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Vanadate activated Akt and promoted S phase entry

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Abstract

Protein kinase B (PKB)/Akt and its upstream signal transducer, phosphatidylinosito-3 kinase (PI3K) play an essential role in control of transcription and translation, which impact cell growth, survival, and metabolism. Transcription factor E2F is a component of the downstream proliferative machinery regulated by Akt. Hyperphosphorylation of retinoblastoma protein (pRb), a pocket protein, leads to release of E2F1, resulting in transition from G_1 to S phase. The present study shows that in normal C141 cells, vanadate treatment increased the percentage of cells at S phase and elevated cyclin E and cyclin A expression. Vanadate treatment triggered phosphorylation of pRb and release of E2F1. Furthermore, vanadate increased Akt kinase activity and caused its phosphorylation at Ser473 and Thr308. Inhibition of Akt by either inhibitors or transfected cells with dominant negative kinase mutant or dominant negative phosphorylation mutant decreased the percentage of the cells at the S phase induced by vanadate, and reduced both cyclin E and E2F1 expression and phosphorylation of pRb. The present study indicates that Akt plays an essential role in vanadate-induced increase in cell number at S phase and transition from G_1 to S phase through E2F-pRb pathway. (Mol Cell Biochem **255**: 227–237, 2004)

Key words: vanadate, PI3K, Akt, pRb, E2F1, cell cycle regulation

Introduction

Mammalian cells require an extracellular proliferative signal directly after mitosis in order to keep on growing and dividing. When cells are faced with a lack of such a signal, they will either die or go into growth arrest in a postmitotic G_1 phase. One of the important intracellular signaling pathways that transduces such proliferative signals is the phosphatidylinositol 3-kinase (PI3K) pathway [1]. The proto-oncogene protein kinase B (PKB), also known as Akt, is a major target of the PI3K signaling pathway that controls cell proliferation. This protein is also involved in anti-apoptotic signaling as well as cell cycle control [2]. PKB/Akt is the cellular homologue of the transforming viral oncogene v-Akt and bears significant homology to PKA and PKC [3]. The three mammalian isoforms, α, β and γ, all contain an N-terminal PH domain, a central kinase domain with an activation-loop Thr308 phosphorylation site, and a conserved, regulatory serine phosphorylation site, Ser473 near the C terminus [4]. For full activation of the Akt kinase requires the phosphorylation at both of these sites is required [4].

Although there has been recent progress in elucidating the mechanisms by which PKB contributes to the regulation of apoptosis, relatively little is known concerning its role in the regulation of cell cycle progression [2]. One recent study has demonstrated that the cell-cycle regulator E2F is a component of the downstream proliferative machinery regulated by Akt [5]. One of the key events in G_1 phase is the activation of E2F, which in turn binds to promoters and trans-activates various genes critical for cell cycle progression, such as cyclin

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E [2]. E2F can activate expression of the S phase regulatory genes necessary for initiating DNA replication [6] and are important regulators of the G_1/S cell cycle transition. E2F acts as a repressor protein when complexed with a member of the retinoblastoma (Rb) family of pocket proteins [7, 8]. The continuation of cell proliferation at various stages of the cell cycle involves inactivation of at least one of three members of the Rb family [1]. The pRb protein is an essential component of the G_1/S checkpoint [9]. pRb is present at relatively constant levels throughout the cell cycle but is hyperphosphorylated by cyclin/cdk complexes and released from E2F1 at the G_1/S transition, allowing continuation through the cell cycle [10].

Vanadate is widely found in occupational and environmental systems. Epidemiological studies have shown a correlation between vanadium exposure and the incidence of lung cancer in humans [11–13]. Vanadium compounds were reported to modify DNA synthesis and repair [11, 13, 14], and induce mutations and DNA-protein crosslinks [14–18]. Vanadate is able to induce an increase in cell number at the G_2/M phase through reactive oxygen species (ROS) in A549 cells [19]. A recent study from our group also indicated that vanadate caused p53-dependent S phase cell number increase in mouse epidermal C141 cells [20]. Our study has shown that vanadate induced expression of both hypoxia-inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) through PI3K/Akt pathway in DU145 human prostate carcinoma cells and ROS play an important role (unpublished observations).

The purposes of the present study are: (a) to investigate whether vanadate is able to promote S phase entry; (b) to study the involvement of cell cycle regulatory proteins in the vanadate-induced cell number increase at S phase; (c) to examine whether vanadate is able to activate Akt; and (d) to explore the effect of Akt on vanadate-promoted S phase entry and its regulation.

Materials and methods

Chemicals

Sodium metavanadate was from Aldrich (Milwaukee, WI, USA). RNase A and Eagle's minimal essential medium (MEM) were from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Gibco BRL (Life Technologies, Gaithersburg, MD, USA). LY294002 and wortmanin were from Calbiochem (San Diego, CA, USA). Dominant negative Akt1 cDNA plasmid was from Upstate (Lake Placid, NY, USA). Antibodies against cyclin E, cyclin A and E2F-1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Rb, Akt and second AP linked anti-rabbit IgG, and Akt kinase assay kit were from Cell Signaling (Beverly, MA, USA).

Cell culture

The JB6 P^+ mouse epidermal cell line, its stable transfection with dominant negative Akt phosphorylation sites mutant (SRα-Akt-T308A/S473A) cells, DN/P cells, and its stable transfection with dominant negative Akt kinase domain mutant (L179M) cells, DN/K cells were cultured in MEM medium containing 5% FBS, 2 mM L-glutamine and 1000 U/ml penicillin-streptomycin in an incubator at 5% CO_2 and 37°C.

Measurement of cell cycle/DNA content

DNA content in G_1/S, G_2/M phase was measured using flow cytometry [21, 22]. C141 cells, DN/K cells and DN/P cells were fixed and permeabilized with 70% ice-cold ethanol, and incubated with the freshly prepared staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml RNase A, and 20 μg/ml PI) for 15 min at room temperature. Cell cycle analysis was performed by flow cytometry with at least 10,000 cells for each sample. The histogram was abstracted and the percentage of cells in the G_1/S and G_2/M phase was then calculated using ModFit LT software.

