Reentry into the Cell Cycle of Contact-inhibited Vascular Endothelial Cells by a Phosphatase Inhibitor

POSSIBLE INVOLVEMENT OF EXTRACELLULAR SIGNAL-REGULATED KINASE AND PHOSPHATIDYLINOSITOL 3-KINASE* (Received for publication, June 14, 1999, and in revised form, November 1, 1999)

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Vascular endothelial cells are unique in that they exit from the cell cycle when they come into contact with each other. Although the phenomenon is called “contact inhibition,” little is known about the cellular mechanisms involved. Here we show that the phosphatase inhibitor sodium orthovanadate (SOV) induced the reentry of contact-inhibited human umbilical vascular endothelial cells (HUVECs) into the cell cycle and that reentry was associated with activation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI 3-K)/Akt pathways. SOV stimulated \(^{3}H\)thymidine uptake of contact-inhibited HUVECs in a time- and dose-dependent manner. SOV-induced increase in \(^{3}H\)thymidine uptake was significantly inhibited by the mitogen-activated protein kinase kinase-inhibitor PD98059 and by the PI 3-K inhibitor LY294002. SOV also stimulated the expression of cyclin D1, cyclin E, and cyclin A, and the activity of CDK2 kinase, whereas it decreased the expression of p27\(^{kip1}\). In marked contrast, growth media alone did not induce these changes. Furthermore, these SOV-induced changes were abolished by pretreatment with PD98059 and LY294002. SOV stimulated phosphorylation of ERK and Akt in contact-inhibited HUVECs, while growth media alone did not. This phosphorylation was associated with inhibition of phosphatase activity in the cells. Finally, overexpression of high cell density-enhanced protein tyrosine phosphatase 1 inhibited c-fos and cyclin A promoter activity. Taken together, our results suggest that in contact-inhibited HUVECs, increased phosphatase activity suppressed the ERK and PI 3-K/Akt pathways, resulting in exit from the cell cycle by down-regulation of cyclin D1, cyclin E, and cyclin A and by up-regulation of p27\(^{kip1}\).

Vascular endothelial cells (ECs)‡ play a variety of pathophysiological roles such as provision of a barrier through which substances are transported into vessel walls, maintenance of vascular tone by releasing vasoactive substances including nitric oxide and endothelin, and oxidation of lipoproteins (1). ECs are unique because they grow as a strict monolayer, and they exit from the cell cycle once they come into contact with each other. Although the phenomenon is well known and called “contact inhibition,” little is known about the molecular mechanisms involved.

Several lines of evidence suggest that up-regulation of phosphatase activity may be implicated in density-dependent growth arrest. NRK-1 cells were transformed by treatment with the phosphatase inhibitor sodium orthovanadate (SOV), and transformation was accompanied by increases in protein phosphorylation in the cells (2). Protein-tyrosine phosphatase (PTPase) activity was increased in human umbilical vein endothelial cells (HUVECs) harvested at high density (3). The increase in PTPase activity was also observed in Swiss 3T3 fibroblasts whose growth was arrested at high density (4). Recently, a novel class of receptor-like PTPases was isolated and named high cell density-enhanced PTPase 1 (DEP-1). DEP-1 has an extracellular domain that contains eight fibronectin type III motifs, a single transmembrane domain, and a single intracellular PTPase domain. The expression level and the PTPase activity of DEP-1 were increased in WI-38 and AG1518 cells harvested at high density (5). Although these findings suggest that increased PTPase activity in the cells might counteract with protein phosphorylation, which leads to cell proliferation, little is known as to which intracellular signaling pathways are affected by the increase in PTPase activity and how the increased PTPase activity finally affects the cell cycle regulatory machinery.

Cell cycle progression is regulated by serine/threonine kinases termed cyclin-dependent kinases (CDKs), the activities of which oscillate during the cell cycle. CDKs are associated with the positive coactivators cyclins and the negative regulators, CDK inhibitors (6, 7). In mammalian cells, cyclin D-CDK4/CDK6, cyclin E-CDK2, cyclin A-CDK2, and cyclin B-Cdc2 are the main cyclin-CDK complexes that regulate the progression of G1, G1/S, S, and G2/M phases, respectively. CDK inhibitors comprise two families, the Ink4 and Cip/Kip families. CDK inhibitors of the Cip/Kip family are of particular interest in that they inhibit the activity of a broader spectrum of CDKs including CDK2, CDK4, and CDK6 (8). The Cip/Kip family is composed of p21\(^{waf1/cip1}\), p27\(^{kip1}\), and p57\(^{kip2}\) (9–12). The expression level of p27\(^{kip1}\) is reportedly increased in growth-arrested cells by contact inhibition or by stimulation

PBS, phosphate-buffered saline; HA, hemagglutinin; PI 3-K, phosphatidylinositol 3-kinase.

