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The G_1 cell cycle progression and the expression of G_1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells

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ABSTRACT

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, anti-apoptosis, tumorigenesis, and angiogenesis. In this study, we demonstrate that the inhibition of PI3K activity by LY294002 inhibited ovarian cancer cell proliferation and induced G_1 cell cycle arrest. This effect was accompanied by the decreased expression of G_1-associated proteins including cyclin D1, CDK4, CDC25A, and Rb phosphorylation at Ser780, Ser795, and Ser807/811. Expression of CDK6 and β-actin was not affected by LY294002. Expression of the cyclin kinase inhibitor $p16^{INK4a}$ was induced by the PI3K inhibitor, while steady state levels of $p21^{CIP1/WAF1}$ were decreased in the same experiment. The inhibition of PI3K activity also inhibited the phosphorylation of AKT and p70S6K1, but not ERK1/2. The G_1 cell cycle arrest induced by LY294002 was restored by the expression of active forms of AKT and p70S6K1 in the cells. Our study shows that PI3K transmits a mitogenic signal through AKT and mTOR to p70S6K1. The mTOR inhibitor rapamycin had similar inhibitory effects on G_1 cell cycle progression and on the expression of cyclin D1, CDK4, CDC25A, and Rb phosphorylation. These results indicate that PI3K mediates G_1 progression and cyclin expression through activation of an AKT/mTOR/p70S6K1 signaling pathway in the ovarian cancer cells.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological malignancy and the fourth most common cause of cancer death among American women (40). Recent observations indicate that the gene encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K) is increased in copy number in approximately 80% of primary ovarian cancer cells and several ovarian epithelial carcinoma cell lines. The PI3K inhibitor LY294002 has been shown to inhibit growth of an ovarian cancer cell line *in vitro* (48). PI3K is a heterodimeric enzyme composed of a 110-kD catalytic and an 85-kD regulatory subunits (3). PI3K phosphorylates the D3 hydroxyl of phosphoinositides and produces phosphatidyl-inositol-3-phosphates. PI3K binds to and is activated by several receptor and nonreceptor protein tyrosine kinases (PTKs) (5, 8, 18, 25). The oncogenic form of PI3K, *v-p3k,* was first discovered in avian sarcoma virus 16 (6). The expression of *v-p3k* can induce cellular transformation and induce tumors when the transformed cells are injected into chickens (6). The best known downstream target of PI3K is the serine-threonine kinase AKT, which transmits survival signals from growth factors (5, 11, 23). v-P3k transmits the angiogenic and oncogenic signals through its downstream target, AKT (1, 20). In addition, overexpression of active forms of PI3K and AKT can directly induce angiogenesis *in vivo* (20).

PI3K is involved in cell proliferation and differentiation. The addition of the PI3K inhibitor LY294002 induced G_1 cell cycle arrest and the expression of the cyclin-dependent kinase inhibitor p27 in melanoma and osteosarcoma cell lines (4, 52). We hypothesized that PI3K and AKT activities were also important for the G_1 cell cycle progression in ovarian cancer cells and for the expression of proteins associated with the G_1 progression.

A downstream target of AKT, the serine/threonine kinase p70S6K1 is a highly conserved element in a wide array of cellular processes including the mitogenic response to growth factors (13, 30, 42, 47). The mTOR-p70S6K1 signaling was also constitutively activated in several cancer cell lines, including small cell lung cancer cells and pancreatic cancer cells (15, 46). Therefore, p70S6K1 may be an important signaling molecule downstream of PI3K and AKT in the cell proliferation and cell cycle progression.

Progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs), whose activity is inhibited by the CDK inhibitors. Cyclins, CDKs, and CDK inhibitors are frequently deregulated in cancers (53). Activation of cyclin/cyclin-dependent kinase (CDK) activities is required for cell cycle progression and G_1/S transition in response to growth factor stimulation. The expression of cyclin D and CDK4/6 in G_1 cell cycle acts as the primary sensors of positive and negative environmental signals (31, 49, 50). The cyclin D/CDK4/6 complexes induce the phosphorylation of retinoblastoma protein (Rb) and the release of E2F, which trigger G_1 cell cycle progression. Normally, Rb binds to the members of the E2F family of transcription factors. In response to the growth factors, the Rb protein is phosphorylated and dissociated from E2F, which triggers G_1 cell cycle progression (21). On the other hand, the up-regulation of CDK inhibitors such as $p21^{CIP1/WAF1}$ and $p16^{INK4a}$, is frequently responsible for the inhibition of G_1 cell cycle progression and for withdrawal from the cell cycle (38, 41, 51). $p16^{INK4a}$ is capable of binding to the cyclin-dependent kinase CDK4, thereby inhibiting the catalytic activity of the CDK4/cyclin D1 enzymes. $p21^{CIP1/WAF1}$ 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 LY294002 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. PI3K inhibitor, LY294002, and mTOR/FRAP 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 (Ser780), phospho-Rb (Ser795), phospho-Rb (Ser807/811), phospho-AKT (Ser473), Phospho-p44/42 MAPK, p70S6K1, phospho-p70S6K1 (Thr389), phospho-p70S6K1 (Thr421/Ser424) 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 RPMI1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 0.2% insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin, and cultured at 37°C in 5% CO_2 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 1×10^5 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator, followed by the treatment of the cells with or without LY294002 (10 μM and 20 μM). After 24 h, 48 h, and 72 h of the treatment, cells were scraped and washed twice with phosphate-buffered saline (PBS), and centrifuged at 1000 rpm for 5 min. Cells were resuspended in 1 ml of Hank's 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 5×10^5 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator. Cells were then treated with or without LY294002 (10 μM and 20 μM) for 6 h to 48 h. For cell cycle analysis, cells were scraped and washed twice with phosphate-buffered saline. 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 1×10^4 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_1, S, and G_2/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 EGFP protein. A constitutive active rapamycin-resistant p70S6K1 with the mutation of F5A, 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 RPMI1640 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 LY294002 (10 μM and 20 μM) for 12 h 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 (PMSF), 1 mM DTT, 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 using Bio-Rad protein assay reagent (Richmond, CA). The total cellular protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS- PAGE), and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (v/v) 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-Rb (Ser780), phospho-Rb (Ser795), phospho-Rb (Ser807/811), phospho-AKT (Ser473), phospho-p44/42 MAPK, p70S6K1, phospho-p70S6K1 (Thr389), phospho-p70S6K1 (Thr421/Ser424). Blots were washed three times in PBS buffer, followed by the incubation with the appropriate HRP-linked IgG. The specific proteins in the blots were visualized 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 cells 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 LY294002. Total cell numbers were counted 24 h, 48 h, and 72 h after the incubation. As shown in Fig. 1A, the number of both OVCAR-3 and A2780/CP70 cells were greatly increased over the period of 72 h in culture in the absence of LY294002. The proliferation of the cells was slightly decreased by LY294002 24 h after the treatment. However, after 48 h and 72 h of the treatment, the proliferation of the cells was significantly inhibited by LY294002 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/CP70.

To determine whether the inhibition of PI3K activity by LY294002 affects cell cycle progression, OVCAR-3 and A2780/CP70 cells were treated as described above, and the cell cycle distribution was analyzed by flow cytometry. A typical histogram was shown in Fig. 1B. The treatment of the OVCAR-3 cells with 10 μM and 20 μM of LY294002 for 24 h increased the percentage of the cell population to 53% and 58% at G_1 phase compared to the control of 42%, respectively (Fig. 1B). The inhibition of PI3K activity decreased the percentage of OVCAR-3 cells at S phase. Similarly, the inhibition of PI3K activity induced G_1 arrest, and decreased the number of cells at S phase in A2780/CP70 cells (Fig. 1B). Moreover, the increased percentage of cells at G_1 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/CP70 cells (Fig. 1C). These data indicate that PI3K is required for the cell cycle entry from the G_1 to S phase, and the increases in the cell population at G_1 phase

due to the inhibition of PI3K activity may account for the decreases in the cell population at S phase in the presence of PI3K inhibitor LY294002 (Fig. 1B and 1C).