Western blot analysis

The cells were seeded in 100 mm dishes. Cells were lysed 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 on ice for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the protein extract. The protein concentration was determined using Bio-Rad protein assay reagent (Richmond, CA, USA). The protein extracts were run by Tris-Glycine SDS gel electrophoresis, and transferred to a PVDF membrane. Western blotting was performed using primary antibodies against cyclin E, cyclinA, E2F, phospho-Rb, Akt and a secondary antibody against anti-rabbit IgG. After reaction with ECF substrate, the signal was detected using a Storm Scanner (Molecular Dynamics, Sunnyvale, CA, USA). The same amount of total protein was loaded in each analysis.

Akt kinase assay

Cells were rinsed once with ice-cold PBS, scraped from the plates, and centrifuged at 4,000 rpm for 5 min. The cell pellets were incubated for 10 min on ice in lysis buffer (150 mM

NaCl, 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) supplemented with 1 mM PMSF, and centrifuged at 11,000 rpm for 10 min to clarify the supernatants. 200 μg of protein extracts were incubated with 20 μl of resuspended immobilized Akt antibody slurry gentle rocking 3 h at 4°C. The pellets were washed twice with lysis buffer and kinase buffer (25 mM Tris (pH 7.5), 5 mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$), respectively. Then the pellets were suspended in 40 μl kinase buffer supplemented with 200 μM ATP and 1 μg GSK-3 fusion protein, and were incubated 30 min at 30°C. 20 μl 3 × SDS sample buffer (187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromphenol blue) was added to terminate the reaction, followed by centrifugation at 11,000 rpm for 2 min. The supernatant was transferred to a new tube and boiled for 5 min. Western blotting was performed to probe for phospho-GSK-3α/β antibody. The same amount of total protein was loaded in each analysis.

Results

Effects of vanadate on cell cycle in C141 cells

DNA content measured by flow cytometry was used to analyze the percentage of the cells in S phase. Figure 1, panel A shows that in C141 cells 50 μM vanadate causes a time-dependent increase in S phase from 0–24 h. The percentage of the cells at S phase is 7.6% at beginning of vanadate stimulation and 34.8% at 24 h, respectively. Similarly, a dose-dependency is observed from 0 to 50 μM vanadate treatment (panel B). The results thus show that vanadate increased the DNA synthesis and promoted S phase entry.

Effects of the vanadate on cell growth regulatory proteins in C141 cells

To study the effects of vanadate on cell growth regulatory proteins, Western blotting was used to measure the expression of

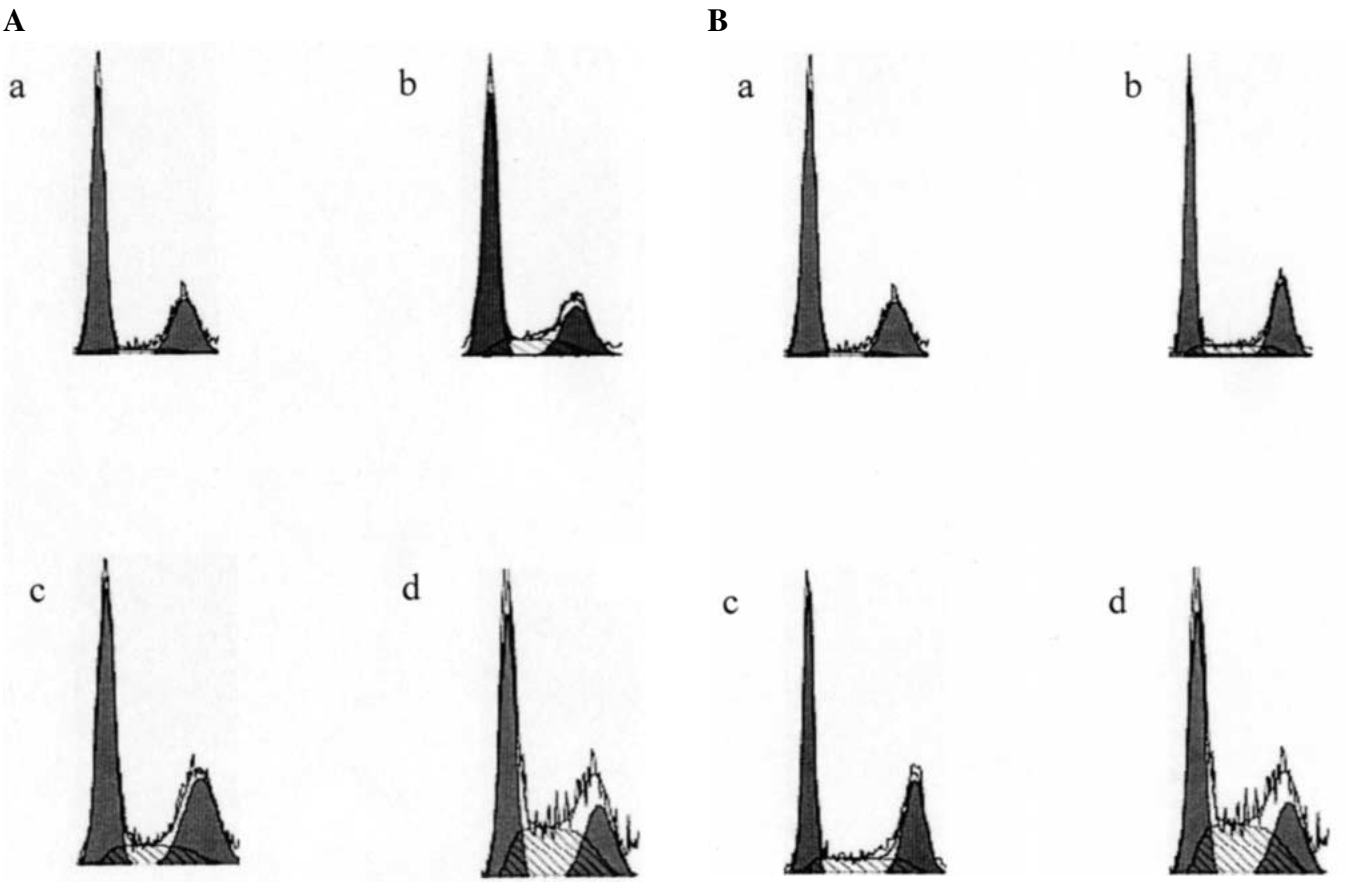

Fig. 1. Effects of vanadate on cell number increase at S phase in C141 cells. C141 cells were seeded in 5% fetal bovine serum (FBS) MEM in a 100 mm dish. After 80% confluence, cells were treated with 50 μM vanadate for a different period of time: a – control; b – 6; c – 12; d – 24 h (Fig. 1, panel A), or with a – control without vanadate; b – 10; c – 25; d – 50 μM vanadate for 24 h (Fig. 1, panel B). DNA content was measured by flow cytometry.