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with transforming growth factor-β (13–17).

Recent evidence suggests that several intracellular signaling pathways are linked to the cell cycle regulatory machinery. Among them, the p21fos (RAS)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway is the most characterized pathway. It is reported that constitutively active MEK is sufficient to transform cells or induce differentiation (18, 19), suggesting that the MEK/ERK pathway is implicated in the cell cycle regulation. Several studies have indicated, using a dominant-negative Ras mutant, that the Ras signaling pathway is involved in the induction of cyclin D1 protein, CDK4 kinase activity, and CDK2 kinase activity and in the down-regulation of p21cip1 (20, 21). Furthermore, the ERK pathway is reportedly implicated in vascular endothelial cell growth factor-induced EC proliferation by stimulating cyclin D1 synthesis and CDK4 kinase activity (22). Protein kinase C (PKC) stimulates cell proliferation or induces cell cycle arrest in vascular ECs, which appears to depend upon the timing of PKC activation during the cell cycle and PKC isoforms expressed in the cells (23–26). The PI 3-K-mediated pathways also seem to be implicated in cell cycle progression, because a retrovirus-encoded PI 3-K could transform fibroblasts (27). However, it remains unclear as to how these intracellular signaling pathways are regulated in contact-inhibited vascular ECs.

To investigate the cellular mechanisms for density-dependent growth arrest and to apply those mechanisms to the regulation of the growth of other cell types, such as vascular smooth muscle cells, we examined in the present study whether treatment with the phosphatase inhibitor SOV induced reentry of contact-inhibited vascular ECs into the cell cycle by examining [3H]thymidine incorporation, expression levels of cyclins, CDKs and CDK inhibitors, and CDK2 kinase activity. We also studied the effects of inhibition of the ERK-, PKC-, and PI 3-K-mediated pathways on SOV-induced cell cycle reentry. Finally, we examined the effect of overexpression of DEP-1 on c-fos and cyclin A promoter activity.

**MATERIALS AND METHODS**

**Reagents**—HUVECs and bovine aortic endothelial cells (BAEcs) were purchased from Sanko Junyaku (Tokyo, Japan). Anti-cyclin D1, -cyclin E, -cyclin A, -CDK4, -CDK2, -p21weg-cip1, -p27kip1, -ERK1, and -Akt1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphospecific anti-ERK1/2 antibody and phosphospecific anti-Akt antibody that recognize catalytically active ERK1/2 and Akt, respectively, were obtained from New England Biolabs (Beverly, MA). Histone H1, histone H2B, and myelin basic protein were obtained from Roche Molecular Biochemicals. Calphostin C, GF109203X, wortmannin, and LY294002 were purchased from Sigma, and PD98059 was obtained from New England Biolabs.

**Cell Culture**—HUVECs were maintained in medium 199 containing 20% fetal bovine serum, 100 μg/ml EC growth supplement, and 50 units/ml heparin. BAEcs were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After reaching confluence, medium was replaced with fresh growth medium, and the cells were further incubated for 48 h. Cells were then stimulated with fresh growth medium in the presence and absence of SOV.

**Preparation of Protein Extracts**—For Western blot analyses and the CDK2 kinase assay, we used Nonidet P-40 cell lysates buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation. Cells were then lysed in Nonidet P-40 cell lysis buffer for 30 min on ice. After centrifugation, the supernatant was used for Western blot analysis. Protein concentration was measured according to Bradford’s method (Bio-Rad).

**In Vitro Kinase Assays**—For the CDK2 kinase assay, 75 μg of each protein extract was preincubated with protein A-agarose beads (Roche Molecular Biochemicals) for 1 h at 4 °C in the Nonidet P-40 cell lysis buffer. The extracts were then incubated with 1 μg of anti-CDK2 antibody for 1 h at 4 °C and with protein A-agarose beads for another 1 h at 4 °C with constant rocking. After centrifugation, the pellets were washed twice with the Nonidet P-40 cell lysis buffer and then incubated with secondary antibodies, which were preincubated with horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at a final dilution of 1:7,000. After final washes with 1× PBS, 0.2% Tween 20, the signals were detected using ECL chemiluminescence reagents (Amersham Pharmacia Biotech).