Inhibition of PI3K led to G_1 arrest through $p16^{INK4a}$/CDK4/cyclin D1/Rb pathway. Cell cycle progression through G_1 is regulated principally by the sequential activation of the cyclinD/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 G_1 phase in OVCAR-3 and A2780/CP70 cells, the cells were cultured and treated as described in the previous section. 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 LY294002 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). Cyclin-dependent kinase (CDK) inhibitors are important negative regulators of cell cycle progression. After binding to cyclin-CDK complexes at the G_1 phase of the cell cycle, these inhibitors block the CDK kinase activity, preventing the phosphorylation of members of the Rb gene family and the transition from G_1 to S phase. To determine which CDK inhibitor was involved in the inhibition of CDK/cyclin complexes, we analyzed the expression of $p16^{INK4a}$, p15, $p27^{KIP1}$, and $p21^{CIP1/WAF1}$ in these two types of ovarian cancer cells. The treatment of cells with LY294002 increased the expression of $p16^{INK4a}$ and decreased the expression of $p21^{CIP1/WAF1}$ in a dose-dependent manner (Fig. 2A). To determine whether serum affects the expression of these proteins associated with G_1 cell cycle progression, OVCAR-3 and A2780/CP70 cells 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 (Ser780, Ser795 and Ser807/811) were induced by serum and inhibited by LY294002 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 LY294002 to the cultured medium (Fig. 2B). The levels of p15 and $p27^{KIP1}$ 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 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 $p21^{CIPI/WAFI}$ by the LY294002 treatment. These data indicate that $p21^{CIP1/WAF1}$ expression may not be relevant for the G_1 arrest induced by PI3K inhibitor LY294002 in the ovarian cancer cells. Since the expression of Rb protein is known to inhibit the $p16^{INK4a}$ transcriptional activation and expression (27, 44), the inhibition of Rb expression by LY294002 may result in the induction of $p16^{INK4a}$ protein levels, which are involved in the LY294002-induced G_1 arrest in the cells (Figs. 1 and 2).

Serum-induced phosphorylation of AKT but not ERK1/2 was inhibited by LY294002 in the ovarian cancer cells. It is known that serum induces the activation and phosphorylation of AKT and ERK1/2 through PI3K signaling (9, 24). To determine whether LY294002 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 LY294002 (20 μM) for various exposure times. The phosphorylation of AKT and ERK1/2 was detected by

western blot. As shown in Figs. 3A and 3B, a high level of AKT phosphorylation was induced by serum in both ovarian cancer cell lines and was inhibited by the addition of LY294002. Total AKT protein levels were not affected by the treatment of serum and LY294002. While the ERK1/2 phosphorylation was also increased by the addition of serum, it was not decreased by the treatment of LY294002. These data indicated that the activation and phosphorylation of AKT, but not ERK, was involved in G_1 cell cycle progression in the ovarian cancer cells. To further confirm that the serum-induced activation of AKT was inhibited by LY294002 during the prolonged treatment, these ovarian cancer cells were treated by the addition of serum without or with LY294002 for 12 h and 24 h. Similarly, AKT phosphorylation was inhibited by the treatment of LY294002 for 12 h and 24 h (Figs. 3C and 3D).

Expression of an active form of AKT reversed cell cycle arrest at the G_1 phase induced by the PI3K inhibitor LY294002. Inhibition of PI3K activity by LY294002 induced G_1 cell cycle arrest. To study whether this inhibition could be reversed by expression of AKT, we made a fusion of GFP into Myr-AKT (GFP-Myr-AKT) driven by the CMV 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 LY294002. Cells expressing GFP proteins were separated from un-transfected cells by fluorescence-activated cell sorting (FACS). When compared to the un-transfected control, the expression of active forms of AKT substituted for PI3K in the cells and reversed the inhibition of LY294002 on cell cycle progression (Fig. 3E). This result further confirmed that AKT is a downstream target of PI3K, which is required for the G_1 cell cycle progression in the ovarian cancer cells.

LY294002 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 Ser389 and Ser421/Thr424. To determine whether the phosphorylation of p70S6K1 was involved in LY294002-induced G_1 cell cycle arrest in the ovarian cancer cells, these cells were treated with serum in the absence or presence of different doses of LY294002 for 12 h, and the phosphorylation of p70S6K1 at Ser389 and Ser421/Thr424 was detected using western blot. As shown in Fig. 4A, LY294002 completely inhibited serum-induced phosphorylation of p70S6K1 at Ser389 and at Ser421/Thr424, 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 G_1 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 P70S6KI in the absence or presence of LY294002, 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 LY294002, and restored the p70S6K1 phosphorylation in the presence of 10 μM of LY294002 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 indicates 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, while total p70S6K1 protein levels were not affected (Fig. 4C). This result indicates that the addition 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 G_1 phase through $p16^{INK4a}$/CDK4/Cyclin D1/Rb pathway. To investigate whether the inhibition of mTOR and p70S6K1 affects G_1 cell cycle arrest, the ovarian cancer cells were treated with the mTOR inhibitor rapamycin and cell cycle progression was analyzed. Rapamycin induced the G_1 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 Ser780, Ser795, and Ser807/811. The levels of cyclin D1, CDK4, Rb, and phospho-Rb (Ser780, 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ 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 $p16^{INK4a}$ may play an

important role in G_1 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 using the PI3K inhibitor and suggest that p70S6K1 was a downstream molecule of PI3K and AKT for regulating G_1 cell cycle arrest and the expression of $p16^{INK4a}$/CDK4/cyclin D1/Rb. The Rb protein negatively regulates the $p16^{INK4a}$ expression in the cells (27, 44), thus the decrease of Rb expression by rapamycin would lead to the induction of $p16^{INK4a}$ protein expression (Fig. 5). These results indicate that p70S6K1 regulates G_1 cell cycle progression through Rb/$p16^{INK4a}$/CDK4/Cyclin D1 pathway.

Expression of an active form of p70S6K1 reversed cell cycle arrest at the G_1 phase induced by PI3K inhibitor LY294002. To determine the role of p70S6K1 in G_1 progression, we co-transfected OVCAR-3 cells with a 1:5 ratio of GFP and the constitutively active form of p70S6K1 (S6K1-F5AE389D3E) 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 LY294002. Cells expressing GFP proteins were separated from un-transfected cells by FACS analysis. The expression of the active form of p70S6K1 reversed LY294002-induced cell cycle progression (Fig. 5C), indicating that p70S6K1 is an essential target of PI3K for cell cycle progression in the cells.

Expression of an active form of p70S6K1 reversed rapamycin-induced G_1 cell cycle arrest. To determine whether the overexpression of rapamycin-resistant p70S6K1 construct can restore rapamycin-induced G_1 cell cycle arrest, 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 the 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). Co-expression 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 LY294002 decreased the cell proliferation and induced the G_1 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 G_1 cell cycle progression, we studied the role of PI3K in the expression of known regulators associated with the G_1 cell cycle in these cells. During the G_1-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 LY294002 greatly decreased the expression of cyclin D1, CDK4, and the phosphorylation of Rb at Ser780, Ser795, and Ser807/811, whereas CDK6 activity remained relatively unchanged. This data indicated that PI3K was required for cyclin D1/CDK4 interactions, which induces the Rb phosphorylation, E2F release, and the G_1 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 $p16^{INK4a}$ and p53 have been shown to regulate the G_1 cell cycle progression through different mechanisms. p53 regulates the cell cycle at the G_1 checkpoint and is primarily stimulated by DNA damage (12). Activation of p53 leads to G_1 arrest through the induction of $p21^{CIP1/WAF1}$ (58). $p16^{INK4a}$ is a member of the INK4 cell cycle proteins. It encodes a protein that inhibits cyclin D 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 LY294002 markedly induced $p16^{INK4a}$ expression in a dose-dependent manner, but

decreased $p21^{CIP1/WAF1}$ expression, suggesting that the LY294002-induced G_1 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 has 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 G_1 arrest induced by blocking PI3K activity in the cells. Our results are consistent with previous reports that indicate 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, 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 16^{INK4a}/CDK4/Cylin D1/ Rb pathway in G_1 cell cycle progression in the ovarian cancer cells.

The results obtained from the present study demonstrate that treatment of cells with LY294002 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 at multiple Ser/Thr residues such as Ser389 and Ser421/Thr424. The phosphorylation of p70S6K1 was markedly inhibited by treatment of cells with the PI3K inhibitor LY294002 and mTOR inhibitor rapamycin. PI3K inhibitor LY294002-induced G_1 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 G_1 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 Ser780, Ser795, and Ser807/811. The level of CDK6 remained relatively unchanged in the presence of rapamycin. These results were similar to those obtained by treatment of cells with LY294002 (Figs.1 and 2). To study the effect of rapamycin on p70S6K1, the rapamycin-resistant p70S6K1 constructs were recently generated by several groups, who demonstrated that expression of rapamycin-resistant p70S6K1 constructs restored rapamycin-induced G_1 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 $p16^{INK4a}$/CDK4/cylin D1/Rb pathways play an important role in G_1 cell cycle arrest induced by rapamycin in ovarian cancer cells.