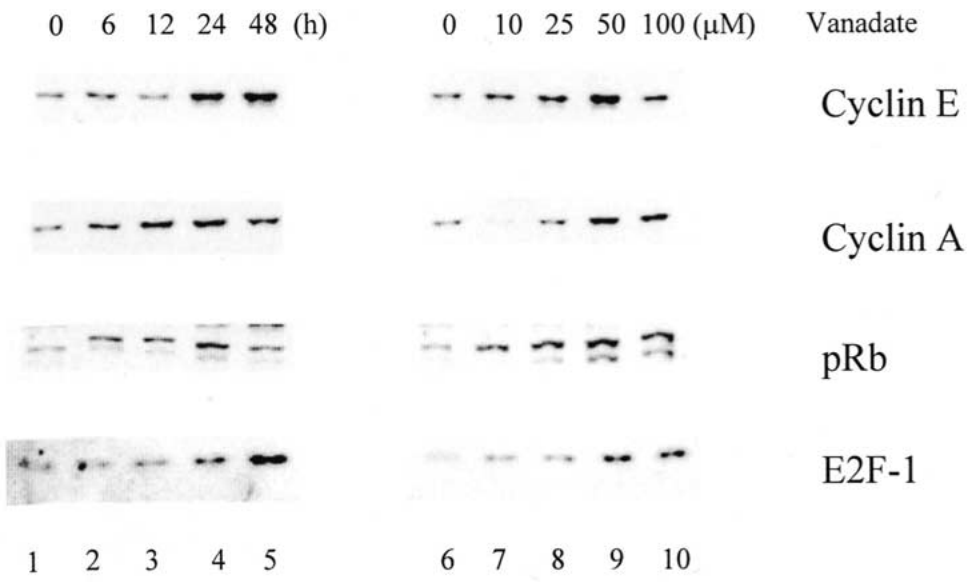

Fig. 2. Effects of vanadate on cell growth regulatory proteins in C141 cells. The cells were treated with 50 μM vanadate for 6, 12, 24, and 48 h and 10, 25, 50, and 100 μM for 24 h. The whole cell lysates were collected for western blotting using specific antibodies against cyclin E, cyclin A, E2F-1 and phospho-Rb. Lanes 1 and 6, control; lane 2, 50 μM, 6 h; lane 3, 50 μM, 12 h; lanes 4 and 9, 50 μM, 24 h; lane 5, 50 μM, 48 h; lane 7, 10 μM, 24 h; lane 8, 25 μM, 24 h; and lane 10, 100 μM, 24 h. Data are from a single preparation representative of three independent experiments.

cyclin E, cyclin A, and E2F1, and phoshpo-Rb in C141 cells. Figure 2 shows that vanadate caused a time- and dose-dependent increase in cyclin E, cyclinA, and E2F1 level, and phosphorylation of Rb.

Effects of vanadate on Akt in C141 cells

Both the kinase assay and Western blotting were used to examine the effects of vanadate on both Akt kinase activity and its phosphorylation in the present study. As shown in Fig. 3, 50 μM vanadate caused a time-dependent increase in Akt activity (phospho-GSK-3α/β) from 1 to 120 min (panel A). Similarly, Akt activity was increased with the increase in vanadate dose (panel A, lanes 7–11). In addition, the phosphorylation of Akt at both Ser473 and Thr308 sites occurred in vanadate-treated cells (panel B). Phosphorylation of Akt increased with the vanadate treatment time (0–240 min at 50 μM). Similar results were obtained when the cells were treated with different doses of vanadate (from 0–100 μM for 120 min).

Effects of Akt on the percentage of cells in S phase

As shown in Fig. 4, panel A, the two PI3K inhibitors, LY294002 and wortmanin significantly inhibited vanadate-promoted S phase entry in C141 cells. As the dose of inhibitors increased, the percentage of cells in S phase decreased. In DN/K cells, although vanadate caused an increase in the number of cells in S phase in a time- and dose-dependent manner, the fold of increase was significantly reduced (panels B and C) compared with that obtained in wild type cells (Fig. 1, panels A and B, S phase range, 7.6–34.8%). The percentage of S phase is 3.8% in untreated cells and the highest is 8.3% in cells treated with 50 μM for 48 h (panel B). A similar effect was also observed in panel C as the cells were treated with different doses (panel C). In the DN/P cells, the percentage of S phase increased from 4.8–15.1% when cells were treated with 50 μM vanadate for 24 h compared to control cells without stimulation (panel D). In the panel E, the percentage of cells at S phase is 4.4, 5.6, 8.1, 11.6, and 13.4% when the cells were stimulated with vanadate at 0, 10, 25, 50, and 100 μM for 24 h, respectively. The percentage of S phase in DN/P cells is between that of wild type cells (Fig. 1, panels A and B, S phase range, 7.6–34.8%) and that of DN/K cells (Fig. 4, panels B and C).