**Western Blot Analysis**—Protein extracts were separated on 10% SDS-polyacrylamide gels and transferred onto nylon membranes (Millipore Corp., Bedford, MA) using a semidry blotting system (Amersham Pharmacia Biotech, Uppsala, Sweden). After blocking in 1× PBS, 5% nonfat dry milk, 0.2% Tween 20 at 4 °C overnight, the membranes were washed with the primary antibody in blocking buffer (1× PBS, 2% nonfat dry milk, 0.2% Tween 20) for 1 h at room temperature. Antibodies were used at a dilution of 1:100, except for phosphospecific anti-ERK1/2 antibody and phosphospecific anti-Akt antibody, which were diluted at 1:5,000. The membranes were washed three times with the blocking buffer and then incubated with secondary antibodies, which were preincubated with horseradish peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at a final dilution of 1:7,000. After final washes with 1× PBS, 0.2% Tween 20, the signals were detected using ECL chemiluminescence reagents (Amersham Pharmacia Biotech).

**Measurement of [3H]Thymidine Incorporation**—Confluent HUVECs were maintained in growth medium for 48 h and restimulated with fresh growth medium for 8, 16, and 24 h in the presence and absence of 50 μM SOV. [3H]Thymidine (2 μCi/ml; Amersham Pharmacia Biotech) was added to each well 2 h before the end of the incubation period. Cells were washed twice with ice-cold 1× PBS and incubated with ice-cold 10% trichloroacetic acid for 30 min. After washing them twice with distilled water, the cells were lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and subjected to liquid scintillation counting.

**Measurement of Phosphatase Activity**—Phosphatase activity was measured as previously reported (28). In brief, protein extracts were incubated in 200 μl of buffer containing 50 mM imidazole (pH 7.5), 0.1% β-mercaptoethanol, and 10 mM p-nitrophenyl phosphate, for 10 min at room temperature. The reaction was stopped by adding 800 μl of 0.25 N NaOH. Absorbance at 410 nm was measured to estimate the amount of hydrolyzed p-nitrophenyl phosphate.

**Phospho-c-fos**—The 5-fluorodeoxy-β-D-arabinofuranoside (c-fos) gene was amplified by polymerase chain reaction. One μg of mouse genomic DNA (Promega, Madison, WI) was subjected to polymerase chain reaction using LA Taq DNA polymerase (Takara shuzo, Osaka, Japan). The polymerase chain reaction conditions were 1 min at 95 °C, 1 min at 63 °C, and 1 min at 72 °C for 35 cycles, with final extension for 10 min at 72 °C. The polymerase chain reaction-amplified product was digested.

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with SacI and XhoI and subcloned in the pGL2 vector (pGL2-c-fos). Primer sequences used for the reaction were as follows: sense primer, 5′-GAGGCTTCTGTCCTCTCCTAATCAGAAGACT-3′; antisense primer: 5′-CTCGAGTCGACATCCGCGTTGAGTGA-3′.

The nucleotide sequence of the construct was confirmed by cycle sequencing using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). pGL2-cyclin A′-3200/+245 encodes approximately 3.2 kilobase pairs of the promoter region of the human cyclin A gene upstream of the luciferase gene (29). pME18S-mouse DEP-1 encodes the full length of mouse DEP-1 cDNA in the expression vector pME18S (30). pcDNA3-HA-mouse RasS17N encodes the amino-terminally HA-tagged mouse Ras cDNA in which Ser17 was replaced with Asn, and pcDNA3-HA-mouse RasG12V encodes the amino-terminally HA-tagged mouse Ras cDNA in which Gly12 was replaced with Val, as described previously (31). PRL-TK, which encodes the sea pansy luciferase gene, was purchased from Toyo Ink (Tokyo, Japan) and used as the internal control for the luciferase assays.

**Translational Transfection Assays—**BECs were transiently co-transfected with pGL2-cyclin A′-3200/+245 or pGL2-c-fos and pRl-TK, along with pcDNA3-HA-mouse RasS17N or pcDNA3-HA-mouse RasG12V or pME18S-mouse DEP-1 using LipofectAMINE (Life Technologies, Inc.). After 48 h, cells were harvested, and the dual luciferase assay was performed using a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany).