In summary, the present study demonstrates that the PI3K inhibitor LY294002 inhibited ovarian cancer cell proliferation by inducing G_1 cell cycle arrest. The inhibition of PI3K activity by

LY294002 inhibited the phosphorylation of AKT and p70S6K1, but not ERK1/2. LY294002 and mTOR inhibitor rapamycin have similar inhibitory effects on the phosphorylation of p70S6K1 at Ser389 and Ser421/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 G_1 cell cycle progression through the increased expression of cyclins and CDKs. The inhibition of PI3K activity is able to induce the expression of $p16^{INK4a}$, 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, 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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FIGURE LEGENDS

Fig. 1. PI3K is required for the proliferation and G_1 progression of ovarian cancer cells. ***A,*** OVCAR-3 and A2780/CP70 cells were seeded in a 60 mm dish at a density of 1×10^5 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator. Cells were then cultured in the absence or presence of PI3K inhibitor LY294002 (10 μM and 20 μM) for different times as indicated. Cells were harvested, stained with trypan blue, and counted using a hemocytometer. The number of total cells was counted and normalized to the number obtained in the control. The data represented the mean and standard error from three independent experiments. The proliferation assays were performed in triplicate. * and ** indicate that the normalized cell number was significantly decreased compared to the control at the same time point with $P<0.05$ and $P<0.01$, respectively (Student's test). ***B,*** Inhibition of PI3K leads to G_1 arrest in OVCAR-3 and A2780/CP70 cells. Cells were cultured in a 100 mm dish at a density of 1×10^6 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator. The cells were then cultured in the absence or presence of LY294002 (10 μM and 20 μM) for 12 h, 24 h, and 48 h. Cells were washed with 1× PBS, fixed, stained, and analyzed by flow cytometry. Typical cell cycle histograms were recorded (Fig. 1B) 24 h after treatment. Gates were configured manually to determine the percentage of cells at G_1, S, and G_2/M phases based on DNA content. ***C,*** The percentage of cells at G_0/G_1, S, and G_2/M phases was obtained from three replicate experiments.

Fig. 2. Role of PI3K in the expression of proteins associated with the cell cycle of OVCAR-3 and A2780/CP70 cells. ***A,*** OVCAR-3 cells were cultured in the RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator, followed by the addition of

solvent alone, 10 μM, or 20 μM of LY294002 for 12 h. The total cellular protein extracts were prepared and subjected to immunoblotting analysis using specific antibodies against phospho-Rb (Ser795, Ser780, and Ser807/811), Rb, cyclin D1, Cdk4, Cdk6, Cdc25A, p21, p16 and β-actin. ***B,*** OVCAR-3 and A2780/CP70 cells were cultured in a 100 mm dish at a density of 1×10^6 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 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 LY294002 for 12 h and 24 h as indicated. The proteins were analyzed by immunoblotting as described above.

Fig. 3. AKT is a downstream target of PI3K in mediating the cell cycle progression. ***A-B,*** Effects of LY294002 on the phosphorylation of AKT and ERK. OVCAR-3 and A2780/CP70 cells were cultured for 24 h in a 100 mm dish at a density of 1×10^6 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator, followed by starvation for 16 h using serum-free media. Cells were switched to the media in the absence or presence of 10% FBS and LY294002 as indicated (20 μM) for 30 min, 1 h, and 2 h, respectively. The whole cell lysates were prepared and subjected to immunoblotting assay using specific antibodies against phospho-AKT at Ser473 and phospho-p44/42 ERK1/2. ***C-D,*** After the cells were cultured in serum-free medium for 16 h, the cells were switched to the media in the absence or presence of 10% FBS and LY294002 as indicated for 12 h (***C***), or 24 h (***D***). The phospho-AKT at Ser473 and total AKT were analyzed by immunoblotting as described above. ***E,*** Over-expression of an active form of AKT reverses the inhibition of PI3K that had led to cell cycle arrest in G_1 in OVCAR-3 cells. The cells were cultured in a 100 mm dish at a density of 1×10^6 cells/dish in RPMI1640 media supplemented with 10% FBS for 24 h at 37°C in a 5% CO_2

incubator. The cells were transfected with GFP alone or GFP-Myr-AKT expression plasmids and cultured for 36 h. The cells were then cultured in the absence or presence of LY294002 (10 μM) for 12 h. The cells expressing GFP proteins were separated and analyzed by FACS analysis. Typical cell cycle histograms were recorded.

Fig. 4. The activation of p70S6K1 was affected by PI3K and mTOR. ***A,*** The phosphorylation of p70S6K1 was inhibited by LY294002. OVCAR-3 and A2780/CP70 cells were cultured and subjected to serum starvation for 16 h, followed by incubation in the presence or absence of 10% FBS, and/or LY294002 for 12 h. The total cellular protein extracts were prepared and analyzed for the phosphorylation of p70S6K1 using specific antibodies against phospho-p70S6K1 at Ser389 and phospho-p70S6K1 at Ser421/Thr424, and against p70S6K1 as a control. ***B,*** OVCAR-3 cells were transfected with pNeoSRα vector alone, or pNeoSRα-Myr-AKT plasmids, followed by the G418 selection for two weeks. The cells expressing vector alone or Myr-AKT were incubated with the solvent, 10 μM, or 20 μM of LY294002 for 12 h. The levels of total p70S6K1 and phosphorylation of p70S6K1 at Ser389 were analyzed by immunoblotting as described above. ***C,*** The cells were cultured and subjected to serum starvation as described above. The cells were switched to the media in the presence or absence of 10% FBS and mTOR inhibitor rapamycin (10 ng/ml and 20 ng) for 12 h. The total cellular protein extracts were prepared and subjected to immunoblot assay using specific antibodies against phospho-p70S6K1 at Ser389, and at Ser421/Thr424, and total p70S6K1.

Fig. 5. PI3K signaling regulated cell cycle progression through p70S6K1. ***A,*** Effects of rapamycin on protein expression associated with G_1 cell cycle in OVCAR-3 and A2780/CP70

cells. The cells were cultured and subjected to serum starvation for 16 h as described above. The cells were switched to the media in the presence or absence of 10% FBS and rapamycin (10 ng/ml and 20 ng) for 12 h. The total cellular protein extracts were prepared and subjected to immunoblot assay. The protein expression associated with G_1 cell cycle was studied using specific antibodies against phospho-Rb at Ser795, Ser780, and Ser807/811, Rb, cyclin D1, Cdk4, Cdk6, Cdc25A, p21, p16, and β-actin. ***B,*** OVCAR-3 cells were cultured in the media supplemented with 10% FBS for 24 h at 37°C in 5% CO_2 incubator, followed by the addition of solvent alone, 10 ng/ml, or 20 ng/ml of rapamycin for 12 h. The cell cycle associated proteins were analyzed by immunoblotting as described above. ***C,*** Over-expression of an active form of p70S6K1 reversed the inhibition of PI3K leading to G_1 arrest in OVCAR-3 cells. The cells were co-transfected with GFP:active p70S6K1 (S6K1-E389D3E) expression plasmids (1:5) and cultured for 36 h. The cells were then cultured in the absence or presence of LY294002 (10 μM) for 12 h. The cells expressing GFP proteins were separated from untransfected cells by FACS. Typical cell cycle histograms were recorded. ***D,*** Expression of an active form of p70S6K1 reversed the inhibition of G_1cell cycle progression induced by rapamycin in OVCAR-3 cells. The cells were transfected with GFP alone or with GFP/p70S6K1 plasmids as described above, then cultured for 36 h after transfection. The cells were treated with or without 10 μM rapamycin for 12 h. The cells expressing GFP were separated from the un-transfected cells by FACS analysis, and the cell cycle progression was analysed as described in Fig. 1B.

Fig. 6. Schematic model for a mechanism of PI3K/AKT/mTOR/p70S6K1 signaling pathway involved in cell proliferation and G_1 cell cycle progression in ovarian cancer cells. The activation of the PI3K signaling pathway in human ovarian cancer cells induces the

activation of AKT and PDK1. AKT and PDK1 transmit the PI3K activation signal to p70S6K1, which in turn induces G_1 cell cycle progression and inhibits $p16^{INK4a}$ expression in the ovarian cancer cells.