Effects of Akt on cell growth regulatory proteins

Both LY294002 and wortmanin reduced vanadate-induced cyclin E, cyclin A, and E2F1 expression, and the phosphorylation of Rb in C141 cells with different potencies (Fig. 5, panel A, lanes 3–6). Both the kinase mutant and the phosphorylation mutant partially reduced vanadate-induced cyclin E expression (Fig. 5, panels B and C). Cyclin E levels increased

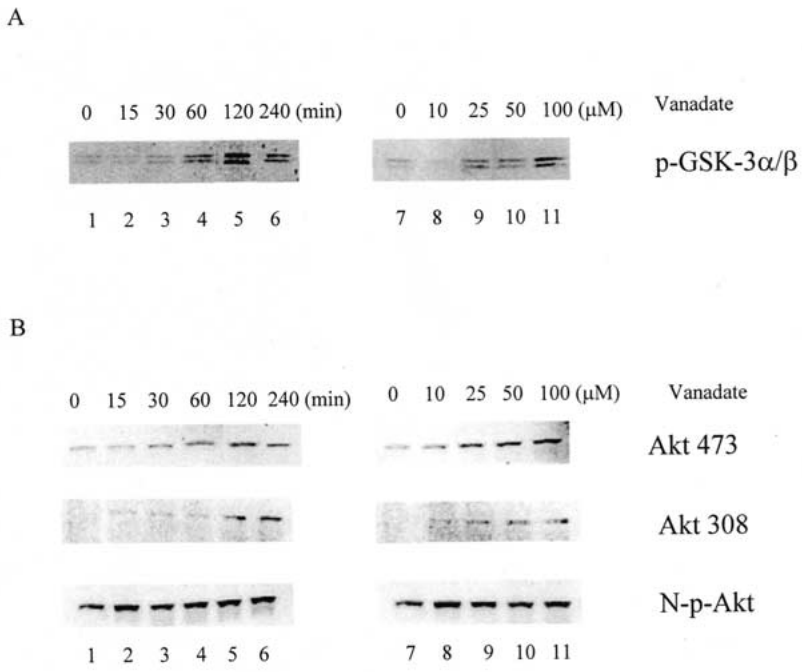

Fig. 3. Effects of vanadate on Akt. The cells were treated with 50 μM vanadate from 0 to 240 min and 10, 25, 50, and 100 μM for 60 min. The whole cell lysates were used to measure the kinase activity and phosphorylation. Panel A and panel B represent Akt kinase activity and phosphorylation of Akt, respectively. Lanes 1 and 7, control; lane 2, 50 μM 15 min; lane 3, 50 μM, 30 min; lanes 4, 50 μM, 60 min; lanes 5 and 10, 50 μM, 120 min; lane 6, 50 μM, 240 min; lane 8, 10 μM, 120 min; lane 9, 25 μM, 120 min; and lane 11, 100 μM, 120 min. Data are from a single preparation representative of three independent experiments.

in response to vanadate treatment in DN/P cells is between that in C141 wild type cells (Fig. 2) and DN/K cells (Fig. 5, panel B and C). In both DN/K and DN/P cells, cyclin A levels had no observable change compared to the C141 wild type cells (Fig. 2). In DN/K cells, both E2F1 expression and phosphorylation of pRb stay at the same low level regardless of vanadate treatment. While E2F1 expression and phosphorylation of pRb slightly increased in DN/P cells treated with vanadate compared to those in wild type cells (Fig. 2), they remained in very low levels.

Effects of PI3K on Akt activity

PI3K is an upstream kinase of Akt. We examined the effect of PI3K on Akt activity. As shown in Fig. 6, wortmanin and LY294002 inhibited Akt activity in vanadate-treated C141 cells (panel A, lanes 3–6). Vanadate had no effect on Akt activity in DN/K cells regardless of the treatment times and doses (panel B, lanes 1–11). Akt activity was slightly increased in DN/P cells treated with vanadate (panel B, lanes 1–11), although the percentage of increase was much reduced compared to that in wild type cells (Fig. 3, panel A).

Effectsof PI3K on Akt phosphorylation

Both LY294002 and wortmanin completely blocked the phosphorylation of Akt at Ser473 (Fig. 7, panel A). LY294002 had no significant inhibitory effect on the phosphorylation of Akt at Thr308 (lanes 3 and 4). In DN/K cells vanadate-induced phosphorylations at Ser473 and Thr308 (Panels B and C, left part) were much lower than that in wild type cells (Fig. 3). In DN/P cells, vanadate failed to activate phosphorylation of Akt at these two major sites (panel B and C, right part).

Discussion

Vanadate compound is widespread in both environmental and biological systems. It exerts toxic and carcinogenic activity. It has been reported that vanadium can regulate growth factor-mediated signal transduction pathways and promote cell transformation [23, 24]. Workers occupationally exposed to vanadium are at risk as respirable particulates may penetrate deep into pulmonary tract. Epidemiological studies have shown a correction between vanadium exposure and lung cancer in humans [15, 17].

