Cell. Mol. Life Sci. (2011) 68:3249–3260
DOI 10.1007/s00018-011-0626-5

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Role of p21 in SP600125-induced cell cycle arrest, endoreduplication, and apoptosis

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Received: 25 January 2010 / Revised: 24 December 2010 / Accepted: 6 January 2011 / Published online: 11 February 2011

Abstract The anti-cancer effect of the c-Jun N-terminal kinase (JNK) inhibitor SP600125 has been well evaluated in human cancer cells. However the role of p21 in SP600125-mediated G_2/M distribution is not fully understood. Our results showed that the transcriptional activation of p21 by SP600125 is mediated through the proximal regions of multiple Sp1 sites in the p21 promoter following ERK-dependent phosphorylation of Sp1. In this process, p21 induces endoreduplication through the inhibition of cyclin E/Cdk2 activity at 24 h but does not directly regulate cyclin B1/Cdc2 activity. Furthermore, SP600125 induces the phosphorylation of p21 at Thr 145 through the PI3K/Akt pathway. Akt-mediated phosphorylation of p21 and protection of apoptosis are completely abolished by inhibitors of PI3K and Akt. In summary using time points, we identified the dual functions of p21 as an inhibitor of cell-cycle progression at 24 h and as an anti-apoptotic factor at 48 h.

Keywords SP600125 · P21 · G_2/M phase · Endoreduplication · Apoptosis

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Introduction

SP600125 is a specific inhibitor of c-Jun N-terminal kinase (JNK) that is commonly used in clinical research for its anti-inflammatory and anti-cancer properties. According to recent studies, the inhibition of the JNK pathway with SP600125 results in G_2/M phase arrest and endoreduplication-related apoptosis in a variety of human cancer cell lines including multiple myeloma, breast cancer, prostate cancer, and erythroleukemia cells [1–5]. Other recent reports showed that SP600125 directly induces p53 expression but that cell-cycle arrest by SP600125 is independently mediated by p53 [4, 6]. SP600125 is also known to upregulate the cyclin-dependent kinase (Cdk) inhibitor p21, a transcriptional target of the p53 tumor suppressor, in KB-3 cells [1, 7, 8]. Since treatment with SP600125 causes induction of p21 [1], it was of interest to investigate whether SP600125-induced cell-cycle arrest, endoreduplication, and apoptosis are influenced by p21 activation. In the present study, we attempt to determine the role of p21 in the response of SP600125 using the p53 null cell line U937 for ruling out the possibility of any p53 involvement.

p21 is a universal inhibitor of cell-cycle progression that selectively inhibits Cdk activity in G_1/S and G_2/M phases [9]. In addition to its role in Cdk regulation, p21 also binds directly to the proliferating cell nuclear antigen (PCNA) [10], thus interfering with the role of PCNA in DNA polymerase δ function and inhibiting DNA replication. The regulation of p21 occurs primarily at the transcriptional and post-transcriptional levels [11–14]. The expression of p21 is mainly induced in a p53-dependent manner in response to DNA damage [15]; however, p21 can also be transcriptionally regulated by Sp1 transcription factor in p53-independent mechanisms. The involvement of Akt, a known inhibitor of apoptosis, in p21 post-transcriptional

regulation recently has been shown. p21 is frequently phosphorylated at Thr 145 and Ser 146 by Akt activation, and these phosphorylations appear to regulate p21 function [16]. The Akt-dependent phosphorylation of p21 prevents the formation of a complex between p21 and PCNA and also decreases binding of p21 to Cdk2 [17].

In this study, we investigated whether the p21 induction confers resistance to SP600125-mediated cell-cycle distribution and cytotoxicity, and we analyzed the role of the PI3K/Akt survival pathway in SP600125-induced regulation of p21. We report that SP600125 induces both an increase in p21 synthesis through an Sp1-dependent pathway and the upregulation of p21 phosphorylation via the regulation of its Akt-dependent phosphorylated status. These effects result in an inhibition of SP600125-induced apoptosis and confer protection against SP600125-induced cell-cycle arrest and endoreduplication.

Materials and methods

Antibodies and reagents

Antibodies against p21, phospho (p)p21, Sp1, Cdc2, Cdk2, cyclin B1, cyclin E, caspase-3, caspase-8, caspase-9, PCNA, Bid, and tensin homolog deleted on chromosome ten (PTEN) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibodies against p-histone H3 (pH3; Ser10), pPI3K, PI3K, pAkt, Akt, PARP, and β-actin were purchased from Cell Signaling Biotechnology (Beverly, MA, USA). SP600125, Cdk2 inhibitor II, and FITC-conjugated caspase substrate z-VAD-FMK-FITC were purchased from Calbiochem (San Diego, CA, USA). p21 siRNA and control siRNA were purchased from Cell Signaling Biotechnology. Peroxidase-labeled anti-rabbit immunoglobulins were purchased from KOMA Biotechnology (Seoul, Republic of Korea).

Cell line and culture

Human leukemic U937 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells were kindly provided from Prof. D.Y. Shin (Dankook University College of Medicine, Chungnam, Republic of Korea). Cells were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in RPMI 1640 culture medium containing 10% heat-inactivated FBS (Gibco BRL, Gaithersburg, MD, USA). Cells were seeded at 1×10^5 cells/ml and treated with SP600125 for the indicated times. The number of cells and their viability were determined by the trypan blue exclusion assay and MTT assay, respectively.

Cell cycle analysis

The cell cycle was analyzed using flow cytometry of propidium iodide (PI)-stained cells. Cells were fixed in 70% ethanol overnight at 4°C. The cells were washed in phosphate-buffered saline with 0.1% BSA and then incubated with 1 U/ml of RNase A (DNase free) and 10 μg/ml of PI (Sigma) overnight at room temperature in the dark. Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA)

In vitro caspase-3 activity assay

The activity of caspase-like protease was measured using a caspase activation kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. This assay is based on spectrophotometric detection of the color reporter molecule Ac-DEVD-*p*NA, which is linked to the end of the caspase-specific substrate. The cleavage of the peptide by the caspase releases the chromophore *p*NA, which can be quantified spectrophotometrically at a wavelength of 405 nm.