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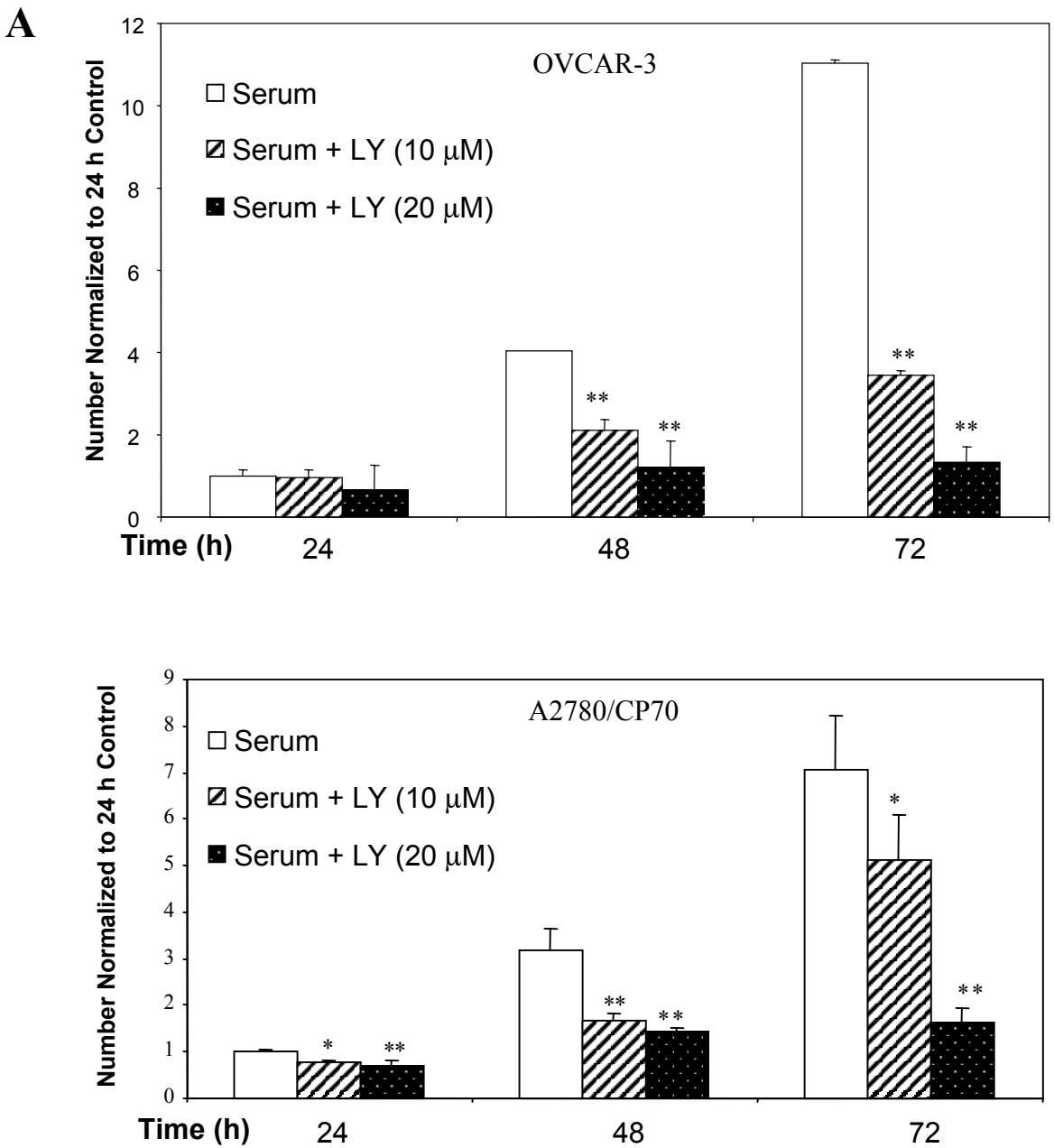

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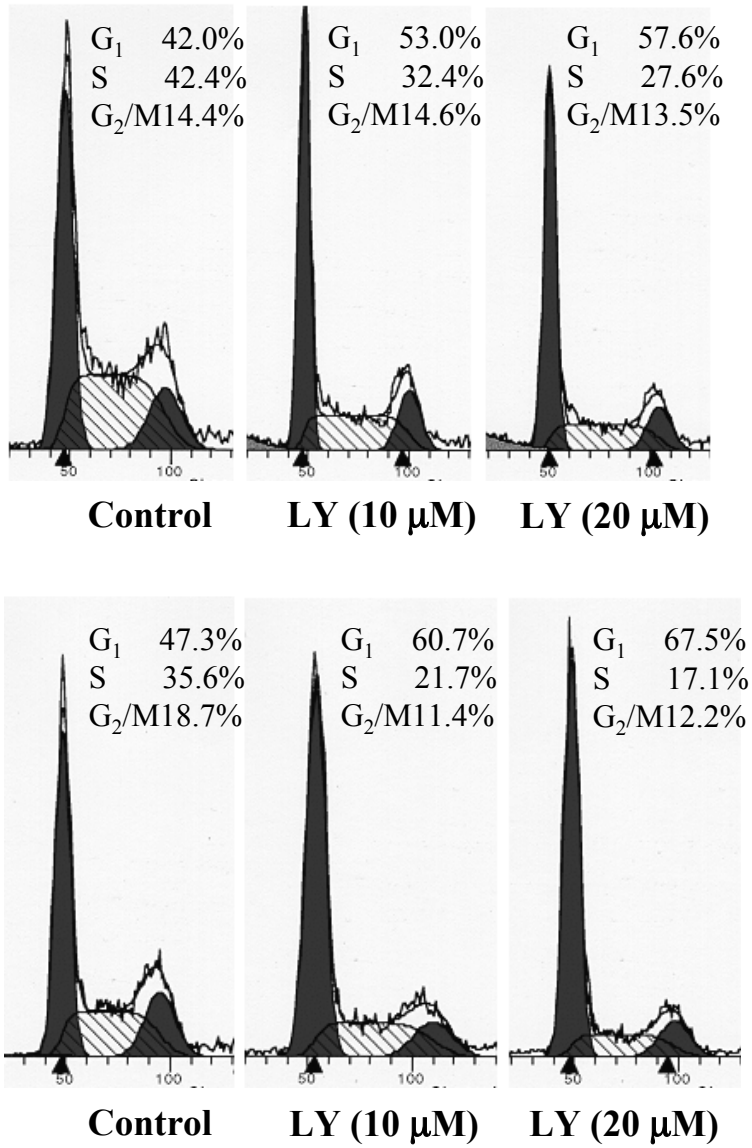

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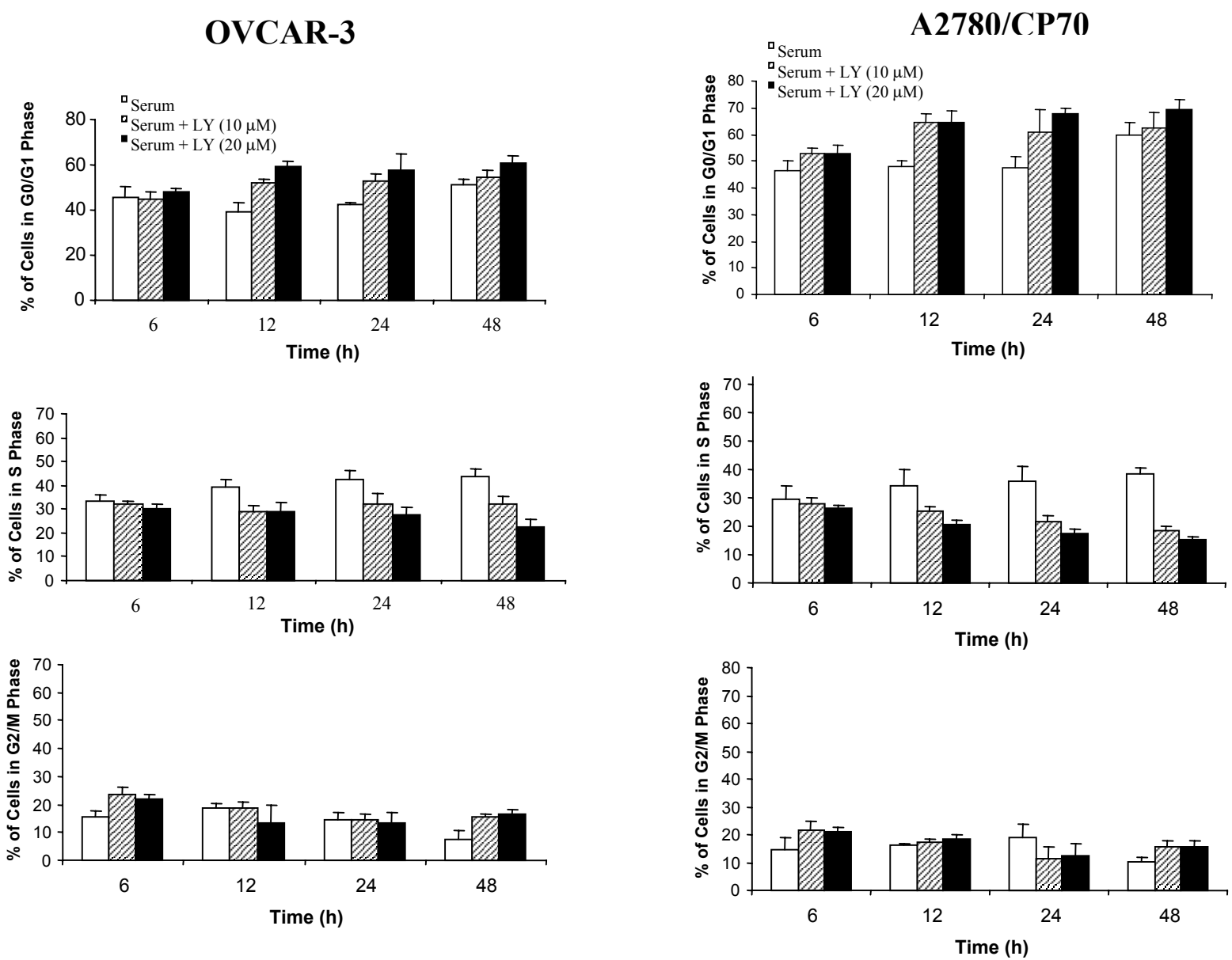

