manner (Fig. 2A). To determine whether serum affects the expression of these proteins associated with G1 cell cycle progression, OVCAR-3 and A2780/CPT0 cells were cultured in the RPMI 1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO₂ incubator, followed by the addition of solvent alone or 10 or 20 μM of LY-294002 (LY) for 12 h. The total cellular protein extracts were prepared and subjected to immunoblotting analysis by using specific antibodies against phospho-retinoblastoma (Rb) (Ser795, Ser780, and Ser807/811), Rb, cyclin D1, cyclin-dependent kinase (CDK) 4, CDK6, CDC25A, p21, p16, and β-actin. A: OVCAR-3 cells were cultured in a 100-mm dish at a density of 1 × 10⁶ cells/dish in RPMI 1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO₂ incubator, followed by the incubation with serum-free media for 16 h. Cells were switched to the media in the presence (+) or absence (−) of 10% FBS and LY-294002 for 12 and 24 h, as indicated. The proteins were analyzed by immunoblotting, as described in MATERIALS AND METHODS.
were cultured in the serum-free medium for 16 h, followed by the addition of serum. As shown in Fig. 2B, the expression levels of cyclin D1, CDK4, CDC25A, p21^{CIP1/WAF1}, Rb, and phospho-Rb (Ser^{780}, Ser^{795}, and Ser^{807/811}) were induced by serum and inhibited by LY-294002 in a dose-dependent manner (Fig. 2B). Levels of CDK6 and β-actin were not induced by serum or inhibited by the PI3K inhibitor. Expression of p16^{INK4a} was induced by the addition of LY-294002 to the cultured medium (Fig. 2B). The levels of p15 and p2^7^kip were below the limits of detection (data not shown). Although the expression of p21^{CIP1/WAF1} is important for the cell cycle arrest induced by DNA-damaging agents, p21^{CIP1/WAF1} expression has been recently demonstrated to be induced by serum and several growth factors in other cell lines in which the p21^{CIP1/WAF1} level is not relevant to cell cycle progression (14, 26, 43, 57). These recent reports are consistent with our study showing the decreased expression of p21^{CIP1/WAF1} by the LY-294002 treatment. These data indicate that p21^{CIP1/WAF1} expression may not be relevant for the G1 arrest induced by the PI3K inhibitor LY-294002 in the ovarian cancer cells. Because the expression of Rb protein is known to inhibit the p16^{INK4a} transcripational activation and expression (27, 44), the inhibition of Rb expression by LY-294002 may result in the induction of p16^{INK4a} protein levels, which are involved in the LY-294002-induced G1 arrest in the cells (Figs. 1 and 2).

**Serum-induced phosphorylation of AKT but not extracellular regulated kinase 1/2 was inhibited by LY-294002 in the ovarian cancer cells.** It is known that serum induces the activation and phosphorylation of AKT and extracellular regulated kinase (ERK) 1/2 through PI3K signaling (9, 24). To determine whether LY-294002 affects the serum-induced activation and phosphorylation of AKT or ERK1/2, the serum-starved ovarian cancer cells were treated by the addition of serum in the presence or absence of LY-294002 (20 μM) for various exposure times. The phosphorylation of AKT and ERK1/2 was detected by Western blot. As shown in Fig. 3, A and B, a high level of AKT phosphorylation was induced by serum in both ovarian cancer cell lines and was inhibited by the addition of LY-294002. Total AKT protein levels were not affected by the treatment of serum and LY-294002. Whereas the ERK1/2 phosphorylation was also increased by the addition of serum, it was not decreased by the treatment of LY-294002. These data indicated that the activation and phosphorylation of AKT, but not ERK, were involved in G1 cell cycle progression in the ovarian cancer cells. To further confirm that the serum-induced activation of AKT was inhibited by LY-294002 during the prolonged treatment, these ovarian cancer cells were treated by the addition of serum without or with LY-294002 for 12 and 24 h. Similarly, AKT phosphorylation was inhibited by the treatment of LY-294002 for 12 and 24 h (Fig. 3C and D).

**Expression of an active form of AKT reversed cell cycle arrest at the G1 phase induced by the PI3K inhibitor LY-294002.** Inhibition of PI3K activity by LY-294002 induced G1 cell cycle arrest. To study whether this inhibition could be reversed by expression of AKT, we made a fusion of green fluorescent protein (GFP) into Myr-AKT (GFP-Myr-AKT) driven by the cytomegalovirus promoter, which is a constitutively active form of AKT in the cells (data not shown). We transfected GFP alone or GFP-Myr-AKT expression plasmid into OVCAR-3 cells. The cells transfected by GFP alone were used as a control. The cells were cultured for 36 h after transfection, followed by treatment for 12 h in the absence or presence of 10 μM of LY-294002. Cells expressing GFP proteins were separated from untransfected cells by FACS. Compared with the untransfected control, the expression of active forms of AKT substituted for PI3K in the cells and reversed the inhibition of LY-294002 on cell cycle progression (Fig. 3E). This result further confirmed that AKT is a downstream target of PI3K, which is required for the G1 cell cycle progression in the ovarian cancer cells.