**Statistical Analyses—**The values are the mean ± S.E. The effects of SOV on cyclins/CDKs/CDK inhibitors, kinase activities, and [3H]thymidine uptake were assessed using analysis of variance followed by the Student-Neumann-Keul test. Differences with a p value of <0.05 were considered statistically significant.

**RESULTS**

**Reentry of Contact-inhibited HUVECs into the Cell Cycle Induced by Sodium Orthovanadate—**We first examined whether the phosphatase inhibitor SOV induced reentry of contact-inhibited HUVECs into the cell cycle. Contact-inhibited HUVECs were stimulated with fresh growth medium in the presence and absence of SOV. SOV, at concentrations of 25 and 50 μM, stimulated [3H]thymidine incorporation in a time- and dose-dependent manner (Fig. 1A). SOV-stimulated [3H]thymidine uptake peaked around 16 h poststimulation (0 μM SOV, 71162 ± 2660 cpm; 25 μM SOV, 183023 ± 11247 cpm, p < 0.0001 versus 0 μM; 50 μM SOV, 217582 ± 5650 cpm, p < 0.0001 versus 0 μM and p < 0.05 versus 25 μM) and remained at increased levels until 24 h (0 μM SOV, 69425 ± 2347 cpm; 25 μM SOV, 148175 ± 9950 cpm, p < 0.0001 versus 0 μM; 50 μM SOV, 172971 ± 20088 cpm, p < 0.0001 versus 0 μM). SOV, at a concentration of 100 μM, had a cytotoxic effect, because almost all cells were rounded up and detached within 3 days. Thus, we used SOV at a concentration of 50 μM in subsequent experiments. We next examined the effects of SOV on protein expression levels of cell cycle regulatory factors, including cyclins, CDKs, and CDK inhibitors, in contact-inhibited HUVECs. The basal levels of cyclin D1, cyclin E, cyclin A, and p21 were minimal. SOV significantly increased the expression of cyclin D1 (2.21 ± 0.30-fold increase, 16 h poststimulation, p < 0.01, n = 4), cyclin E (2.54 ± 0.63-fold increase, 16 h poststimulation, p < 0.05, n = 3), cyclin A (5.17 ± 0.87-fold increase, 24 h poststimulation, p < 0.01, n = 4), and p21 (1.76 ± 0.18-fold increase, 16 h poststimulation, p < 0.01, n = 3) in a time-dependent fashion, whereas it significantly down-regulated that of p27 (0.63-fold increase, 16 h poststimulation, p < 0.01, n = 4) and 24 h (5.66 ± 1.65-fold increase versus 0 h, p < 0.05, n = 4) poststimulation (Fig. 2B, left panels). In contrast, growth medium alone did not induce CDK2 kinase activity (Fig. 2B, right panels). Collectively, the results described above suggest that growth medium containing 50 μM SOV induced reentry of contact-inhibited HUVECs into the cell cycle, whereas growth medium alone did not.

**Effects of MEK and PI 3-K Inhibition on SOV-induced Cell Cycle Reentry in Contact-inhibited HUVECs—**The results described above suggested that inhibition of phosphatases by SOV might result in stimulation of phosphorylation of a variety of proteins that are involved in transmitting mitogenic signals. We therefore examined the intracellular signaling pathways that might be implicated in reentry of contact-inhibited HUVECs into the cell cycle induced by SOV. We studied the effects of the MEK1/2 inhibitor PD98059, the PKC inhibitor calphostin C, and the PI 3-K inhibitor LY294002 on [3H]thymidine uptake after stimulation of contact-inhibited HUVECs by 50 μM SOV. The changes in [3H]thymidine incorporation were assessed at 16 h poststimulation. SOV-induced increase in [3H]thymidine uptake in confluent HUVECs was significantly suppressed by pretreatment with PD98059 and LY294002 in a

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dose-dependent fashion (Fig. 1B). We also used another PI 3-K inhibitor, wortmannin, and obtained basically the same results (data not shown). Calphostin C, at a concentration of 100 nM, which seems to be sufficient to inhibit PKC (32), tended to inhibit [3H]thymidine uptake; however, the difference was not statistically significant. Calphostin C, at a concentration of 200 nM, significantly inhibited [3H]thymidine uptake (data not shown). However, some cells were rounded up and detached in 48 h, probably because of its cytotoxic effects at that concentration. We also used GF109203X, another PKC inhibitor. GF109203X, at concentrations varying from 0 to 3.5 μM, did not significantly inhibit [3H]thymidine uptake (data not shown). Overall, PKC inhibitors used in the experiments did not significantly inhibit SOV-induced changes in [3H]thymidine uptake.