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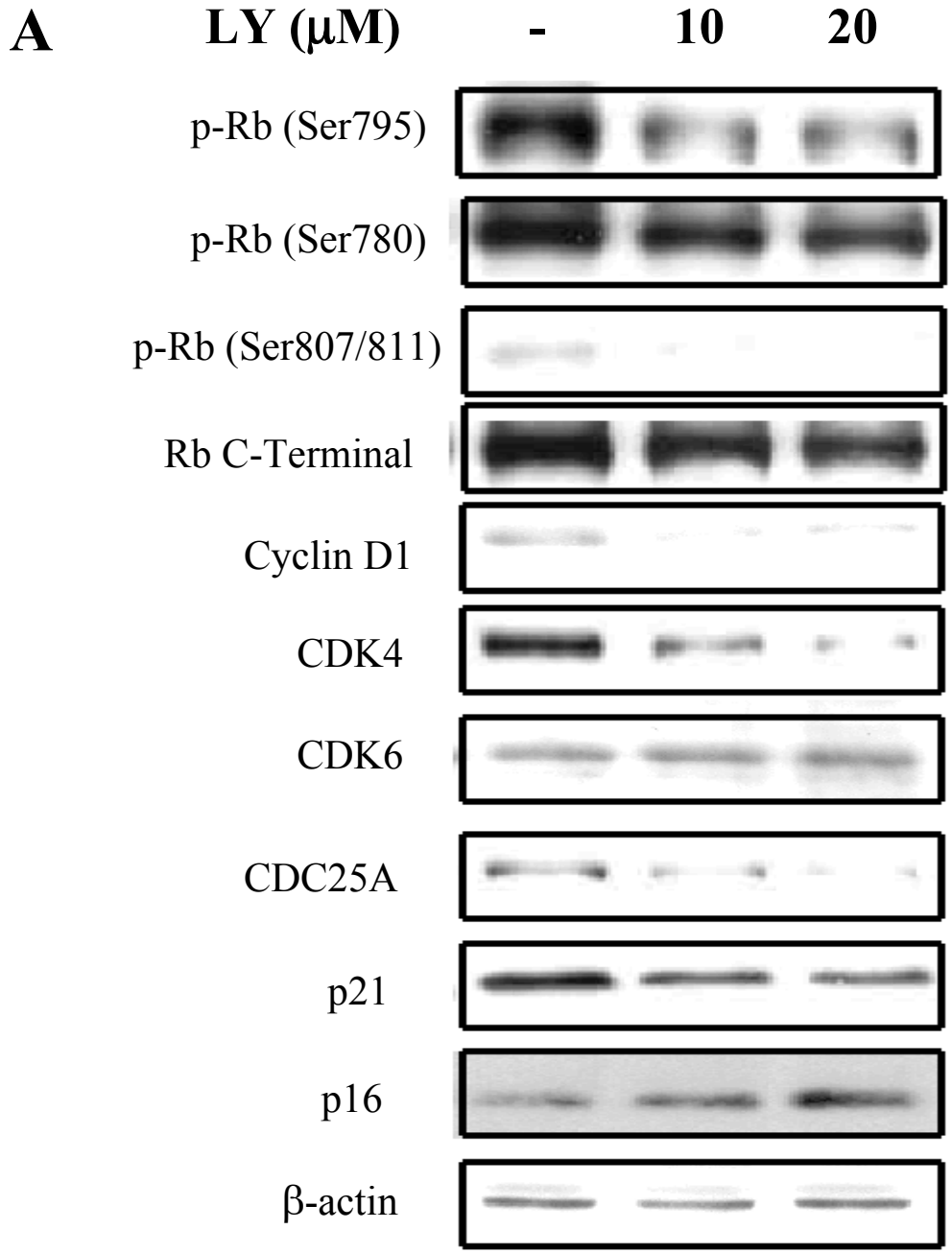

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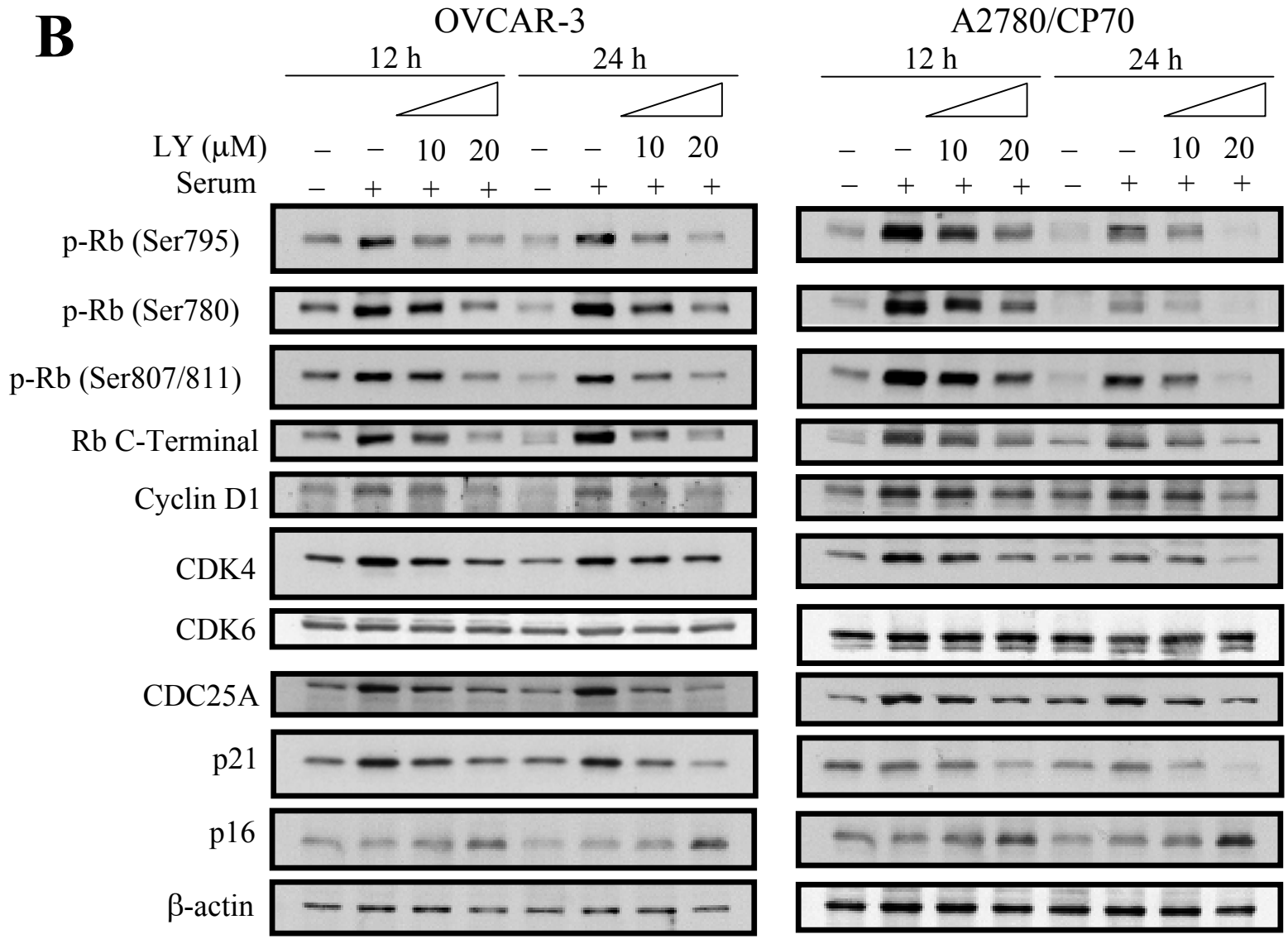