**LY-294002 inhibited phosphorylation of p70S6K1 in the ovarian cancer cells.** p70S6K1 is a downstream target of PI3K and AKT. The activation of p70S6K1 depends on the phosphorylation of several residues in the pseudosubstrate region, such as Ser^{389} and Ser^{421}/Thr^{424}. To determine whether the phosphorylation of p70S6K1 was involved in LY-294002-induced G1 cell cycle arrest in the ovarian cancer cells, these cells were treated with serum in the absence or presence of different doses of LY-294002 for 12 h, and the phosphorylation of p70S6K1 at Ser^{389} and at Ser^{421}/Thr^{424} was detected by using Western blot. As shown in Fig. 4A, LY-294002 completely inhibited serum-induced phosphorylation of p70S6K1 at Ser^{389} and at Ser^{421}/Thr^{424} whereas it did not affect the expression of p70S6K1 total protein. These results suggest that the p70S6K1 activation was associated with the inhibition of PI3K activity and the G1 growth arrest in the cells.

**p70S6K1 is a potential target of AKT that mediates cell cycle progression.** To study whether the overexpression of AKT affects the activation of p70S6K1 in the absence or presence of LY-294002, the OVCAR-3 cells were transfected with an active form of AKT, Myr-AKT, to establish a stable cell line expressing Myr-AKT. The cells were also transfected by the vector to establish a cell line expressing the vector alone as a control. As shown in Fig. 4B, overexpression of AKT greatly increased the p70S6K1 phosphorylation in the absence of LY-294002 and restored the p70S6K1 phosphorylation in the presence of 10 μM of LY-294002 to the similar level of vector control without the inhibitor (Fig. 4B). This result is consistent with that of the cell cycle progression observed in Fig. 3E. These data indicate that p70S6K1 is potentially involved in PI3K- and AKT-mediated cell cycle progression.

To test whether mTOR mediates p70S6K1 activity in ovarian cancer cell cycle progression, the cells were treated by the mTOR inhibitor rapamycin, as indicated, for 12 h (Fig. 4C). The p70S6K1 phosphorylation indicates the activation of its protein. The p70S6K1 phosphorylation was induced by serum and inhibited in the rapamycin-treated cells, whereas total p70S6K1 protein levels were not affected (Fig. 4C). This result indicates that the activation of rapamycin is sufficient to inhibit p70S6K1 activity in the ovarian cancer cells.

**Inhibition of the mTOR-p70S6K1 activity led to cell cycle arrest in the G1 phase through p16^{INK4a}/CDK4/cyclin D1/Rb pathway.** To investigate whether the inhibition of mTOR and p70S6K1 affects G1 cell cycle arrest, the ovarian cancer cells were treated with the mTOR inhibitor rapamycin, and cell cycle progression was analyzed. Rapamycin induced the G1 cell cycle arrest in both OVCAR-3 and A2780/CP70 cells (data not shown). The total cellular proteins were prepared and subjected to immunoblotting assay for cyclin D1, CDK4, CDK6, Rb, and phospho-Rb at Ser^{780}, Ser^{795}, and Ser^{807-811}. The levels of cyclin D1, CDK4, Rb, and phospho-Rb (Ser^{780},
Ser795, and Ser807/811) were increased by serum in both cell lines (Fig. 5A). Treatment of cells with rapamycin resulted in a decrease in expression of cyclin D1, CDK4, Rb, and phospho-Rb (Ser780, Ser795, and Ser807/811) induced by serum, whereas the levels of CDK6 and β-actin remained unchanged (Fig. 5A). The expression of p16INK4a and p21^{CIP1/WAF1} was also analyzed in the ovarian cancer cells in a similar experimental condition. The results indicate that the treatment of cells with rapamycin increased the expression of p16INK4a in a dose-dependent manner, whereas the expression of p21^{CIP1/WAF1} was decreased, suggesting that, rather than expression of p21^{CIP1/WAF1}, expression of p16INK4a may play an important role in G1 cell cycle arrest.