Western blot analysis, immunoprecipitation, and kinase activity

Cells were washed with PBS and lysed in ice-cold lysis. For Western blot analysis, proteins were resolved by SDS-PAGE electrophoresis and transferred on to nitrocellulose membranes by semidry blotting. The membranes were hybridized with specific antibodies. For equal loading control, the blot was probed with actin antibody. For immunoprecipitation, rabbit polyclonal p21 antibodies were added to pre-cleared cell lysates and incubated at 4°C for 12 h. Immune complexes were recovered with protein A Sepharose (Sigma) and washed three times with lysis buffer. Immune complexes were separated on 12.5% SDS-PAGE and revealed with mouse polyclonal Cdc2, Cdk2, and PCNA antibodies. Cdc2 and Cdk2 activity was measured by MESACUP Cdc2 kinase assay kit (MBL, Woburn, MA, USA) and Cdk2/cyclin E kinase assay kit (Reaction Biology, Malvern, PA, USA) according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA that was reverse transcribed from 1 μg of total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology, Sungnam, Republic of Korea). The PCR reaction was initiated at 94°C for 2 min followed by 28 cycles of

94°C for 1 min, 1min annealing temperature, 72°C for 1 min followed by a final extension at 72°C for 5 min. The annealing temperatures for p21 and glyceraldehyde-3-phophate dehydrogenase (GAPDH) were 56°C. After amplification, PCR products were separated on 1.2% agarose gels.

Electrophoretic mobility shift assay

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary Sp1 (5′-ATT CGA TC**G GGG CGG GGC** GAG C-3′) binding oligonucleotides (Santa Cruz Biotechnology) were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris-borate/EDTA before being transferred onto a positively charged nylon membrane (HybondTM-N+) in 0.5× Tris-borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using the EZ-Chip assay kit according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). In brief, 1×10^5 cells were treated with and without SP600125 for 24 h. Cells were cross-linked, lysed, and sonicated. Then, immunoprecipitation was carried out by incubating cell lysate with anti-Sp1 antibody (with normal mouse IgG served as the negative control and with anti-RNA polymerase antibody as the equal loading control in the assay) with rotation at 4°C overnight. The immunocomplex beads were pelleted by centrifugation and washed. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and precipitated with ethanol. The pellets were resuspended in TE buffer and subjected to PCR amplification using specific p21 promoter primers and GAPDH promoter primer: the primers used for the amplification of the Sp1 binding site of p21 promoter region were 5′-GTA AAT CCT TGC CTG CCA GA-3′ (forward) and 5′-GGC TCC ACA AGG AAC TGA CT-3′ (reverse) (433 bp DNA product) [18]; the primers used for the amplification of the RNA polymerase binding site of GAPDH promoter region were 5′-TAC TAG CGG TTT TAC GGG CG-3′ (forward) and 5′-TCG AAC AGG AGG AGC AGA GAG CGA-3′ (reverse) (150 bp DNA product). The samples were run on a 2% agarose gel and visualized by ethidium bromide (EtBr) staining.

Statistical analysis

All data are presented as mean ± SD. Significant differences between the groups were determined using the unpaired Student's t test. A value of $P < 0.05$ was accepted as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments with a similar pattern.

Results

SP600125 increases p21 mRNA expression through Sp1 activation

To determine the relationship between increased p21 and cell-cycle arrest induced by SP600125, we first analyzed the sensitivity of U937 cells to SP600125 using trypan blue exclusion assay. SP600125 completely blocked U937 cell proliferation in all the time periods studied (Fig. 1a). During the analysis, SP600125 significantly induced p21 expression in mRNA and protein levels in a dose-dependent (Fig. 1b) and a time-dependent (Fig. 1c) manner. Maximum activation of p21 was reached at 24 h after treatment with 20 μM of SP600125, and a time-response analysis showed that 20 μM SP600125 promoted a significant accumulation of p21 at 24 h after treatment. Although it is well known that p21 can be induced transcriptionally by the p53 tumor suppressor protein [15], we ruled out the possibility of p53-related p21 regulation because U937 cells are known to be p53-null. Recently, many factors, including transforming growth factor-β1 (TGF-β1), phorbol esters, tamoxifen, and histone deacetylase inhibitors, have been shown to upregulate the p21 promoter (base pairs −210 to +1), which contains several closely spaced G/C-rich motifs that serve as binding sites for members of the Sp1 family of transcription factors [19]. Therefore, to determine if the increase in p21 expression by SP600125 is due to the transcriptional activation of the p21 promoter through Sp1 activation, we analyzed the nuclear translocation of Sp1 using SP600125-treated nuclear extracts. We found that SP600125 induced Sp1 translocation with Sp1 phosphorylation to the nucleus in a time-dependent manner (Fig. 1d). Furthermore, to determine if SP600125 affects the DNA-binding activity of Sp1, an electrophoretic mobility shift assay (EMSA) was performed by incubating nuclear extracts from cells exposed to SP600125 with a biotin-labeled Sp1 response element containing oligonucleotide. Treatment with SP600125 increased the specific DNA-binding activity of Sp1 in a time-dependent manner (Fig. 1e). Recent studies have shown that ERK1/2 leads to the phosphorylation and activation of several transcriptional factors including Sp1