Confluent HUVECs were pretreated with 50 μM PD98059 and stimulated with 50 μM SOV for the indicated periods. Seventy-five μg of each protein extract was immunoprecipitated with anti-CDK2 antibody, and CDK2 kinase activity was measured using histone H1 as the substrate. SOV, at a concentration of 50 μM, stimulated the kinase activities of ERK and Akt.

Activation of ERK and Akt by SOV Treatment in Contact-inhibited HUVECs—The results described above suggest that the ERK- and PI 3-K-mediated pathways were activated by SOV in contact-inhibited HUVECs, resulting in the activation of the cell cycle regulatory machinery. To confirm this hypothesis, we examined whether ERK and Akt, a major downstream target of PI 3-K, were activated by SOV in contact-inhibited HUVECs. Neither ERK nor Akt was significantly phosphorylated under basal conditions. SOV, at a concentration of 50 μM, stimulated phosphorylation of ERK1/2 and Akt in a time-dependent manner. Phosphorylation of ERK1/2 and Akt peaked around 15 min poststimulation (Fig. 5A, left panels). In marked contrast, neither ERK nor Akt was significantly phosphorylated in the absence of SOV in contact-inhibited HUVECs, although fresh growth medium was added (Fig. 5A, right panels), suggesting that both pathways were reversibly shut down in contact-inhibited HUVECs. We also examined the kinase activities of ERK1 and Akt using myelin basic protein and histone H2B, respectively, as the substrate. SOV, at a concentration of 50 μM, stimulated the kinase activities of ERK and Akt in a time-dependent fashion (Fig. 5B, left panels). The kinase activities of ERK1 and Akt peaked around 15 min poststimu-
induced changes in CDK2 kinase activity in contact-inhibited HUVECs. Confluent HUVECs were pretreated with 50 μM PD98059 and stimulated with 50 μM SOV for the indicated periods. Seventy-five μg of each protein extract was immunoprecipitated with anti-CDK2 antibody, and CDK2 kinase activity was measured using histone H1 as the substrate (upper panel). The same protein extracts were immunoprecipitated and immunoblotted with anti-CDK2 antibody as the internal control (lower panel). Shown are the results of a representative experiment among three independent experiments in which the same results were obtained. B, time course of changes in CDK2 kinase activity in the presence and absence of LY294002. Confluent HUVECs were pretreated with 50 μM LY294002 and stimulated with 50 μM SOV for the indicated periods. Experiments were performed in the same way as in A. Shown are the results of a representative experiment among three independent experiments in which the same results were obtained.

Overexpression of DEP-1 Inhibits c-fos and Cyclin A Promoter Activity in BAECs—To examine whether increased phosphorylation could affect the intracellular signaling pathway and inhibit cell cycle progression in vascular ECs, vascular ECs were transiently co-transfected with the luciferase gene, mouse c-fos promoter, and mouse DEP-1 cDNA. In this case, we used BAECs instead of HUVECs, because transfection efficiency was poor in HUVECs. We used c-fos promoter activity as an indicator for the activity of the Ras/MEK/ERK-dependent pathway. It was also reported that c-fos promoter activity was increased via the PI 3-K-dependent pathway (33). As shown in Fig. 8A, c-fos promoter activity was significantly suppressed to 57% of the control level by co-transfection with DEP-1 (p < 0.0001). The activity was restored by co-transfection with the constitutively active Ras mutant RasG12V. We also examined the effect of a dominant-negative Ras mutant on c-fos promoter activity. The RasS17N mutant inhibited c-fos promoter activity to 32% of the control level (p < 0.0001). We next examined the effect of DEP-1 overexpression on cyclin A promoter activity. We used this promoter activity as an indicator for S phase progression, because a previous report showed that cyclin A promoter activity was strikingly down-regulated in contact-inhibited BAECs (29). As shown in Fig. 8B, cyclin A promoter activity was significantly inhibited to 42% of the control level by co-transfection with DEP-1 (p < 0.001). The suppression was overcome by co-transfection with RasG12V. The RasS17N mutant inhibited cyclin A promoter activity to 5% of the control.
ERK and PI 3-K in Contact-inhibited HUVECs