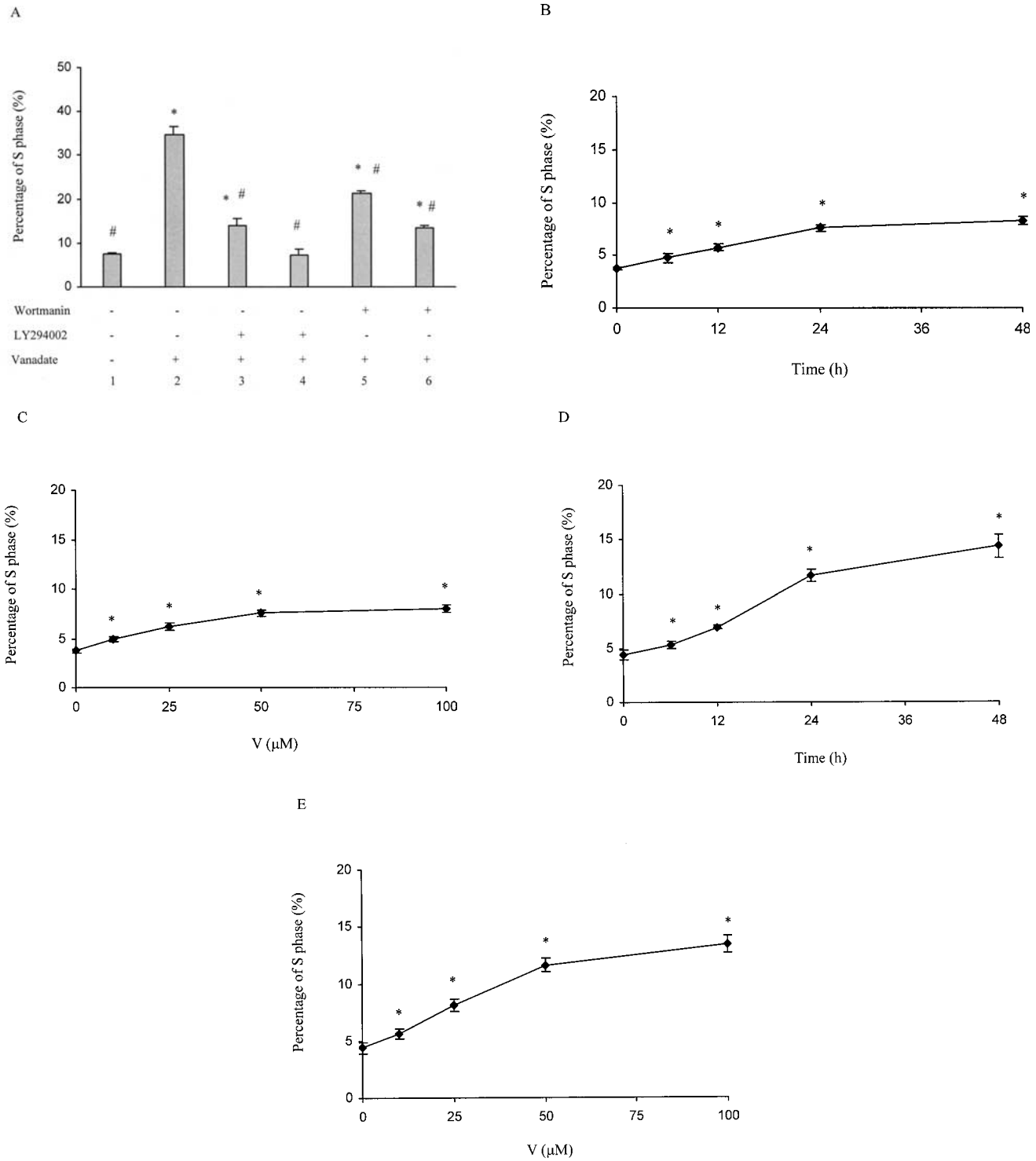

Fig. 4. Effects of Akt on S phase entry. The C141 cells were pretreated with different concentrations of LY294004 and wortmanin for 30 min prior to vanadate treatment (50 μM for 24 h). Both DN/K cells and DN/P cells were treated for different amounts of time (6, 12, 24, and 48 h) and different doses of vanadate (10, 25, 50, and 100 μM for 24 h). DNA content was used to measure the percentage of the cells at S phase. Panel A: lane 1, control; lane 2, 50 μM vanadate; lane 3, 50 μM vanadate + 10 μM LY; lane 4, 50 μM vanadate + 20 μM LY; lane 5, 50 μM vanadate + 50 nM wortmanin; and lane 6, 50 μM vanadate + 100 nM wortmanin. Panel B and panel D represent time-dependency in both DN/K cells and DN/P cells, respectively. The cells were treated with 50 μM vanadate for 0, 6, 12, 24, and 48 h. Panel C and panel E represent dose-response in both DN/K cells and DN/P cells, respectively. The cells were treated with 0, 10, 25, 50, and 100 μM vanadate for 24 h. Each point represents mean ± S.D. of three independent experiments. *$p < 0.05$ compared to control (one-way ANOVA with Scheffe's test).