Similar expression levels of the cell cycle-associated proteins were inhibited by treatment of cells with rapamycin in the normal cultured condition (Fig. 5B). These results are consistent with the data obtained above with the use of the PI3K inhibitor and suggest that p70S6K1 was a downstream molecule of PI3K and AKT for regulating G1 cell cycle arrest and the expression of p16INK4a/CDK4/cyclin D1/Rb. The Rb protein negatively regulates the p16INK4a expression in the cells (27, 44); thus the decrease of Rb expression by rapamycin would lead to the induction of p16INK4a protein expression (Fig. 5). These results indicate that p70S6K1 regulates G1 cell cycle progression through the Rb/p16INK4a/CDK4/cyclin D1 pathway.
Expression of an active form of p70S6K1 reversed cell cycle arrest at the G₁ phase induced by PI3K inhibitor LY-294002. To determine the role of p70S6K1 in G₁ progression, we cotransfected OVCAR-3 cells with a 1:5 ratio of GFP and the constitutively active form of p70S6K1 expression plasmids. The cells transfected by GFP alone were used as a control. The cells were cultured for 36 h after transfection, followed by incubation for 12 h in the absence or presence of 10 μM of rapamycin. Cells expressing GFP proteins were selected by FACS analysis. The expression of the active form of p70S6K1 reversed rapamycin-induced cell cycle arrest (Fig. 5D), indicating that p70S6K1 is an essential target of rapamycin for inhibiting the cell cycle progression.

**DISCUSSION**

PI3K is involved in many cellular functions in response to growth factors. PI3K was initially observed to be required for cellular transformation induced by several viral oncoproteins such as v-Src and v-Abl (16, 28, 56). Recent studies indicate that the gene encoding the p110α catalytic subunit of PI3K is increased in copy number in primary ovarian cancer cells and several ovarian carcinoma cell lines (48). PI3K and AKT signaling has been shown to play an important role in DNA repair and apoptosis induced by chemotherapy agents, including cisplatin and paclitaxel in human ovarian cancer cells (34). Coexpression of an active form of Akt with Myc and Ras was demonstrated to be sufficient to induce ovarian tumor formation in a mouse model by using an avian retroviral gene delivery technique (37). An oncogenic form of PI3K, v-p3k was discovered recently in avian sarcoma virus 16, and v-p3k is the viral homolog of a gene encoding for PI3K catalytic subunit (6), which is amplified in human ovarian carcinomas (48). Therefore, further study of PI3K could provide a better understanding of the molecular mechanism for ovarian carcinoma development.

In this study, we have shown that inhibition of PI3K activity using LY-294002 decreased the cell proliferation and induced the G₁ cell cycle arrest in the ovarian cancer cells OVCAR-3 and A2780/CP70. The data are consistent with previous studies, suggesting that PI3K was required for cell proliferation in different cell types, such as neutrophils, endothelial cells, and breast cancer cells (10, 22, 39). To understand the mechanism by which PI3K regulated the G₁ cell cycle progression, we studied the role of PI3K in the expression of known regulators associated with the G₁ cell cycle in these cells. During the G₁-to-S cell cycle progression in response to a mitogen, levels of D-type cyclins increase, bind to, and activate CDK4 and CDK6 (32). Our studies indicated that inhibition of PI3K by LY-294002 greatly decreased the expression of cyclin D1 and CDK4 and the phosphorylation of Rb at Ser²⁸⁰, Ser²⁹⁵, and Ser²⁰³⁷/³⁸³¹, whereas CDK6 activity remained relatively unchanged. These data indicated that PI3K was required for cyclin D1/CDK4 interactions, which induces the Rb phosphorylation, E2F release, and the G₁ cell cycle progression. These results are consistent with recent studies in other cell lines, indicating that PI3K/AKT was required for the induction of cyclin D1 expression (36) and expression of E2F (2).

The tumor suppressor genes p16INK4a and p53 have been shown to regulate the G₁ cell cycle progression through different mechanisms. The p53 regulates the cell cycle at the G₁ checkpoint and is primarily stimulated by DNA damage (12). Activation of p53 leads to G₁ arrest through the induction of p21CIP1/WAF1 (58). p16INK4a is a member of the INK4 cell cycle proteins. It encodes a protein that inhibits cyclin D
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**C**