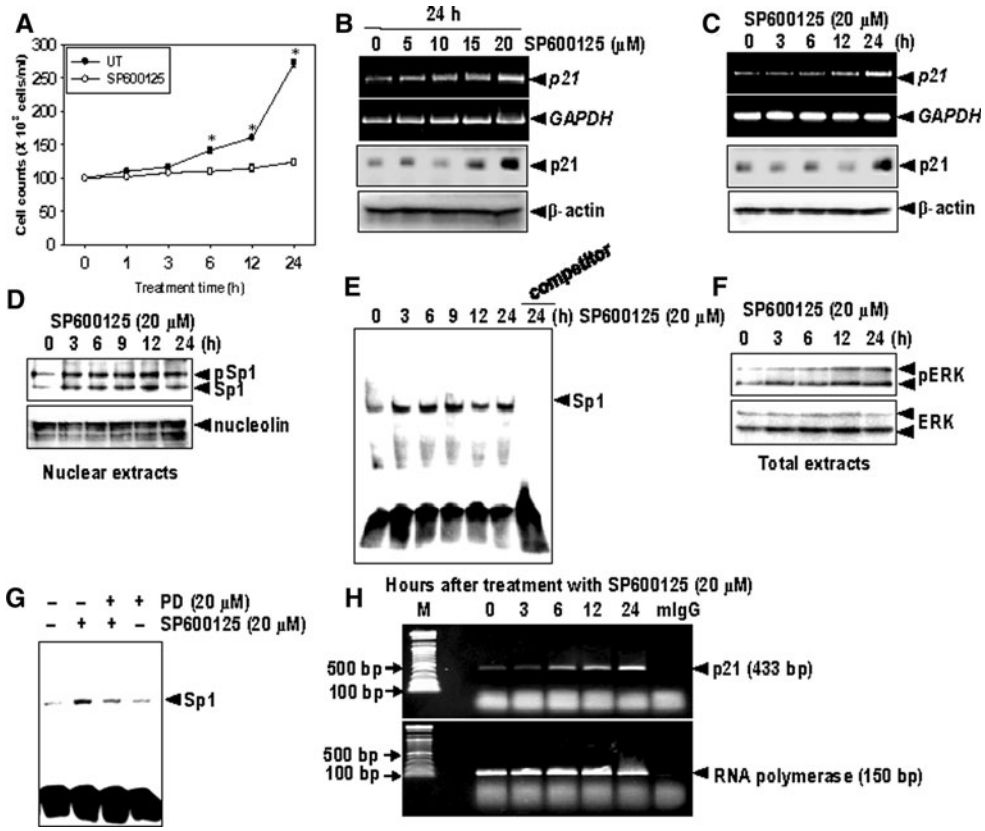

Fig. 1a–h SP600125 induces p21 expression through ERK-dependent Sp1 phosphorylation. U937 cells were seeded at 1×10^5 cells/ml and incubated with SP600125 for 24 h. **a** Cells were treated with 20 μM SP600125 for the indicated times. Cell viability was determined by hemocytometer counts of trypan blue-excluding cells. **b**, **c** Cells were treated with various concentrations of SP600125 for the indicated times. Total RNA was isolated and reverse-transcribed. The resulting cDNAs were subjected to semi-quantitative PCR assay with p21-specific primers, and the reaction products were subjected to electrophoresis in 1.2% agarose gel and visualized by EtBr staining (*upper panel*). GAPDH was used as the internal standard. In the parallel experiment, equal amounts of cell lysates were resolved with SDS-PAGE, transferred to nitrocellulose, and probed with specific antibody against p21. Equal protein loading was evaluated by β-actin (*lower panel*). **d** Cells were treated with 20 μM SP600125 for the indicated times. Nuclear extracts were prepared, fractionated on SDS-PAGE gels, and electrotransferred to a nitrocellulose membrane. Western blot analysis was done with specific antibody against Sp1. Nucleolin was used as the internal standard. **e** Cells were pretreated with 20 μM SP600125 for the indicated times, and nuclear extracts were then prepared and assayed for Sp1 by EMSA. **f** Equal amounts of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibody against pERK or ERK. **g** After incubation with SP600125 for 1 h after pre-treatment of PD98059, the DNA binding activity of Sp1 was analyzed using LightShift Chemiluminescent EMSA kit. **h** A ChIP assay was performed using antibodies against Sp1 and primers against the p21 promoter region. Positive loading control was performed using antibody against RNA polymerase. Data from three independent experiments are expressed as overall mean ± SD. Statistical significance was determined by the Student *t* test (*$P < 0.05$ vs. vehicle control)

[20]. On the basis of these results, we tested whether SP600125 is able to activate the ERK1/2 signaling pathway by exposing U937 cells to SP600125 for the indicated times, followed by Western blot analysis with ERK-specific antibodies. Our results showed that SP600125 slightly increases the phosphorylation of ERK1/2 from 3 to 24 h (Fig. 1f). However, preincubation of PD98059, a specific ERK1/2 inhibitor, revealed a significant decrease in Sp1 DNA-binding activity induced by SP600125 (Fig. 1g). In addition, to further investigate the exact role of SP600125 in the regulation of p21 expression, a ChIP assay was performed to examine the binding of Sp1 on the p21 promoter regions. Our results showed that SP600125 upregulates Sp1 binding to the promoter regions of p21 (Fig. 1h). Taken together, these results suggest that SP600125 activates p21 transcription by increasing the binding of the Sp1 transcription factor to the minimal promoter region of the p21 promoter.

Upregulation of p21 has a critical role in SP600125-induced cell-cycle arrest

Next, we confirmed that decreased cell number is associated with cell-cycle arrest. In our study, we found that

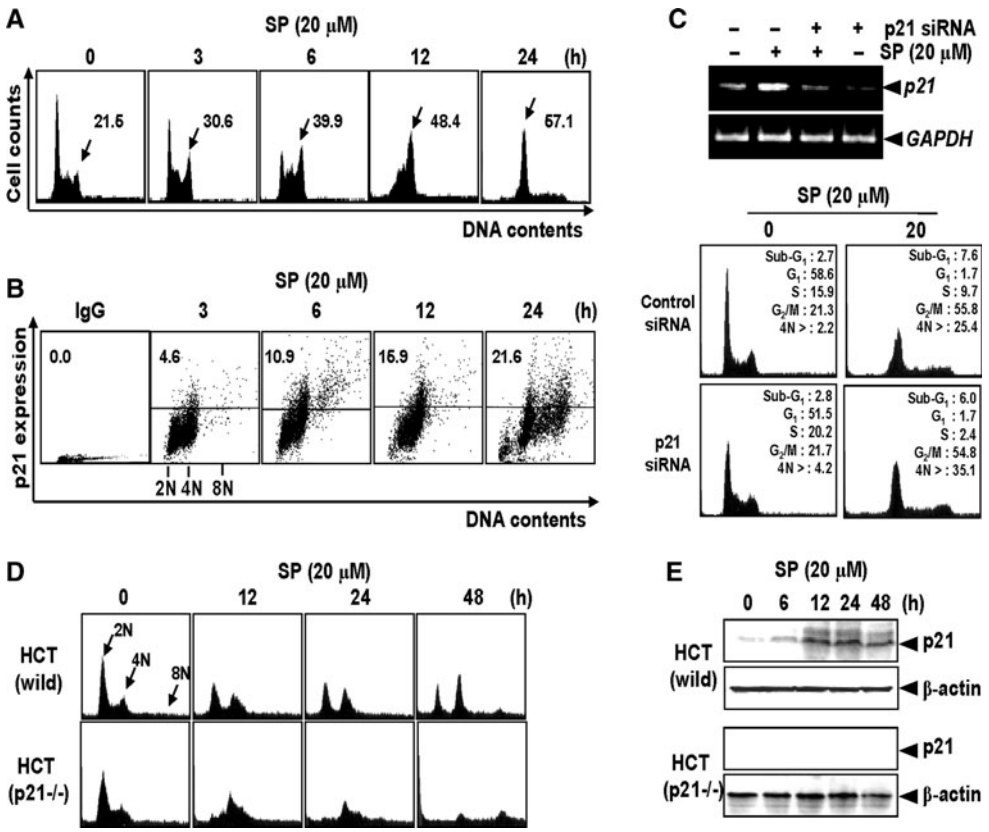

Fig. 2a–e SP600125-induced p21 regulates G2/M phase and endoreduplication. U937 cells were seeded at 1×10^5 cells/ml and incubated with 20 μM SP600125 for 24 h. **a** Cells were harvested, and 10,000 events were analyzed for each sample. The *x*-axis represents DNA content, and the *y*-axis represents the number of cells counted. **b** Cells were measured by dual analysis of p21 expression and DNA content in control and SP600125-treated cells. **c** After transient transfection with a control siRNA or p21-targeted siRNA, cells were treated with 20 μM SP600125. RT-PCR for p21 was performed (*upper panel*), and DNA content was analyzed using a flow cytometer (*lower panel*). **d** In parallel experiments, flow cytometric analyses were conducted in HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells treated with 20 μM SP600125 for the indicated times. **e** Western blot analysis of p21 proteins were performed for HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells at 24 h after treatment with SP600125. Data from three independent experiments are represented