**DISCUSSION**

In the present study, we have shown that contact-inhibited HUVECs reentered the cell cycle in the presence of SOV by examining [3H]thymidine incorporation, protein expression levels of cyclins, CDKs, and CDK inhibitors, and CDK2 kinase activity. We have also demonstrated that SOV-induced cell cycle reentry of contact-inhibited HUVECs was associated with activation of ERK and Akt and that SOV-induced cell cycle reentry was inhibited by pretreatment with the MEK1/2 inhibitor PD98059 and the PI 3-K inhibitor LY294002. Furthermore, we have shown that overexpression of DEP-1 inhibited c-fos and cyclin A promoter activity in BAECs, which was restored by co-expression of the constitutively active Ras mutant RasG12V. A previous report has suggested that at an appropriate concentration, SOV could transform cells and that transformation was associated with increased protein phosphorylation in the cells (2). However, little is known as to which intracellular signaling pathways are activated by SOV and how treatment with SOV is finally linked to the cell cycle regulatory machinery. Our results showed down-regulation of cyclin D1, cyclin E, and cyclin A, an increased CDK2 kinase activity, and down-regulation of p27kip1, all of which could potentially stimulate cells to reenter the cell cycle. Recent reports suggested that up-regulation of p27kip1 was associated with density-dependent growth arrest or growth arrest induced by transforming growth factor-β (13–17). However, it is also reported that p27kip1(−)−(−) cells were growth-arrested by contact inhibition (34), suggesting that up-regulation of p27kip1 was not the sole factor that induced growth arrest. In this regard, it is of interest to note that the transcript level and promoter activity of cyclin A were reduced in contact-inhibited BAECs (29). Thus, it is possible that the down-regulation of cyclin D1, cyclin E, and cyclin A contributes to the growth arrest by contact inhibition in HUVECs. Although p21cip1 expression level was also increased by SOV, this was transient. It has been reported that p21cip1 expression was transiently increased by mitogens (35). Our results are compatible with those of that report.

Our results indicated that inhibition of the MEK- and PI 3-K-mediated pathways resulted in suppression of SOV-induced up-regulation of cyclin D1, cyclin E, cyclin A, and CDK2 kinase activity and restoration of SOV-induced down-regulation of p27kip1. Several lines of evidence have suggested that the Ras/MEK/ERK- and PI 3-K-mediated pathways are linked to the cell cycle regulatory machinery. A constitutively active MEK could transform cells or induce differentiation, which depended upon cell types (18, 19). In several studies, it was demonstrated, using a dominant negative Ras mutant, that Ras signaling pathways were involved in the up-regulation of cyclin D1, cyclin A, and CDK2 kinase activity and in down-regulation of p27kip1 (20, 21). Involvement of MEK/ERK in cyclin D1 up-regulation was also suggested in a report in which PD98059 was used to inhibit platelet-derived growth factor-induced ERK activation (36). PI 3-K also appears to be implicated in cell cycle progression. A retrovirus-encoded PI 3-K may induce cell cycle progression (27). PI 3-K may induce cell cycle progression via activation of p70S6K, which seems to play roles in the initiation of protein synthesis (37, 38). A recent report showed a direct link between Akt activation and stabilization of

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**FIG. 6.** Effects of PD98059 and LY294002 on SOV-induced phosphorylation of ERK and Akt in contact-inhibited HUVECs. A, effects of PD98059 (PD) and LY294002 (LY) on SOV-induced phosphorylation of ERK in contact-inhibited HUVECs. Confluent HUVECs were pretreated with 50 μM PD98059 or 50 μM LY294002 for 1 h and stimulated with 50 μM SOV for the indicated periods. Experiments were performed in the same way as in Fig. 5A. Shown are the results of a representative experiment among three independent experiments in which the same results were obtained. B, effects of PD98059 and LY294002 on SOV-induced phosphorylation of Akt in contact-inhibited HUVECs. Experiments were performed in the same way as in A. Shown are the results of a representative experiment among three independent experiments in which the same results were obtained. Level (p < 0.001). The result suggested that increased phosphatase activity could potentially inhibit cell cycle progression in vascular ECs, although the effect of DEP-1 appeared to be weaker than that of the dominant negative Ras mutant.

