Puromycin-insensitive leucyl-specific aminopeptidase (PILSAP) binds and catalyzes PDK1, allowing VEGF-stimulated activation of S6K for endothelial cell proliferation and angiogenesis

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Puromycin-insensitive leucyl-specific aminopeptidase (PILSAP) plays an important role in angiogenesis by regulating the proliferation and migration of endothelial cells (ECs). Here we characterize the mechanism by which PILSAP regulates the vascular endothelial growth factor (VEGF)–stimulated proliferation of ECs. The specific elimination of *PILSAP* expression or its enzymatic activity inhibited VEGF-stimulated G1/S transition in ECs. This G1 arrest correlated with reduced cyclin dependent kinase 4/6 (CDK4/6) activity and retinoblastoma (Rb) protein phosphorylation. Analyses of signaling molecules upstream of CDK4/6 revealed that S6 kinase (S6K) activation was affected by PILSAP, whereas that of phosphatidylinositol-3 kinase (PI3K), Akt, and extracellular signal-related kinase 1/2 (ERK1/2) was not. We further demonstrated that PILSAP bound phosphatidylinositol-dependent kinase 1 (PDK1) and removed 9 amino acids from its N-terminus, which allowed S6K to associate with PDK1 and PILSAP upon VEGF stimulation. We constructed mutant PILSAP, which lacked the aminopeptidase activity but bound PDK1. Mutant PILSAP abrogated S6K activation upon VEGF stimulation in a dominant-negative manner. An N-terminal truncated form of PDK1 abolished the dominant-negative effect of mutant PILSAP. Finally, the introduction of a mutated *PILSAP* gene in ECs inhibited angiogenesis and retarded tumor growth in vivo. These results indicate that PILSAP plays a crucial role in the cell cycle progression of ECs and angiogenesis via the binding and modification of PDK1. (Blood. 2004;104:2345-2352)

Introduction

Blood vessels are normally quiescent in the adult mammal but have the ability to form neovessels under certain conditions. This so-called angiogenesis plays a critical role in various pathophysiologic conditions including the growth of solid tumors, diabetic retinopathy, and rheumatoid arthritis.[1] Numerous factors are reported to be involved in angiogenesis. Of these, the endothelial cell (EC)–tropic factors and their corresponding receptors have been extensively studied including vascular endothelial growth factor (VEGF), VEGF receptor 1/fms-liked tyrosine kinase 1 (VEGFR-1/Flt-1), and VEGFR-2 (kinase domain receptor/fetal liver kinase 1 [KDR/Flk-1]). VEGF-induced signals are known to activate a diverse array of intracellular signaling molecules such as phosphatidylinositol-3 kinase (PI3K), with its downstream Akt/protein kinase B and p70 S6 kinase (S6K), phospholipase C-γ (PLC-γ), along with the downstream protein kinase C (PKC), and the mitogen-activated protein kinases (MAPKs).[2] However, the entire complex array of intracellular signaling pathways involved in the realization of VEGF effects in ECs has yet to be adequately elucidated.

In order to explore as yet unknown mechanisms of angiogenesis, we decided to isolate genes exclusively expressed in ECs during differentiation. Using a subtraction strategy, we isolated a mouse version of puromycin-insensitive leucyl-specific aminopeptidase (PILSAP), the expression of which is augmented in mouse embryonic stem (ES) cells during differentiation to ECs in vitro.[3] Aminopeptidases, a group of enzymes that catalyze the N-termini of proteins, play important roles in posttranslational modification of proteins during the maturation, activation, modulation, and degradation of bioactive peptides, as well as protein stability.[4] PILSAP belongs to the M1 subfamily of aminopeptidases, which contains a consensus HEXXH(18X)E motif and a central Zn^{2+} ion essential for enzymatic activity.[3] Among this aminopeptidase family, an ectoenzyme aminopeptidase, N/CD13, has been reported to be involved in angiogenesis.[5,6]

Initial study revealed that PILSAP expression is induced in ECs upon stimulation with VEGF. This expression was confirmed in ECs at the site of angiogenesis in vivo. Specific elimination of PILSAP expression revealed a crucial role in VEGF-stimulated migration and proliferation of ECs in vitro as well as angiogenesis in vivo.[3] Subsequent study further demonstrated PILSAP to be involved in endothelial integrin function essential to cell adhesion and cell migration.[7]