SP600125 induces G_2/M cell-cycle arrest in a time-dependent manner, and endoreduplication occurred at 24 h. Cell-cycle arrest was observed at 3 h after the addition of SP600125 (Fig. 2a), whereas the increase in p21 expression occurred 12–24 h after the addition of SP600125 (Fig. 1c), suggesting that the increase in p21 expression is a consequence of cell-cycle arrest. We then investigated whether the increase in p21 expression was directly due to SP600125 or whether it reflected an alteration in cell-cycle distribution. Thus, cells were analyzed by flow cytometry for both DNA and p21 content. Figure 2b represents the expression of p21 in control and SP600125-treated U937 cells as a function of their position in the cell cycle. We found that the treatment of U937 cells with SP600125 for 24 h not only led to an accumulation of cells in the G_2/M phase (57.1% of treated cells compared to 21.5% of control cells) but also an induced endoreduplicated cell proportion. The SP600125-induced increase in p21 expression was therefore assumed to be specific and an increase in p21 was found in G_2/M phase (4N) and endoreduplication stage (8N). Next, we attempted to determine the precise mechanism by which p21 induction affects SP600125-induced cell-cycle arrest. Transient transfection of U937 cells with a p21-targeted small interfering RNA (siRNA) resulted in almost complete loss of p21 RNA induced by SP600125 for 24 h (Fig. 2c). The effect of SP600125 treatment on cell-cycle distribution was determined by flow cytometry. SP600125-mediated G_2/M phase cell-cycle arrest was not affected by p21-targeted siRNA; however, p21-targeted siRNA induced a relatively greater endoreduplicated-cell population caused by SP600125 (35.1% compared to 25.4% in control cells). To confirm the exact mechanism of p21 on the p21-dependent inhibition of endoreduplication, HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells were treated with SP600125. After 48 h of SP600125 treatment, the HCT116 $p21^{+/+}$ cells maintained a persistent 4N DNA content as assessed by flow cytometric analysis (Fig. 2d). In contrast, SP600125-treated HCT116 $p21^{-/-}$ cells

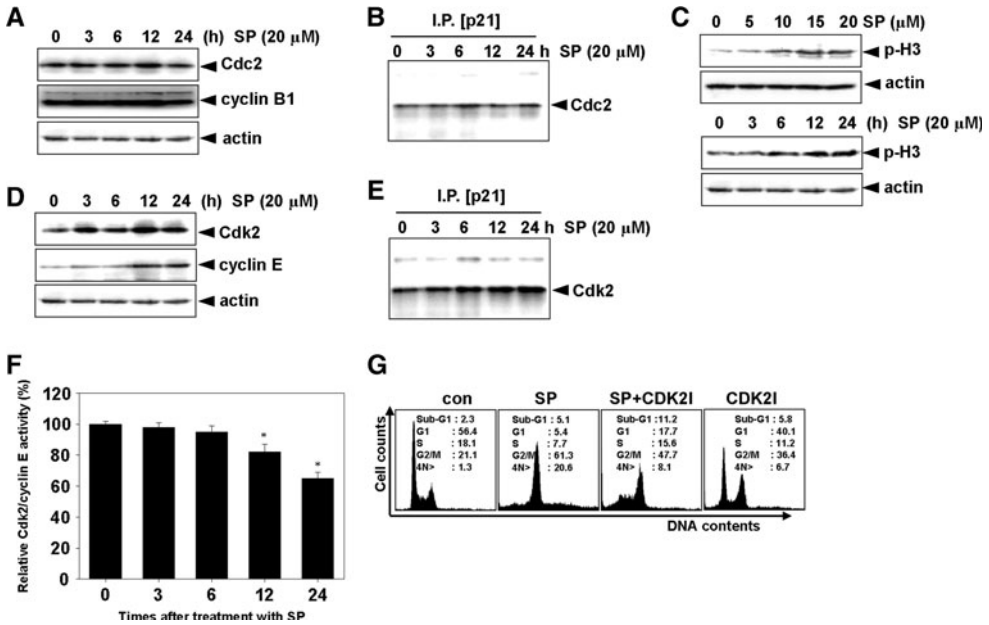

Fig. 3a–g p21 results in decreased cyclin E/Cdk2 kinase activity. U937 cells were seeded at 1×10^5 cells/ml and incubated with 20 μM SP600125 for the indicated times. Cell extracts were prepared for Western blot analysis using specific antibodies against Cdc2 and cyclin B1 (**a**); and against Cdk2 and cyclin E (**d**). **c** Phospho-histone H3 expression was determined by Western blot analysis for different times and doses. Whole-cell lysates from U937 cells were immunoprecipitated with anti-p21, separated by SDS-PAGE, and analyzed for co-immunoprecipitation of Cdc2 (**b**) and Cdk2 (**e**) by Western blot analysis. **f** In a parallel experiment, cyclin E/Cdk2 kinase activity was determined according to the manufacturer's protocol. **g** Cdk2 inhibitor II (CDK2I) was pretreated for 1 h and then 20 μM SP600125 was incubated for 24 h. DNA content was analyzed by flow cytometry. Data from three independent experiments are expressed as overall mean ± SD. Statistical significance was determined by the Student *t* test (*$P < 0.05$ vs. vehicle control)

rapidly underwent endoreduplication with low G_2/M phase cell-cycle arrest. HCT116 $p21^{-/-}$ cells had 4N DNA content through 24 h of SP600125 treatment; however, by 48 h, they had accumulated >4N. Finally, we analyzed the expression of p21 in HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells and found that p21 expression gradually increased in HCT116 $p21^{+/+}$ cells, accompanied by G_2/M arrest and endoreduplication (Fig. 2e). The onset of G_2/M phase cell-cycle arrest and endoreduplication in HCT116 $p21^{-/-}$ cells coincided with the timing of p21 protein induction in SP600125-treated HCT116 $p21^{+/+}$ cells. These results suggest that SP600125-induced p21 protein blocks mitotic exit, thus preventing endoreduplication.