Un-transfected Cells

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<td>46.53%</td>
<td>30.59%</td>
<td>22.88%</td>
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kinases, CDK4, and CDK6 (32, 33). CDK4 and CDK6 are required for the phosphorylation of Rb. In the present study, we found that the treatment of cells with LY-294002 markedly induced p16\(^{INK4a}\) expression in a dose-dependent manner, but decreased p21\(^{CIP1/WAF1}\) expression, suggesting that the LY-294002-induced G1 cell cycle arrest in ovarian cancer cells requires increased p16\(^{INK4a}\) expression, but not p21\(^{CIP1/WAF1}\) expression. The expression of p21\(^{CIP1/WAF1}\) is known to be induced by p53-induced cell cycle arrest, associated with the treatment of DNA-damaging agents. However, p21\(^{CIP1/WAF1}\) expression was recently demonstrated to be induced by serum and several growth factors in different cell lines and regulated independently of the p53 level in the cells and of cell cycle progression (14, 26, 43, 57). These results and our study indicate that neither p21\(^{CIP1/WAF1}\) nor p53 is involved in the G1 arrest induced by blocking PI3K activity in the cells. Our results are consistent with previous reports that indicate that overexpression of p16\(^{INK4a}\), alone is more effective than p53 and p21\(^{CIP1/WAF1}\) in the inhibition of the cancer cell growth (35, 45). The Rb protein is known to inhibit the transcriptional activation of p16\(^{INK4a}\) expression in the cells, and the genetic mutation of the pRb gene resulted in high levels of p16\(^{INK4a}\) expression (27, 44). Our data showed that the inhibition of PI3K and p70S6K1 activities in the cells decreased the expression of Rb protein levels and induced the expression of p16\(^{INK4a}\) expression (Figs. 2 and 5). These data are consistent with other recent reports, suggesting the negative regulatory role of Rb on the p16\(^{INK4a}\) expression. The above results indicated that PI3K regulated p70S6K1, which, in turn, mediates the p16\(^{INK4a}\)/CDK4/cyclin D1/Rb pathway in G1 cell cycle progression in the ovarian cancer cells.

The results obtained from the present study demonstrate that treatment of cells with LY-294002 markedly inhibited AKT phosphorylation, whereas it has no effect on the activation of ERK1/2, suggesting that the activation of AKT but not ERK1/2 was involved in PI3K-dependent cell cycle regulation. A major downstream target of AKT is mTOR, which regulates p70S6K1, p70S6K1 was found to be constitutively phosphorylated in the ovarian cancer cells by the addition of serum. The activation of p70S6K1 is accompanied by the phosphorylation of RB at multiple Ser/Thr residues, such as Ser\(^{389}\) and Ser\(^{21}/\text{Thr}^{22}\). The phosphorylation of p70S6K1 was markedly inhibited by treatment of cells with the PI3K inhibitor LY-294002 and mTOR inhibitor rapamycin. PI3K inhibitor LY-294002-induced G1 cell cycle arrest was restored by the forced expression of active forms of AKT and p70S6K1 in the cells. These results suggest that mTOR and p70S6K1 are downstream of PI3K and AKT in regulating G1 cell cycle progression in the ovarian cancer cells. We predicted that the inhibition of mTOR and p70S6K1 would have a similar effect on the inhibition of PI3K and AKT in these ovarian cancer cells. This is further confirmed by our studies that indicated treatment of the cells with rapamycin markedly induced p16\(^{INK4a}\) expression in a dose-dependent manner and inhibited p21\(^{CIP1/WAF1}\), cyclin D1, CDK4, and the phosphorylation of Rb at Ser\(^{780}\), Ser\(^{782}\), and Ser\(^{807/811}\) (Fig. 5). The level of CDK6 remained relatively unchanged in the presence of rapamycin. These results were similar to those obtained by treatment of cells with LY-294002 (Figs. 1 and 2). To study the effect of rapamycin on p70S6K1, the rapamycin-resistant p70S6K1 constructs were recently generated by several groups, which demonstrated that expression of rapamycin-resistant p70S6K1 constructs restored rapamycin-induced G1 cell cycle arrest (7, 19, 29, 54). Our result indicated that rapamycin-resistant p70S6K1 restored rapamycin-inhibited cell cycle progression (Fig. 5D). Our data are
consistent with these studies, supporting a role of p70S6K1 in PI3K-mediated cell cycle progression. These data suggest that p70S6K1 and p16INK4a/CDK4/cyclin D1/Rb pathways play an important role in G1 cell cycle arrest induced by rapamycin in ovarian cancer cells.

In summary, the present study demonstrates that the PI3K inhibitor LY-294002 inhibited ovarian cancer cell proliferation by inducing G1 cell cycle arrest. The inhibition of PI3K activity by LY-294002 inhibited the phosphorylation of AKT and p70S6K1, but not ERK1/2. LY-294002 and mTOR inhibitor rapamycin have similar inhibitory effects on the phosphorylation of p70S6K1 at Ser389 and Ser422/Thr424, on the expression of CDK4 and cyclin D1, and on Rb phosphorylation at Ser780, Ser795, and Ser807/811. Our results indicate that PI3K signaling regulates G1 cell cycle progression through the increased expression of cyclins and CDKs. The inhibition of PI3K activity is able to induce the expression of p16INK4a, leading to inhibition of the expression of CDK4 and cyclin D1, and Rb phosphorylation in the ovarian cancer cells. PI3K transmits the mitogenic signal through AKT and mTOR to p70S6K1 (Fig. 6). These results suggest that the PI3K/AKT/mTOR/p70S6K1 signaling pathway could serve as a novel target for therapeutic intervention in the ovarian cancer.

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