![Image](https://example.com/image2.png)

**FIG. 7.** Phosphatase activity in HUVECs. A, correlation of phosphatase activity with the amount of protein input. Increasing amounts of protein extract were incubated in the presence and absence of 200 μM SOV, and total (closed circles) and SOV-resistant (open circles) phosphatase activities were calculated by measuring absorbance at 410 nm. B, phosphatase activity was inhibited by SOV treatment. Confluent HUVECs were treated with 50 μM SOV for the indicated periods. SOV-sensitive phosphatase activity was calculated by subtracting SOV-resistant phosphatase activity from total phosphatase activity. * and **, p < 0.05 and p < 0.01, respectively, versus control (n = 3).
p27Kip1, because LY294002 and wortmannin restored the 3-K-mediated pathway was implicated in p27Kip1/cyclin D1 (39). Furthermore, it was suggested that the PI 3-K/Akt pathway alone with the inhibition of the other pathways, although it was not clear whether the SOV-sensitive phosphatases might be involved in the down-regulation of those pathways, although it was not clear whether the SOV-sensitive phosphatases inhibited phosphorylation of molecules such as Shc, which leads to the activation of the MEK/ERK and PI 3-K/Akt pathways, or stimulated dephosphorylation of ERK and Akt or both. It should be noted that the phosphorylation of ERK was reportedly decreased in contact-inhibited vascular endothelial cells, while activation of Ras and MEK was not impaired (41). The results indicate that dephosphorylation of ERK was stimulated and that the pathways located upstream of ERK were intact in contact-inhibited vascular endothelial cells. Recent reports have suggested that PKC-ζ, a PKC isozyme that belongs to a class of atypical PKC, directly phosphorylates and activates MEK, resulting in activation of ERK (42, 43). It has also been reported that PKC-ζ is a downstream target of PI 3-K (44, 45). We therefore examined whether LY294002 inhibited ERK phosphorylation via inhibition of PI 3-K/PKC-ζ. Our results, however, suggested that ERK phosphorylation occurred in the presence of LY294002, indicating that the involvement of PKC-ζ was not the major pathway of activation of the ERK pathway by SOV. Our results also showed that PDS8095 did not inhibit Akt phosphorylation, suggesting that activation of either the ERK pathway alone or the PI 3-K/Akt pathway alone with the inhibition of the other pathway was not sufficient to induce cell cycle reentry in contact-inhibited HUVECs.

To study the role of a specific phosphatase in vascular ECs, we examined the effects of DEP-1 overexpression on c-fos and cyclin A promoter activity in vascular ECs. Our results suggest that overexpression of DEP-1 could potentially inhibit cell cycle progression in vascular ECs. These results were compatible with those of a previous study in which growth of breast cancer cells was inhibited by overexpressed DEP-1 (46). Although we did not exclude the possibility that RasG12V inhibited DEP-1 activity, it is possible that the inhibitory effect of DEP-1 on cell cycle progression was mediated by, at least in part, suppression of the Ras-dependent pathways such as ERK and PI 3-K, because RasG12V restored the activity of the cyclin A promoter. Our results showed that the inhibitory effect of DEP-1 on cyclin A promoter activity was weaker than that of RasS17N. However, this did not mean that the role of phosphatases in density-dependent growth arrest was small, because DEP-1 was not the only phosphatase whose activity was increased in density-dependent growth arrest (3, 4).

Taken together, our results and those of other authors (3–5, 41) suggest the following scenario. The increased phosphatase activities in contact-inhibited vascular ECs cause down-regulation of the MEK/ERK and PI 3-K/Akt pathways, which in turn leads to the exit from the cell cycle due to down-regulation of cyclin D1, cyclin E, and cyclin A expression, and CDK2 kinase activity, and up-regulation of p27kip1 expression. However, these changes are reversible, and once the phosphatases are inhibited, contact-inhibited vascular ECs reenter the cell cycle. Thus, phosphatases, especially those whose activities are increased in density-dependent growth arrest, can be used to control the growth of other cell types, such as vascular smooth muscle cells. Further studies are required to identify other specific phosphatases that are implicated in density-dependent growth arrest and to elucidate the mechanisms by which the MEK/ERK and PI 3-K/Akt pathways are down-regulated by phosphatases.

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Reentry into the Cell Cycle of Contact-inhibited Vascular Endothelial Cells by a Phosphatase Inhibitor: POSSIBLE INVOLVEMENT OF EXTRACELLULAR SIGNAL-REGULATED KINASE AND PHOSPHATIDYLINOSITOL 3-KINASE

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