Cdk2 activity is a major target of p21 induced by SP600125

To determine the exact role of p21 in the regulation of the G_2/M transition, cyclin B1 and Cdc2 protein levels and function were evaluated following SP600125 treatment. The cyclin B1/Cdc2 kinase complex is a known regulator of the G_2/M transition during entry into, progress through, and exit from mitosis [21]. After 24 h of SP600125 treatment, cyclin B1 and Cdc2 protein levels did not change (Fig. 3a). Additionally, there was no significant change in Cdc2 kinase activity (data not shown), a finding that is consistent with continuous Cdc2 expression. Binding of p21 to the cyclin B1/Cdc2 complex did not change in the Cdc2 immunoprecipitates at all of the time points examined (Fig. 3b). These data suggest that p21 induced by SP600125 may not regulate delayed G_2/M cell-cycle through direct interaction with the cyclin B1/Cdc2 complex, even though SP600125 increased the phosphorylation of histone H3, a specific mitosis marker (Fig. 3c).

Since combined results of the p21 siRNA and flow cytometric analysis in HCT116 $p21^{-/-}$ cells more sensitively led to endoreduplication following SP600125 treatment compared to U937 cells treated with p21 siRNA (Fig. 2c, d), we hypothesized that p21 prevented endoreduplication via inhibition of the cyclin E/Cdk2 complex, thus preventing the subsequent S-phase retransition. To confirm this hypothesis, cyclin E and Cdk2 expression and kinase activity were analyzed by Western blot analysis and kinase assay, respectively. SP600125 moderately induced both cyclin E and Cdk2 protein expression in a time-dependent manner (Fig. 3d). To determine if the decrease in Cdk2 kinase activity in SP600125-treated U937 cells was due to p21 binding to the Cdk2 complex, p21 immunoprecipitation (IP) was performed, followed by Western blot analysis to evaluate co-immunoprecipitated Cdk2. After SP600125 treatment, there was a time-dependent

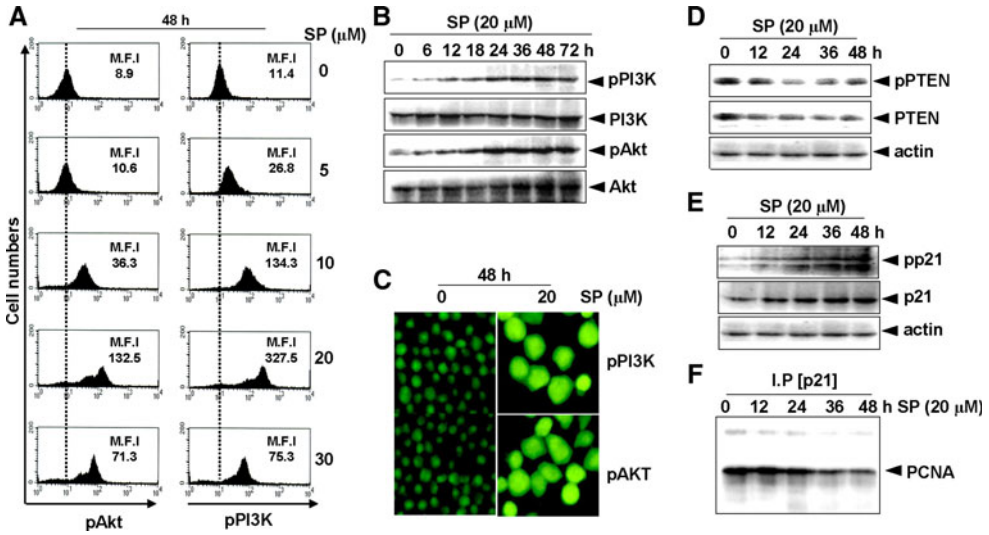

Fig. 4a–f SP600125 induced phosphorylation of p21 through the PI3K/Akt signaling pathway. U937 cells were seeded at 1×10^5 cells/ml and incubated with 20 μM SP600125 for the indicated times. **a** Flow cytometric analysis of pPI3K and pAkt was performed using SP600125-treated U937 cells. Whole-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with pPI3K, PI3K, pAkt and Akt (**b**); pPTEN and PTEN (**d**); and pp21 and p21 (**e**). *β*-Actin was used as a loading control. **c** Cells were fixed, permeabilized, and stained with anti-pPI3K or anti-pAkt monoclonal antibody. The monoclonal antibody was detected using an anti-mouse secondary antibody conjugated with Alexa Fluor 488. Stained cells were then observed under a fluorescent microscope (×400). **f** Whole-cell lysates from U937 cells were immunoprecipitated with specific antibody against anti-p21, separated by SDS-PAGE, and analyzed for co-immunoprecipitation of PCNA by Western blot analysis. Data from three independent experiments are represented

increase in the level of Cdk2 associated with the p21 (Fig. 3e). However, Cdk2 kinase activity was inhibited to <30% of control levels after 24 h (Fig. 3f). To determine the exact role of Cdk2 in regulating endoreduplication, we analyzed the effects of Cdk2 inhibitor II on SP600125-induced endoreduplication. Pre-treatment of Cdk2 inhibitor II significantly blocked SP600125-induced endoreduplication (Fig. 3g). These data indicate that Cdk2 is a potential target of p21 in SP600125-induced endoreduplication through the delay of S-phase.

SP600125 enhances phosphorylation of p21 via the PI3K/Akt pathway

A recent study has shown that p21 is phosphorylated by Akt at a consensus threonine residue (Thr 145), leading to cytoplasmic localization [22] and preventing the formation of a complex between p21 and PCNA [16]. To address the mechanism of p21 at the post-translational level, we first analyzed the change in PI3K and Akt phosphorylation status using flow cytometry, Western blot analysis, and immunocytochemistry assay. We observed that SP600125 increased the phosphorylation of PI3K and Akt in a dose-dependent and time-dependent manner (Fig. 4a–c). This increase in phosphorylation was not associated with an increase in total PI3K/Akt expression. PI3K/Akt phosphorylation was observed from 24 to 72 h after SP600125 treatment.