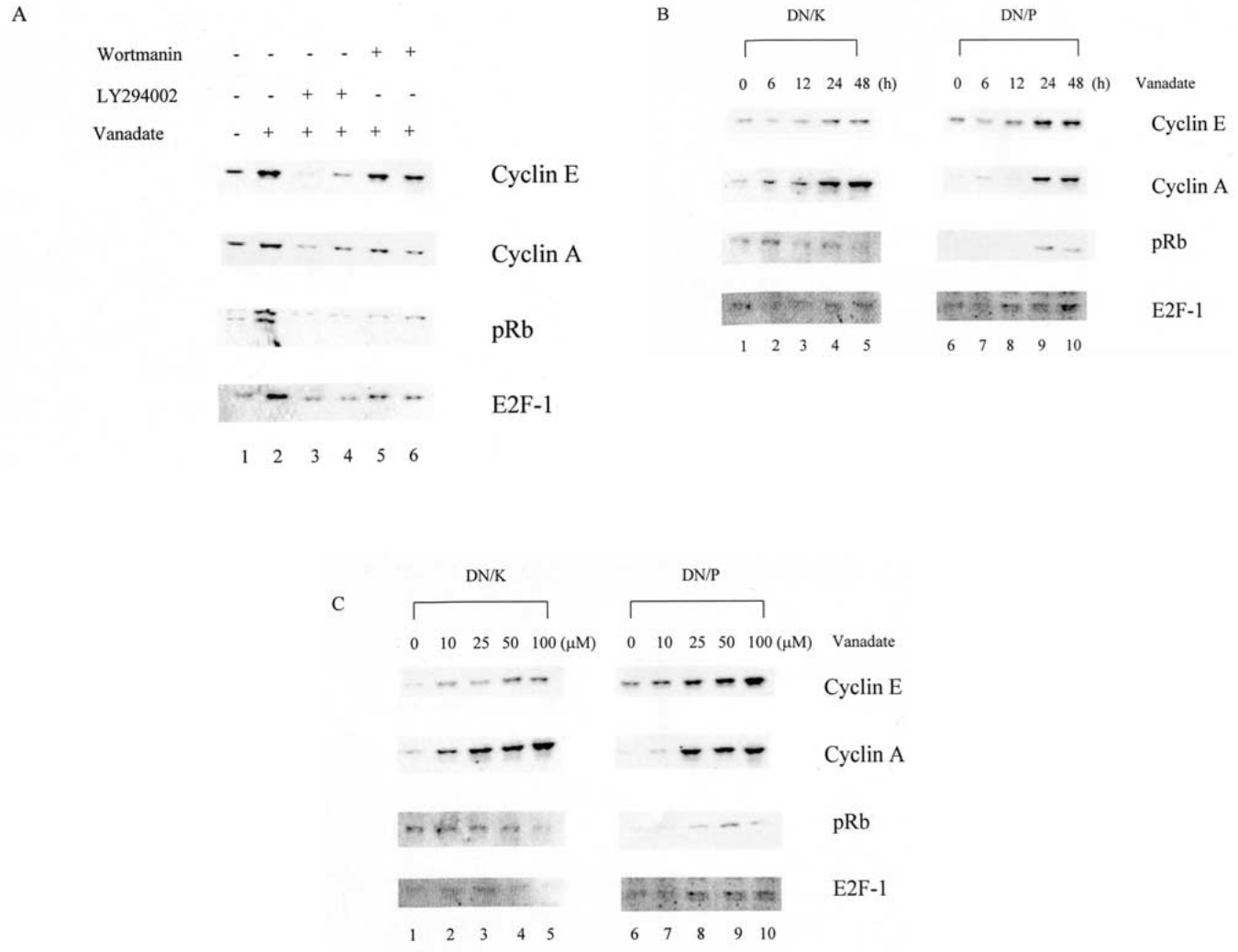

Fig. 5. Effects of Akt on cell growth regulatory proteins. The C141 cells were pretreated with different concentrations of LY294004, wortmanin for 30 min before vanadate treatment (50 μM for 24 h). The DN/K cells and DN/P cells were treated with different times and doses. Western blotting was performed to examine the protein levels of cyclin E, cyclin A, E2F-1 and phosphorylation of Rb. Panel A: lane 1, control without vanadate stimulation; lane 2, vanadate; lane 3, vanadate + 10 μM LY; lane 4, vanadate + 20 μM LY; lane 5, vanadate + 50 nM wortmanin; and lane 6, vanadate + 100 nM wortmanin. In both panel B and panel C, left part and right part represent DN/K cells and DN/P cells, respectively. Panel B: lanes 1 and 6, control; lanes 2 and 7, 50 μM 6 h; lanes 3 and 8, 50 μM, 12 h; lanes 4 and 9, 50 μM, 24 h; and lanes 5 and 10, 50 μM, 48 h. Panel C: lanes 1 and 6, control; lanes 2 and 7, 10 μM 24 h; lanes 3 and 8, 25 μM, 24h; lanes 4 and 9, 50 μM, 24 h; and lanes 5 and 10, 100 μM, 24 h. Data are from a single preparation representative of three independent experiments.

Characterization of cell cycle regulation is important for understanding how extracellular stimuli affect on cell proliferation. Previous studies have shown that vanadate is able to increase S phase cell number in JB6 P^+ mouse epidermal cells [20]. This S phase cell number is p53-dependent through activation of p21 and the mitogen-activated protein kinase (MAPK) signal transduction pathway [20]. The present study shows that vanadate treatment caused increase in percentage of the cells at S phase in a dose- and time-dependent manner. The Cyclin E/cdk2 complex plays a crucial role in the G_1/S phase transition [25]. The expression and activity of cyclin E follows that of cyclin D, with can increase in cyclin E expression occurring in the nuclus during early G_1, peaking at the G_1/S border, and declining thereafter [26, 27]. In contrast, cyclin A activity is thought to contribute to the G_1/S transition, S phase progression and G_2/M transition [25]. The present study shows that vanadate treatment is able to cause increase in cyclin E and cyclin A expression in a dose- and time-dependent manner.

PKB/Akt is activated via a multistep process by a variety of signals. In the early steps of this process, PI3K-generated

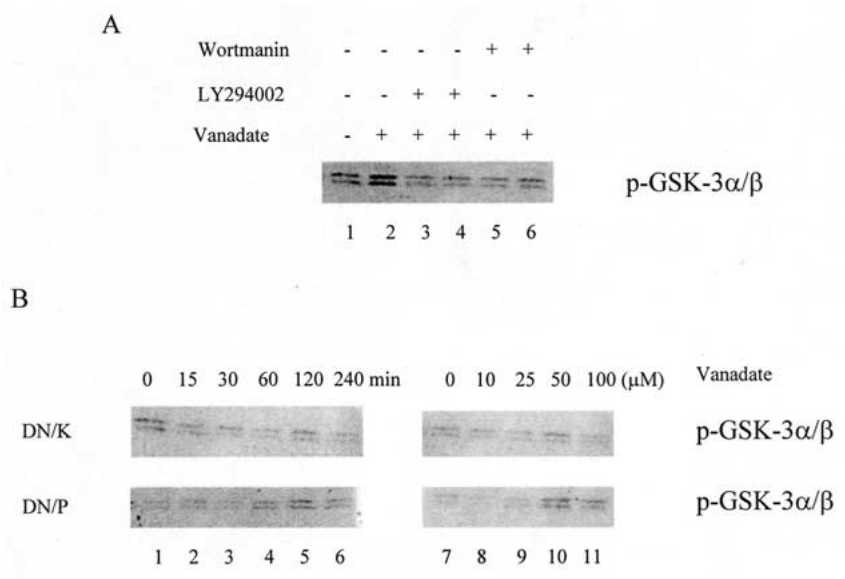

Fig. 6. Effects of PI3K on Akt activity. The C141 cells were seeded in 100 mm dishes. After 80% confluence, the cells were pre-treated with different concentrations of LY294002, and wortmanin before vanadate treatment (50 μM). Panel A: lane 1, control without vanadate stimulation, 60 min; lane 2, vanadate 60 min; lane 3, vanadate + 10 μM LY, 60 min; lane 4, vanadate + 20 μM LY, 60 min; lane 5, vanadate + 50 nM wortmanin, 60 min; and lane 6, vanadate + 100 nM wortmanin, 60 min. Panel B: lanes 1 and 7, control; lane 2, 50 μM 15 min; lane 3, 50 μM, 30 min; lanes 4, 50 μM, 60 min; lanes 5 and 10, 50 μM, 120 min; lane 6, 50 μM, 240 min; lane 8, 10 μM, 120 min; lane 9, 25 μM, 120 min; and lane 11, 100 μM, 120 min. Data are from a single preparation representative of three independent experiments.