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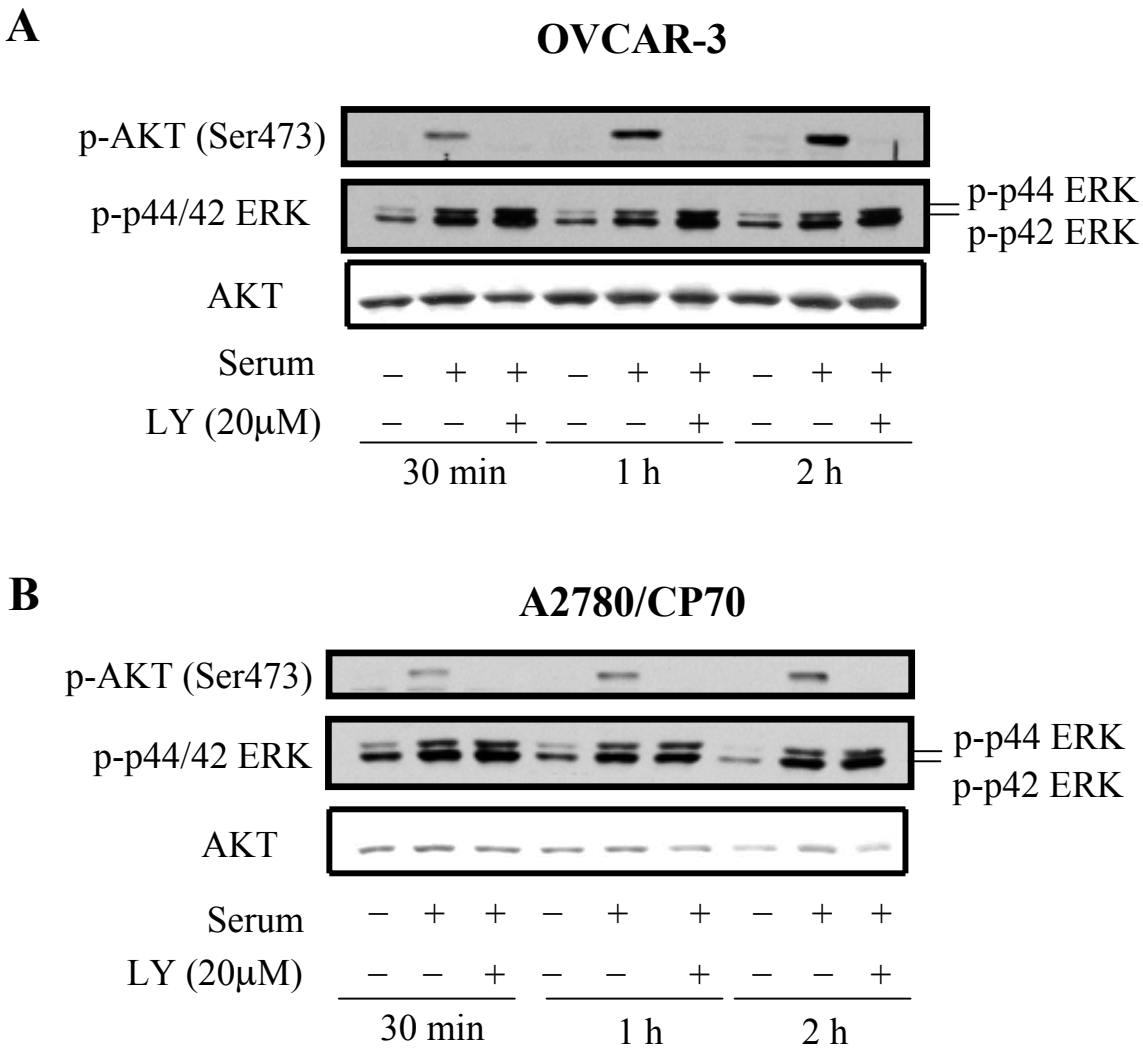

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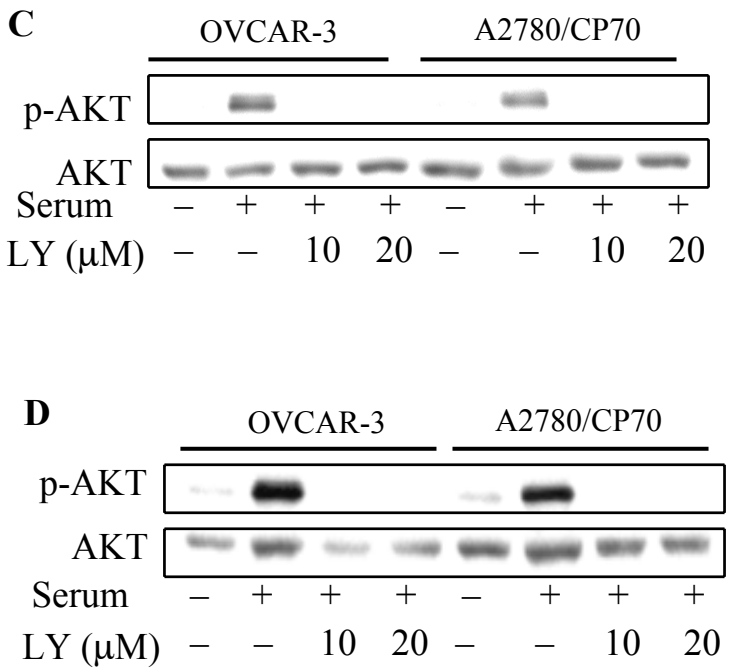
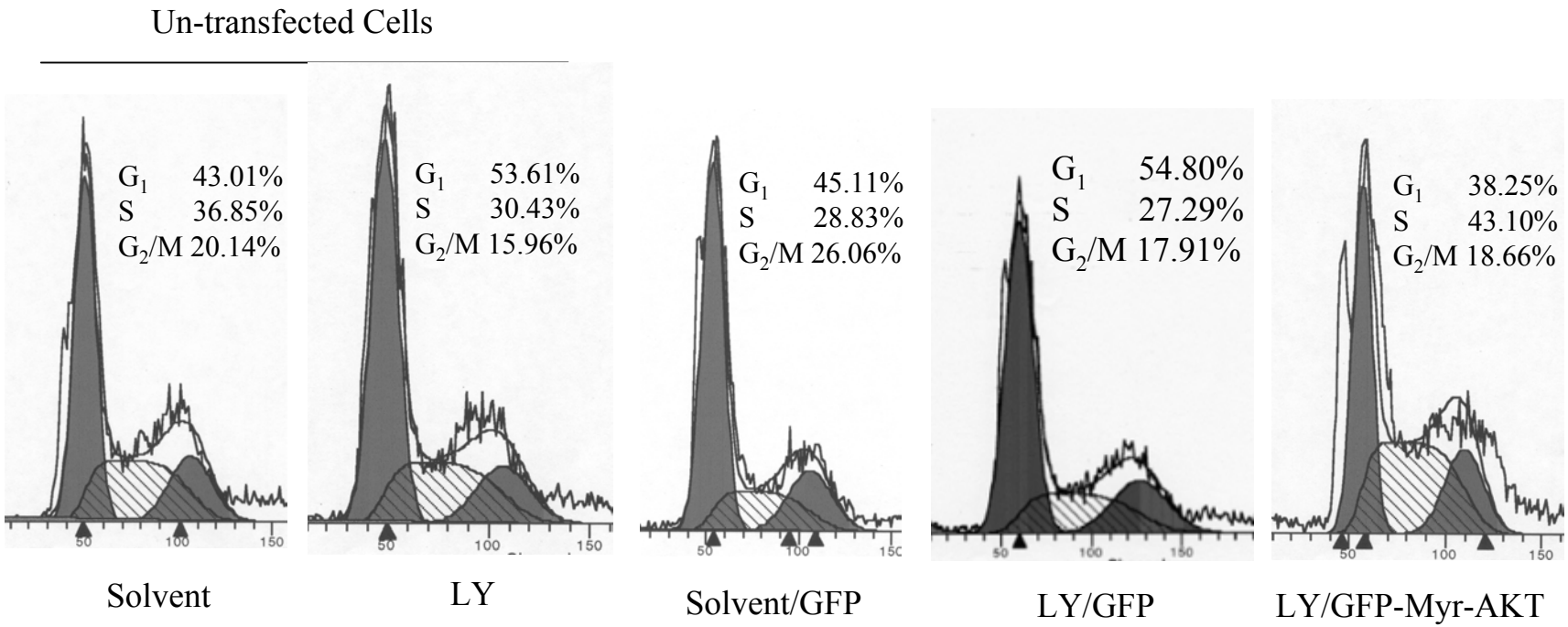
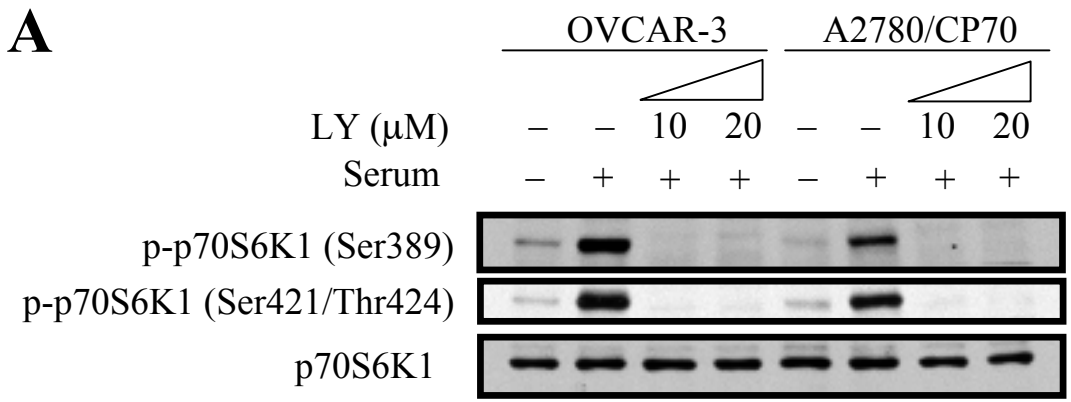