It is well known that the activation of PI3K/Akt is antagonized by PTEN, which dephosphorylates phosphate-inositol 3-phosphate (PIP3) and thereby inhibits PI3K/Akt-mediated survival signaling [23]. To investigate whether induction of PI3K and Akt phosphorylation is associated with a decrease in PTEN expression and phosphorylation, we tested PTEN levels by Western blot analysis. SP600125 downregulates PTEN expression and its phosphorylation (Fig. 4d). The cell-cycle inhibitor p21 has an Akt-specific phosphorylation site at Thr 145 [22]. Using an antibody directed against p21-Thr 145 phosphorylation, we tested whether SP600125 (via the Akt pathway) would confer an increase in phosphorylated p21. In our study, SP600125 increased the phosphorylation of p21 from 24 h, a finding consistent with the PI3K/Akt activation time (Fig. 4e).

It is also well known that phosphorylated p21 can not bind to PCNA because its phosphorylation site overlaps with the PCNA binding site [16]. We tested whether Akt-dependent p21 phosphorylation had functional effects on PCNA binding. We found that the SP600125-induced phosphorylation of p21 inhibited binding to PCNA (Fig. 4f). These data indicate that the phosphorylation of PI3K/Akt-dependent p21 prevents PCNA binding with p21.

The PI3K/Akt pathway is involved in SP600125-induced endoreduplication but not in G_2/M arrest

As previously discussed, increased p21 expression induced by SP600125 prevents endoreduplication through inhibition of Cdk2 kinase activity at 24 h (Fig. 2). To investigate the effects of the phosphorylation of p21 on cell-cycle transition,

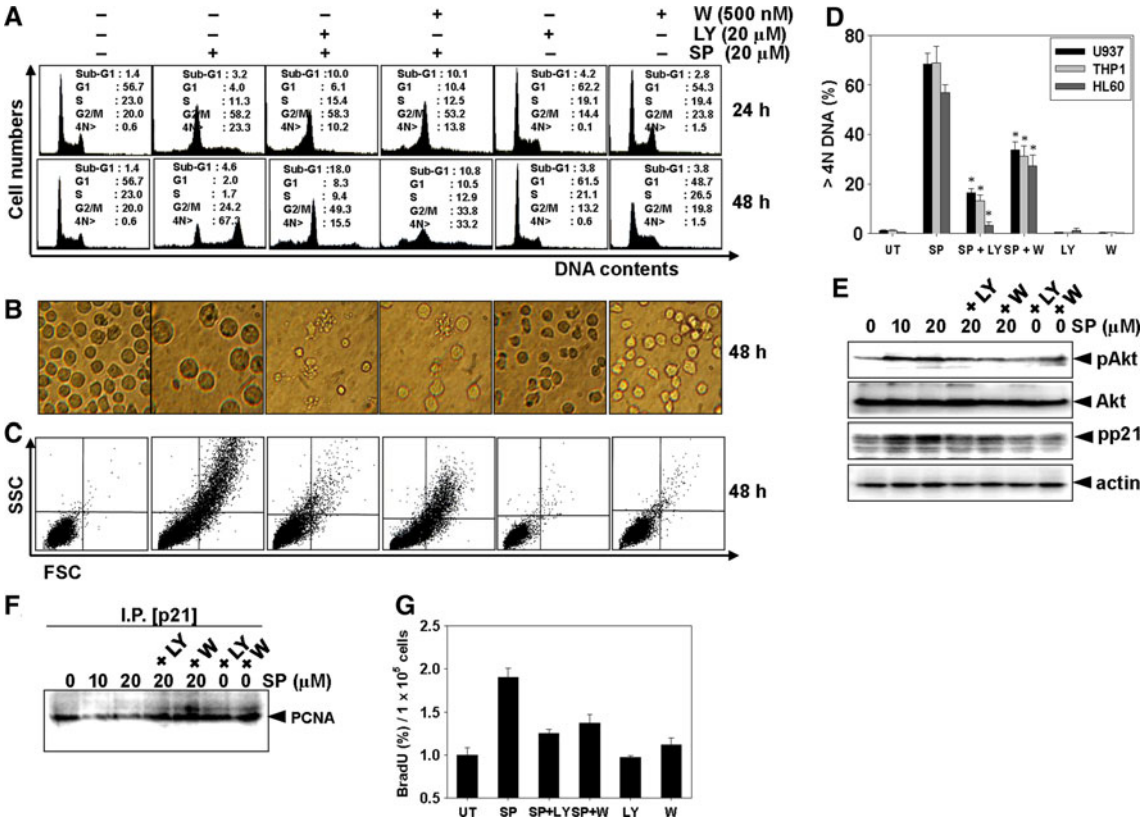

Fig. 5a–g The PI3K/Akt pathway is involved in SP600125-induced endoreduplication. LY294002 (LY, 20 μM) and Wortmannin (W, 500 nM) were pretreated for 1 h and then 20 μM SP600125 was incubated for 48 h. **a** DNA content was analyzed by flow cytometry. **b** Cell morphology was examined using light microscopy (×400). **c** Cell size (FSC) and intracellular granules were detected by flow cytometric analysis. **d** A histogram indicates the percentage of endoreduplicated cells. **e** Whole-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies against pAkt, Akt, and pp21. **f** Whole-cell lysates from U937 cells were immunoprecipitated with specific antibody against p21, separated by SDS-PAGE, and analyzed for co-immunoprecipitation of PCNA by Western blot analysis. **g** Cells (1×10^5) were collected, and BrdU incorporation was analyzed according to the manufacturer's protocol. Data from three independent experiments are expressed as overall mean ± SD. Statistical significance was determined by the Student *t* test (*$P < 0.05$ vs. vehicle control)

we tested the effect of inhibition of PI3K/Akt by the inhibitors LY294002 and Wortmannin by adding them to SP600125-treated U937 cells. Our analysis showed that pretreatment with LY294002 or Wortmannin at 24 h and 48 h decreased the endoreduplicated-cell population induced by SP600125 but did not reduce the G_2/M-cell population (Fig. 5a). Additionally, inhibition of the PI3K/Akt signaling pathway significantly decreased large-sized cells induced by SP600125 (Fig. 5b, c). Furthermore, these effects were not limited to a specific cell type in human leukemia cells (Fig. 5d). To confirm that this effect was specific to the Akt-dependent p21 pathway, the phosphorylation state of Akt was assessed by Western blot analysis after treatment with LY294002 or Wortmannin. We found that both inhibitors partially reversed the phosphorylation of Akt and p21 induced by SP600125 (Fig. 5e). In addition, SP600125-induced release of p21 from PCNA was also reversed by pretreatment with LY294002 or Wortmannin (Fig. 5f).