D3-phosphorylated phosphoinositides bind to the Akt PH domain and induce the translocation of the kinase to the plasma membrane where it co-localizes with phosphoinositide-dependent kinase-1. D3-phosphorylated phosphoinositides also appear to induce conformational changes that permit phosphoinositide-dependent kinase-1 to phosphorylate the activation loop of Akt [28]. Signals induced by stress as well as by beta-adrenergic receptor agonists such as isoproterenol and cAMP have been shown to activate Akt in a PI3K independent manner [29–31]. However, it has been reported that the activation of Akt by stress such as hydrogen peroxide, a key member of the ROS family, or heat shock was PI3K-dependent [32]. UV induced phosphorylation of Akt at Ser473 and Thr308 in C141 cells was through hydrogen peroxide [33]. Vanadate was able to generate ROS, which were involved in vanadate-induced G_2/M phase arrest [19]. A recent study from our group also shows that vanadate induced HIF-1 and VEGF expression was through ROS and the PI3K/Akt pathway (unpublished observations). The present study shows that vanadate stimulation not only increased Akt kinase activity, but also caused phosphorylation of Akt at Ser473 and Thr308 in a dose- and time-dependent manner. Addition of two PI3K inhibitors, LY294002 and wortmanin, reduced vanadate-induced Akt kinase activity and its phosphorylation. These results indicate that vanadate-induced Akt activation was PI3K-dependent. Moreover, the Akt activity induced by vanadate was blocked in cells transfected with dominant negative Akt kinase mutant plasmid. In the Akt kinase mutant plasmid transfected cells, vanadate failed to phosphorylate Akt at Thr308 because activation loop of Thr308 is located in the kinase domain, and mutation at the kinase domain affects the function of phosphorylation. The phosphorylation at Ser473 was also affected in this type of cells. There are two models of Akt activation: (a) Akt is cytosolic, and moves to the plasma membrane in response to PI3K induction [34, 35], where Ser473 phosphorylation occurs, forming forming a docking site for protein dependent kinase-1 (PDK1), which binds and phosphorylates Thr308 [36]. (b) Cytosolic Akt and PDK1 colocalize, however, Akt is inactive due to constraints imposed by its PH domain [37]. Growth factors then stimulate PI3K, which draws both PDK1 and Akt to the plasma membrane. The PH domain of Akt binds to phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5P_3), unmasking the activation loop and allowing PDK1 to phosphorylate Thr308 [37]. Subsequent elevation of Akt activity promotes autophosphorylation of Ser473 or phosphorylation by a third-party enzyme, PDK2 [38], fully activating Akt [39]. It is likely that vanadate-activated Akt is through the later model. Akt kinase domain affects its phosphorylation at Thr308, which in turn affects phosphorylation at Ser473. Similarly, transfection with the dominant negative mutant SRα-Akt-T308A/S473A made the cells unable to be phosphorylated by vanadate. Again the induction of Akt activity by vanadate in this type of cell is less potent than in wild type cells. That is be-