Gao et al., Figure 3

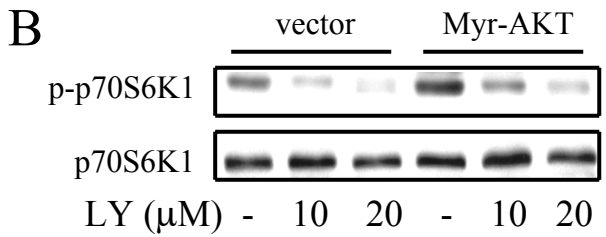
E

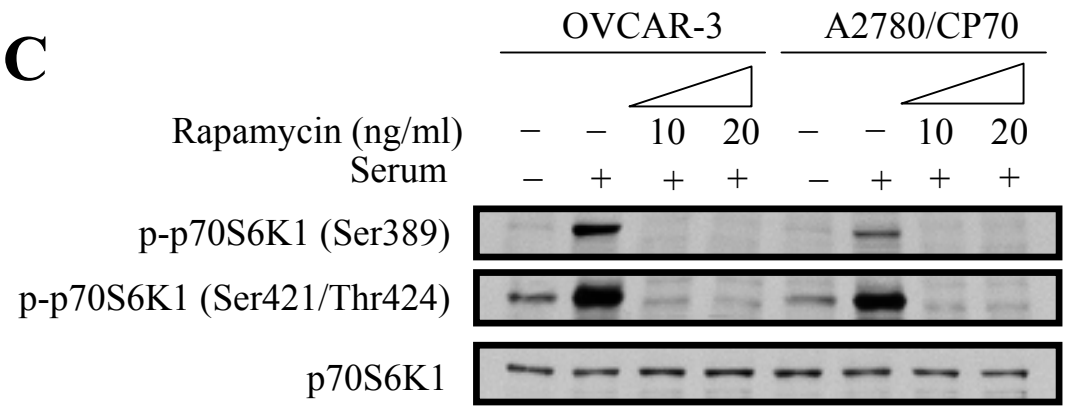


Gao et al., Figure 3

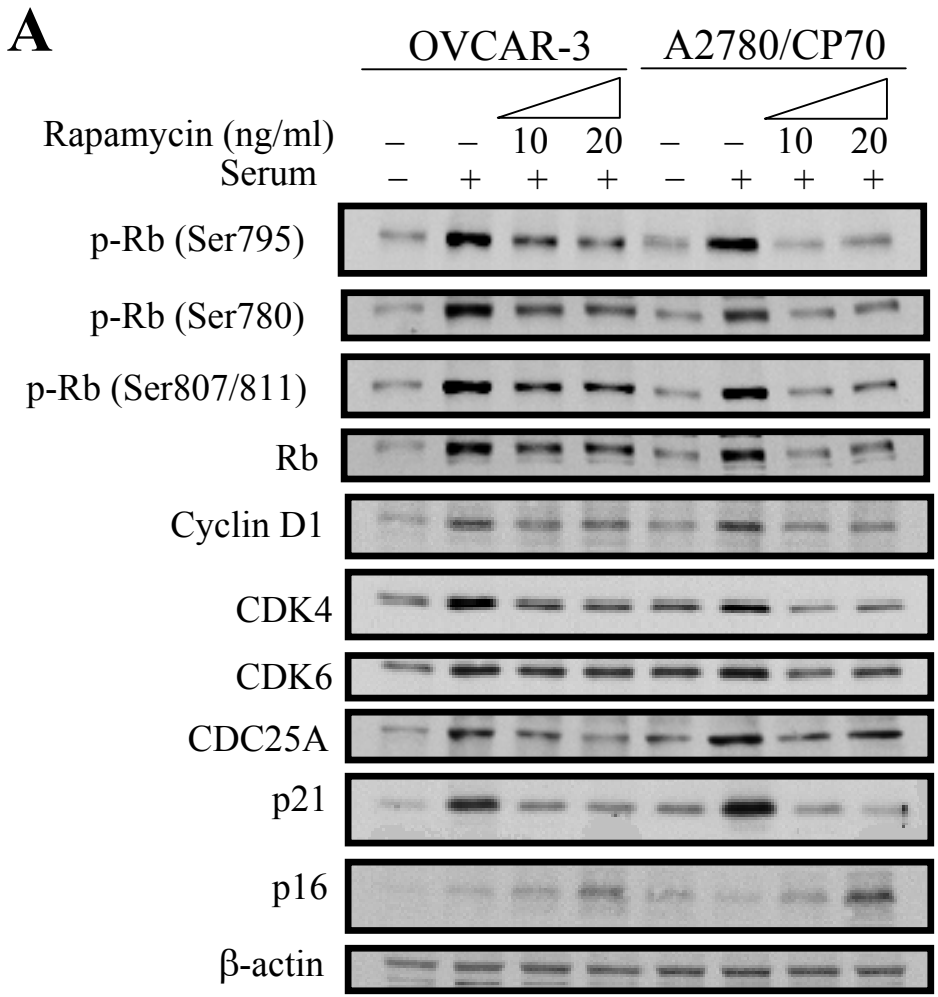


Gao et al., Figure 4

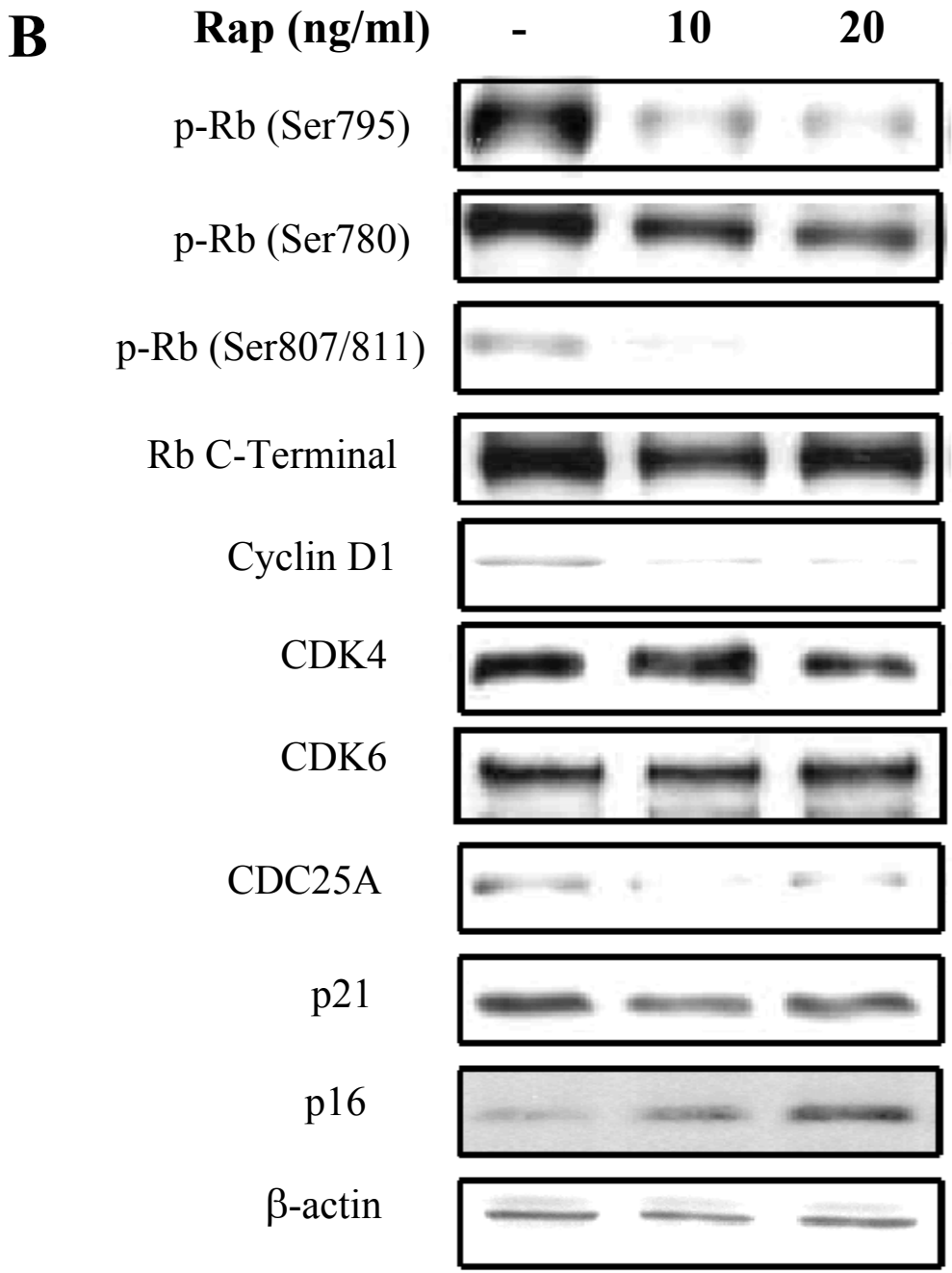




Gao et al., Figure 4



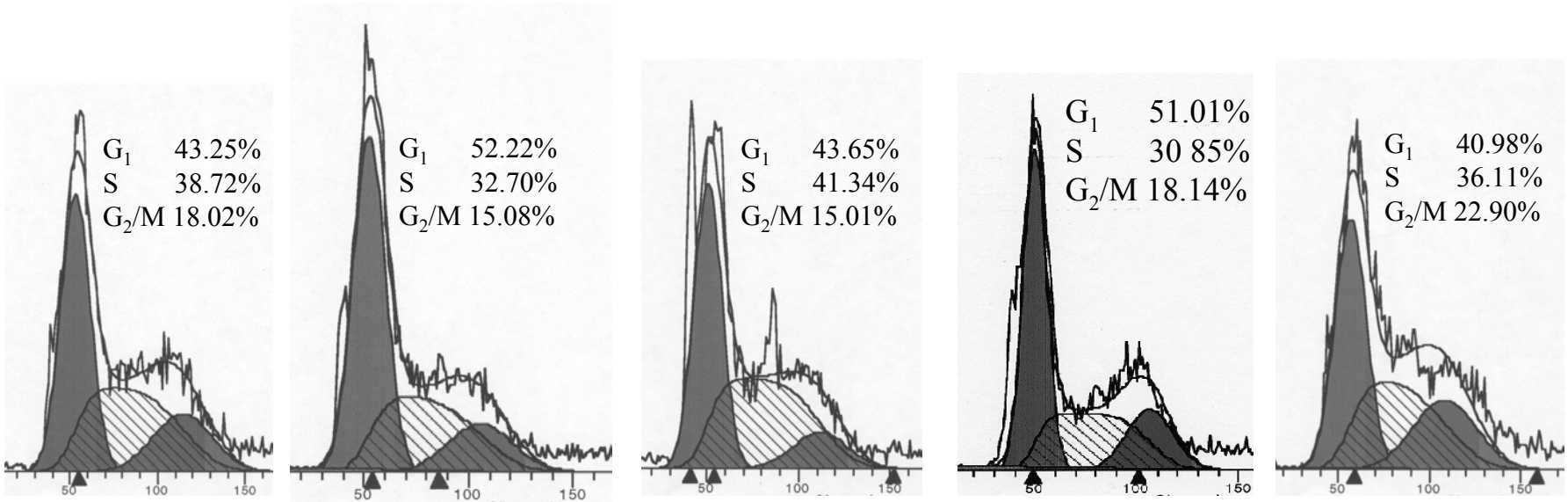