It is well known that the release of PCNA from p21 enables PCNA to exert its essential function during the process of DNA replication [24]. We tested whether PI3K/Akt signaling affects DNA replication using BrdU incorporation. Our analysis revealed that the inhibition of PI3K/Akt decreased SP600125-induced DNA synthesis (Fig. 5g). Taken together, these data suggest that the PI3K/Akt pathway is closely related to the rapid induction of endoreduplication through increasing DNA replication.

The PI3K/Akt pathway protects cell death and induces endoreduplication

Recent studies have shown that p21 functions not only as a cell-cycle inhibitor but also as an inhibitor of caspase-3 and apoptotic cell death [25–27]. Therefore, we confirmed the hypothesis that PI3K/Akt-dependent phosphorylation of p21 induced by SP600125 would prevent apoptosis in

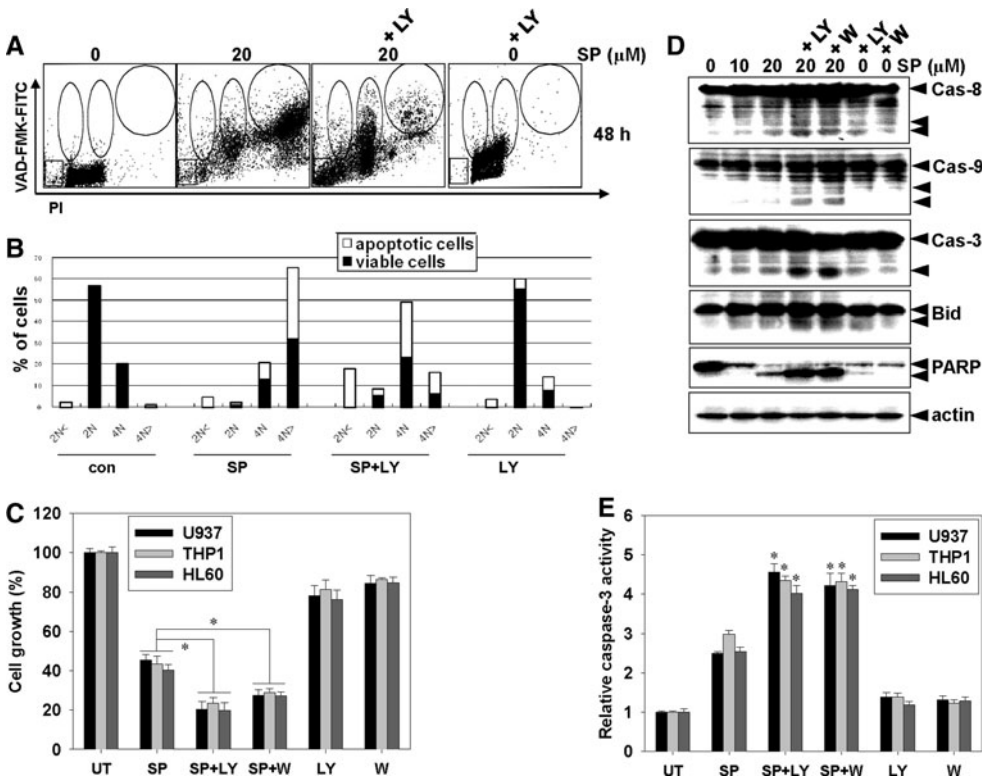

Fig. 6a–e Inhibition of PI3K/Akt sensitized SP600125-induced apoptosis. LY294002 (LY, 20 μM) and Wortmannin (W, 500 nM) were pretreated for 1 h, and then 20 μM SP600125 was incubated for 48 h. **a** Dual analysis of apoptosis was performed using z-VAD-FMK-FITC and the DNA content of SP600125-treated cells. **b** A histogram indicates the percentage of sub-G_1, G_1, G_2/M phases and endoreduplicated cells in U937 cells. **c** Cell viability was determined by MTT assay. **d** Equal amounts of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies against caspase-3, caspase-8, caspase-9, Bid, and PARP. **e** Caspase-3 activity was determined according to the manufacturer's protocol. Data from three independent experiments are expressed as overall mean ± SD. Statistical significance was determined by the Student *t* test (*$P < 0.05$ vs. vehicle control)

leukemia cells. To detect the exact apoptotic cell population induced by SP600125 in various cell-cycle phases, cells were co-stained with PI solution to determine the cell cycle and FITC-conjugated caspase substrate z-VAD-FMK to detect apoptotic cells. SP600125 treatment for 48 h induced apoptosis, mainly in the endoreduplicated-cell population (Fig. 6a). However, the pretreatment of cells with PI3K/Akt inhibitors reversed these effects. Interestingly, z-VAD-FMK-positive cell population appearing in the endoreduplication phase with SP600125 treatment was found to occur in the G_2/M phase with LY294002 treatment. Consistent with these effects, inhibition of the PI3K/Akt signaling pathway sensitized SP600125-induced cell death in endoreduplication phase (Fig. 6b) and growth inhibition in U937, THP-1, and HL60 leukemia cells (Fig. 6e). Pretreatment with a PI3K/Akt inhibitor also enhanced the cleavage of caspase-3, caspase-8, caspase-9, Bid, and PARP induced by SP600125 (Fig. 6d) with increasing caspase-3 activity (Fig. 6e). Finally, to explore the underlying mechanisms by which PI3K/Akt inhibition enhances SP600125-induced apoptosis in U937 cells, we examined the levels of anti-apoptotic protein expression. No significant differences in the protein levels of the tested inhibitor of apoptosis (IAP) proteins (XIAP and IAP1) were noted following treatment with 20 μM SP600125 alone; however, pretreatment with LY294002 or Wortmannin downregulated IAP2 proteins (data not shown). These data indicate that the PI3K/Akt pathway prevents SP600125-induced cell death and strongly induces endoreduplication.