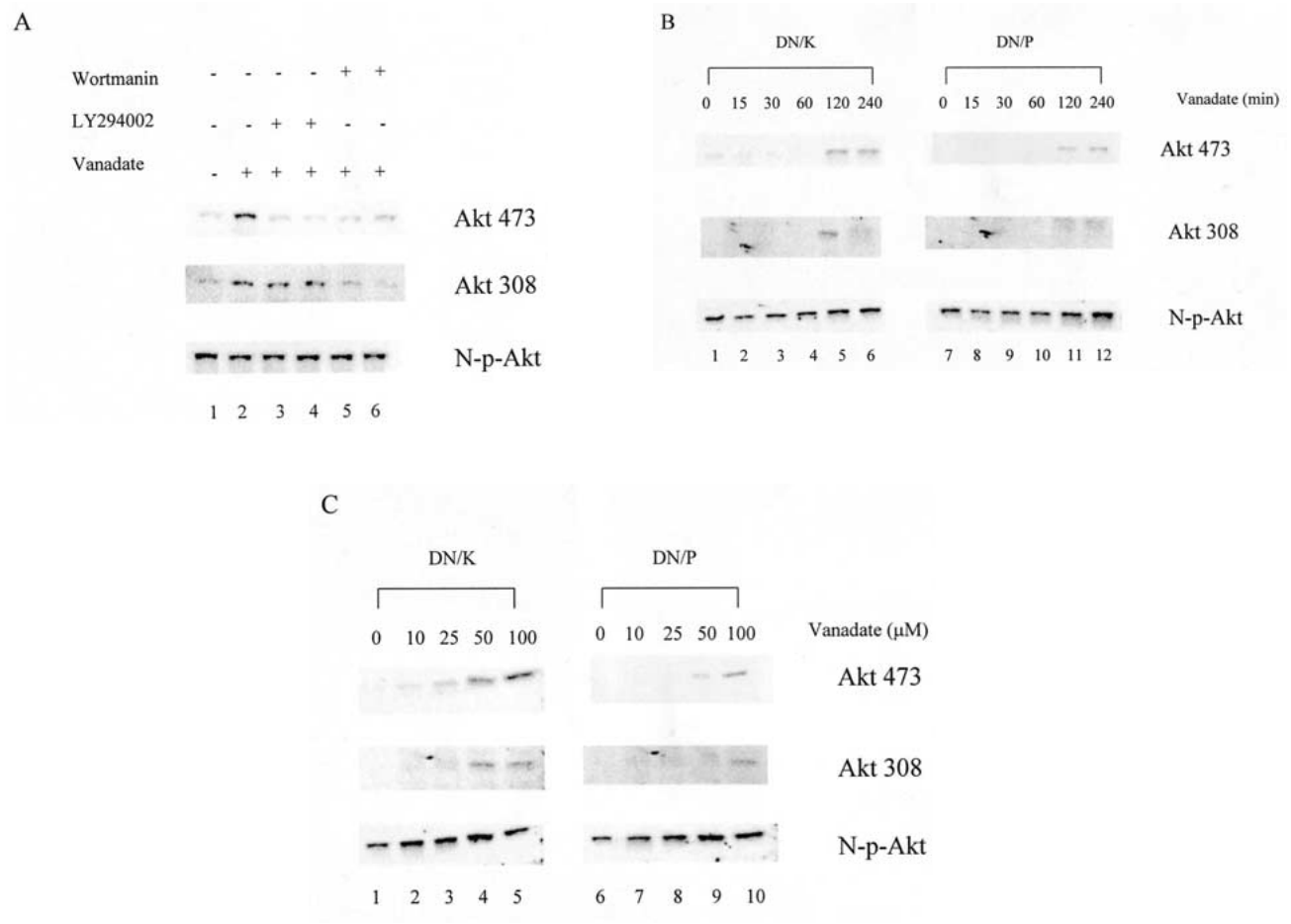

Fig. 7. Effects of PI3K on Akt phosphorylation. The C141 cells were spread in 100 mm dishes. After 80% confluence, the cells were pre-treated with different concentrations of LY294002, and wortmanin prior to vanadate treatment (50 μM). Western blotting was performed in order to measure Akt phosphorylation. Panel A: lane 1, control without vanadate stimulation, 120 min; lane 2, vanadate 120 min; lane 3, vanadate + 10 μM LY, 120 min; lane 4, vanadate + 20 μM LY, 120 min; lane 5, vanadate + 50 nM wortmanin, 120 min; and lane 6, vanadate + 100 nM wortmanin, 120 min. Left part and right part in panel B and panel C represent DN/K cells and DN/P cells, respectively. Panel B: lanes 1 and 7, control; lanes 2 and 8, 50 μM 15 min; lanes 3 and 9, 50 μM, 30 min; lanes 4 and 10, 50 μM, 60 min; lanes 5 and 11, 50 μM, 120 min; and lanes 6 and 12, 50 μM, 240 min. Panel C: lanes 1 and 6, control; lanes 2 and 7, 10 μM, 120 min; lanes 3 and 8, 25 ∝M, 120 min; lanes 4 and 9, 50 ∝M, 120 min; and lanes 5 and 10, 100 μM, 120 min. Data are from a single preparation representative of three independent experiments.

cause Thr308 is located in the kinase domain, and mutation of Thr308 affects the kinase activity.

The mammalian cell cycle is a highly regulated process that is influenced by both positive and negative growth-regulatory signals during the G_1 stage [40]. These signals act by controlling the transcriptional activity of a cellular transcription factor E2F. Activation of E2F is sufficient to irreversibly commit cells to undergo DNA replication. Thus E2F is crucial in the control of cellular proliferation in both normal and tumor cells [41]. The E2F family determines whether or not a cell will divide by controlling the expression of key cell-cycle regulators. These encoded cell cycle regulators include cyclin E, cyclin A, cdc2, cdc25A, pRb, and E2F-6. The E2F family is divided into two distinct groups: E2Fs (E2F1–6) and the DPs (DP1 and DP2) [41]. E2F1 is believed to act as a tumor suppressor through its ability to induce apoptosis and cell cycle regulation. It participates in the repression of E2F-responsive genes through recruitment of pRb [42]. However, recent studies indicate that E2F1 might also be involved in the DNA-damage-response pathway [43]. Other studies showed that E2F1 is phosphorylated by the DNA-damage-response kinase, ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), leading to its stabilization. In the present study, vanadate treatment caused phosphorylation of pRb, which in turn caused itself to be inactivated, leading to release of E2F1 [44]. Activated E2F therefore triggers the regulation of cyclin E and cyclin A.

It has been shown that dominant negative PI3K and pharmacological inhibitors of PI3K both abrogate IL-2 induction of E2F almost completely [5]. Furthermore, expression of gag-PKB also induced a strong transcriptional activation of E2F, suggesting that these proliferation effects are indeed mediated via Akt. Another study showed that activated PI3K induced cyclin D1 transcription and E2F activity, at least in part mediated by Akt, suggesting that the PI3K/Akt pathway contributed to the G_1 cell cycle progression [45]. In addition, it has been reported that Ras-induced increase in E2F1 levels is dependent on Akt, and it is pRb-independent in HEK293 cells [46]. The present study shows that in vanadate stimulation, cyclin E/cdk2 complex may trigger the phosphorylation of pRb. The hyperphosphorylated pRb in turn caused release of E2F1, promoting the transition of G_1 to S phase. Addition of either LY294002 or wortamnin reduced the expression of cyclin E and cyclin A, and decreased phosphorylation of pRb induced by vanadate, resulting in an inability to release E2F1. In addition, cyclin level in DN/P cells was higher than that in DN/K cells, but lower than that in wild type cells. However, the mutation of kinase domain or phosphorylation sites seemed to have no effect on the cyclin A level. Inhibition of kinase activity by transfection of the cells with dominant negative kinase mutant made vanadate unable to phosphorylate pRb. In contrast, inhibition of phosphorylation could not completely block phosphorylation of pRb or E2F1 expression induced by vanadate. These results are consistent with the expression of cyclin E. It is likely that other phosphorylation sites beyond Ser473 and Thr308 may regulate cyclin E. Due to the decreased cyclin E level after inhibition of kinase activity or phosphorylation, the percentage of cells at S phase induced by vanadate decreased in both DN/K cells and DN/P cells compared to the wild type cells. Moreover, in the control cells without stimulation, transfection with either dominant negative Akt mutant plasmid or dominant negative T308A/S473A mutant plasmid decreased the S phase arrest compared to the wild type cells. These observations indicate that both transition of G_1/S phase and S phase progression are mediated by Akt.

In conclusion, (a) vanadate caused Akt phosphorylation at Ser473 and Thr308 and activated Akt; (b) vanadate-induced Akt activation was PI3-dependent; (c) activated Akt phosphorylated pRb and caused E2F1 release; and (d) the released E2F1 up-regulated cyclin E and cyclin A, leading to the increase in cell number at S phase.

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