Gao et al., Figure 5



Gao et al., Figure 5

C

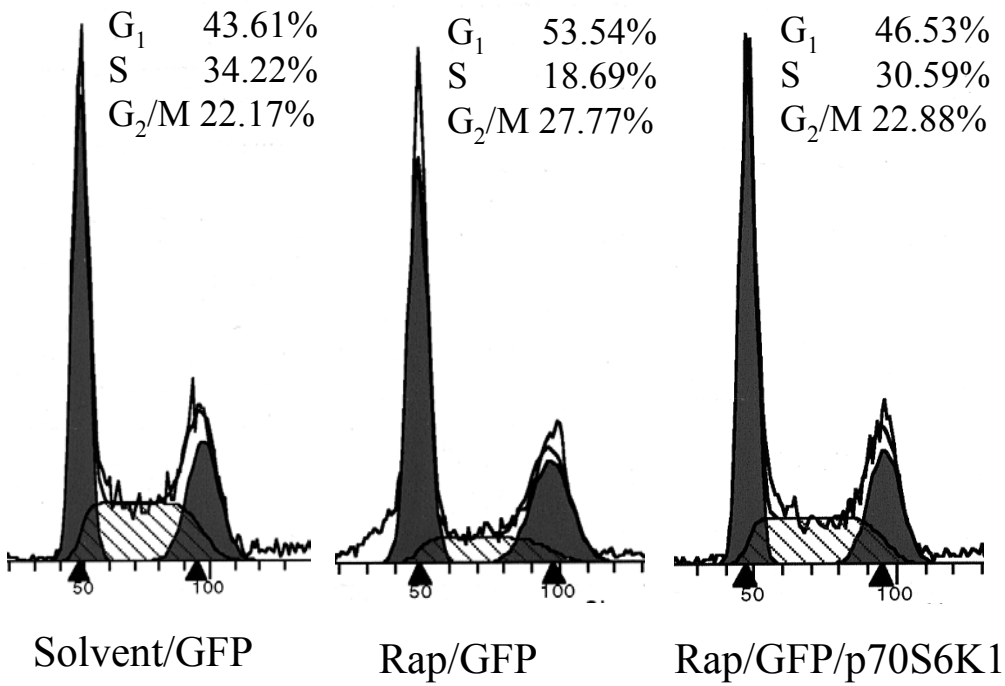
Un-transfected Cells



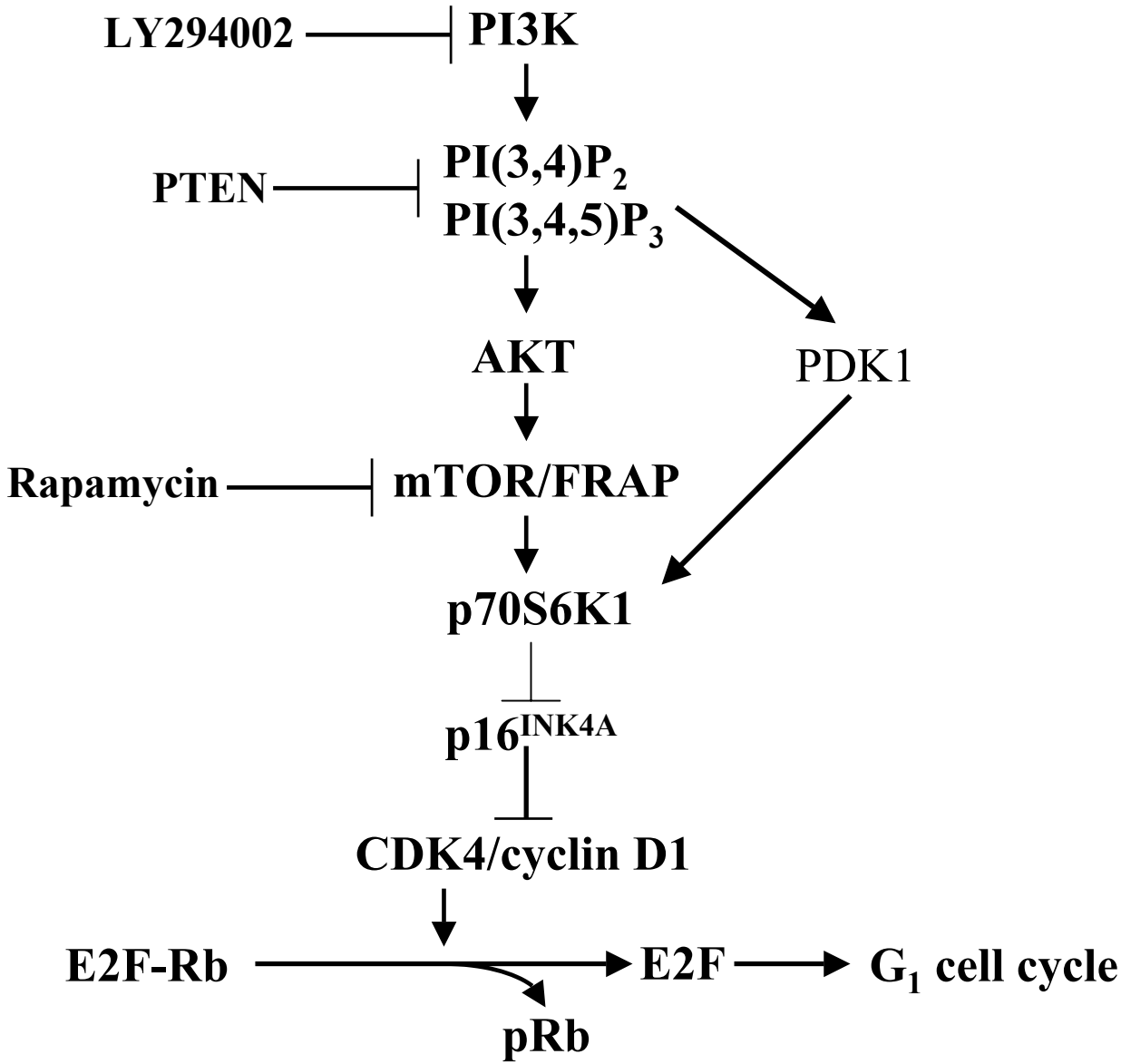
Solvent LY Solvent/GFP LY/GFP LY/p70S6K1

Gao et al., Figure 5

D



Gao et al., Figure 5



Gao et al., Figure 6