Discussion

Recent studies have shown that JNK activity plays a critical role in cell proliferation and tumor progression [28, 29], and the specific JNK inhibitor SP600125 is known to induce G_2/M-phase arrest, endoreduplication, and apoptosis [1–5]. However, the role of p21 in SP600125-mediated responses remains unknown. In the present study, we determined that SP600125 promotes G_2/M-phase arrest, endoreduplication, and apoptosis through the transcriptional and post-translational modification of p21 expression (Fig. 7). Furthermore, the activation of p21 mRNA expression requires a slight

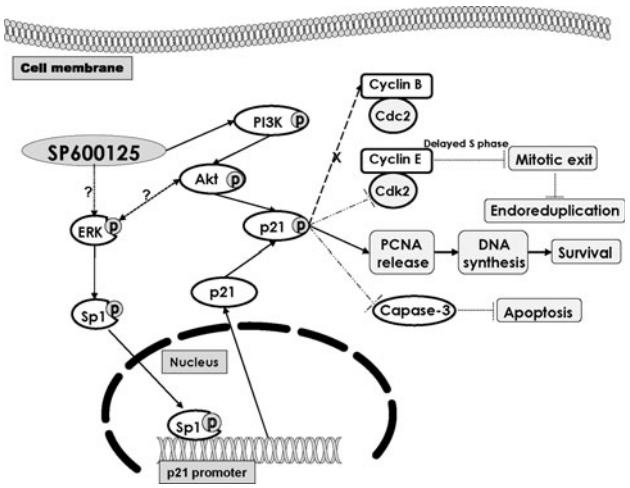

Fig. 7 Scheme for p21 pathway in cell-cycle distribution and cell death. ERK-dependent Sp1 phosphorylation regulates p21 expression in transcriptional levels. Expressed p21 was directly phosphorylated via the PI3K/Akt pathway. Phosphorylated p21 leads to (1) down-regulation of cyclin E/Cdk2 activity, which prevents cells from mitotic exit; (2) a decrease in the apoptotic signal through modulation of caspase activity; and (3) release of PCNA from p21, enhancing DNA synthesis in the endoreduplication phase

ERK activation, which in turn phosphorylates the transcription factor Sp1, thereby promoting increased Sp1 DNA-binding to the p21 promoter in a p53-deficient cell line. Nevertheless, this study highlights that it is still unclear whether the ERK signal pathways are essentially involved in SP600125-induced Sp1 phosphorylation, because ERK inhibitor PD98059 acts by binding to the inactivated form of MEK, thereby preventing its phosphorylation at 2–7 μM. We can not rule out the possibility that PD98059 nonspecifically inhibits Sp1 activation. Furthermore, p21 underwent post-translational modification through the PI3K/Akt signal pathway, and the induction of p21 protein is sufficient to prevent SP600125-induced endoreduplication though p21-mediated temporal inhibition of cyclin E/Cdk2 activity at 24 h. However, we found that SP600125-induced p21 did not affect cyclin B1/Cdc2 activity associated with G_2/M cell-cycle transition. Consistent with our results, previous studies have shown that p21 is not associated with cyclin B1/Cdc2 complexes in transformed cells or p53-deficient cells from patients with Li-Fraumeni syndrome [30]. Based on these findings, we propose that p21 is necessary to regulate cyclin E/Cdk2 kinase activity and prevent mitotic slippage and re-entry to S-phase. Indeed, we show that knockdown or depletion of p21 serve an important role during mitotic exit. Taken together, the results of the current study suggest an ordered biochemical pathway in which p21 plays a critical role in preventing endoreduplication after the aberrant mitotic exit of cells that occurs 24 h after SP600125 treatment. Nevertheless, further studies will be needed because we used different cell lines to elucidate the role of p21. We can not rule out the different specificity of two cell lines.

Akt phosphorylates p21 at Thr 145, resulting in the release of PCNA from p21 [16]. As a functional consequence, phosphorylation of p21 abrogates the inhibitory effect of p21 on cell-cycle progression that appeared at 24 h, thereby mediating the proliferative effect of Akt signaling in U937 cells. Interestingly, phosphorylation of p21 not only reduces its cell-cycle inhibitory effect but even stimulates DNA synthesis. Nevertheless, Anderson and his colleagues reported that SP600125 did not alter phosphorylation of Akt with upregulation of p21 and p27 [2]. In contrast, many researches showed that inhibition of JNK correlates with increased Akt activation to regulate the cell-cycle distribution [31–33]. In spite of discrepancies, inhibition of the PI3K/Akt signaling pathway with LY294002 or Wortmannin significantly blocked not only Akt-induced p21 phosphorylation but also SP600125-induced endoreduplication via an increase in 21 expression and phosphorylation. These data indicate that the JNK signal pathway is cross-talking the Akt signal pathway to regulate cell cycle distribution. Additionally, we previously investigated the inhibition of Bcl-2 by its inhibitor HA14-1, recently identified as another upstream stimulator of Akt that can block SP600125-induced endoreduplication [34]. In the present study, we also found that inhibition of the NF-κB signaling pathway, a component of the Akt downstream signaling cascade, significantly repressed endoreduplication induced by SP600125 (data not shown). Although we suggest that the Akt signaling pathway is strongly involved in SP600125-mediated endoreduplication, further studies are needed to elucidate whether the NF-κB signaling pathway is directly involved in G_2/M arrest and endoreduplication.

Recent studies have shown that phosphorylated p21 may directly inactivate caspase-3 by forming a complex with procaspase-3 in mitochondria. For example, p21 phosphorylated by protein kinase A interacts with procaspase-3 and provides resistance to Fas-mediated apoptosis [34, 35]. In addition, it has been shown that p21 inhibits an initiator caspase [36] and inhibits apoptosis by binding to and inhibiting the stress-activated ASK1 kinase [37]. These facts suggest that phosphorylated p21 may acquire an anti-apoptotic function and may increase resistance to apoptosis. In our study, we found that the inhibition of p21 phosphorylation by LY294002 or Wortmannin significantly elevated caspase-3 cleavage and enzymatic activity and sensitized SP600125-induced apoptosis. Taken together, the results show that early induction (at 24 h) of p21 protein by SP600125 protects mitotic exit through the inhibition of cyclin E/Cdk2 activity. However, at a later time period (24–48 h), p21 phosphorylated Akt mediates, at least in part, the proliferative effect of Akt. It is well

known that Akt phosphorylates several proteins during cell-cycle progression at the boundary of G_1/S phases [38]. Maddika et al. [39] reported that Akt-mediated phosphorylation of Cdk2 regulates its dual role in cell-cycle progression and apoptosis.

In conclusion, SP600125 confers upregulation of *p21* gene expression through ERK-dependent Sp1 phosphorylation and elevation of phosphorylation that may be due to cytoplasmic translocation of p21 via the PI3K/Akt signaling pathway. In the process, p21 plays a central role at the beginning of G_1/S phase by preventing aberrant mitotic exit. In addition, an understanding of how JNK is regulated is thought to offer a potential target for cancer treatment because JNK activity is differentially controlled between normal and cancer cells.

Acknowledgments This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0016098). We greatly appreciate the gift of HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells from Prof. Deung Y. Shin (Dankook University College of Medicine, Chungnam, Republic of Korea).

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