Recently, 2 molecules with distinct functions were reported to be identical to PILSAP (ie, aminopeptidase regulator of tumor necrosis factor [TNF] receptor-1 shedding [ARTS-1][8] and the aminopeptidase associated with antigen processing in the endoplasmic reticulum [ERAAP]).[9] ARTS-1 is expressed as a type II integral membrane protein in pulmonary epithelial and endothelial

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cells. This protein forms a molecular complex with TNF receptor-1 and promotes TNF receptor-1 sheddase activity.[8] ERAAP is expressed in killer T cells and trims intracellular pathogens for major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum.[9] Thus, PILSAP seems to have multiple functions. However, precise mechanisms on how PILSAP exhibits these functions are not characterized.

We examined the mechanism of PILSAP regulation in VEGF-stimulated proliferation of ECs. The results indicate that PILSAP plays a crucial role in VEGF-stimulated G1/S-phase transition in ECs via the binding and modification of phosphatidylinositol-dependent kinase 1 (PDK1).

Materials and methods

Materials

Alpha-minimum essential medium (α-MEM), Opti-MEM, Lipofectin, and LipofectAMINE were purchased from Invitrogen (Carlsbad, CA). Dulbecco modified Eagle medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan); VEGF was from R&D Systems (Minneapolis, MN); anti-S6K, anti-PDK1, anti–cyclin dependent kinase 4 (anti-CDK4) and anti-CDK6 antibodies, S6 peptide, and partial retinoblastoma (Rb) protein were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Rb antibody was from Pharmingen (San Diego, CA); growth factor–reduced Matrigel was from Collaborative Research (Bedford, MA); and anti–von Willebrand Factor (anti-VWF) antibody was from DAKO Cytomation (Kyoto, Japan). Unless indicated, all other chemicals were purchased from Sigma (St Louis, MO).

Cell culture

MSS31 cells were cultured in α-MEM containing 10% fetal calf serum (FCS) as described previously.[3,6] HEK293 cells and COS-7 cells were cultured in DMEM containing 10% FCS, L-glutamine (2 mM), and kanamycin (100 μg/mL). B16F10 melanoma cells were cultured in RPMI 1640 with 10% FCS, L-glutamine (2 mM), and kanamycin (100 μg/mL).

Plasmids and oligodeoxynucleotides

The sense oligodeoxynucleotides (S-ODNs) and antisense ODNs (AS-ODNs) to PILSAP have been described previously.[3,6] Constructs for S6K, PDK1, and a modified PDK1 truncated of 10 amino acids from the N-terminus were generated by reverse transcription–polymerase chain reaction (RT-PCR) using mouse liver total RNA as a template and were cloned into pCR2.1 (Invitrogen). Thereafter, obtained cDNAs were inserted into pGEX-4T-3 (Amersham Bioscience, Piscataway, NJ), pcDNA3.1 (Invitrogen), or pcDNA3.1/myc-His (Invitrogen). cDNAs inserted into pGEX-4T-3 were expressed in BL21 *Escherichia coli* (Invitrogen), and glutathione-*S*–transferase (GST)–tagged S6K proteins were purified by glutathione–sepharose 4B (Amersham Bioscience). Constructs for the various domains of PILSAP were generated by PCR. H1 (amino acids 1-308) was generated using the "5′-primer," 5′-GCG AAT CCA ACC ATG CCC TCT CTT CTT-3′, and "3′-primer," 5′-CCT CGA GCA GCA CCA GAC TGA AAG TC-3′. H2 (amino acids 1-465, containing the aminopeptidase motif) was generated using 5′-primer, 5′-GCG AAT CCA ACC ATG CCC TCT CTT CTT-3′, and 3′-primer, 5′-CCT CGA GCC TCG TTT TTT GTG TTT TT-3′. T1 (amino acids 458-930) was generated using 5′-primer, 5′-TGG AAT TAT GAG TTA TAA AAA CAC AAA-3′, and 3′-primer, 5′-CCT CGA GCA GCA ACT TTG GCT TTT CTT-3′. T2 (amino acids 308-930, containing the aminopeptidase motif) was generated using 5′-primer, 5′-TGG AAT TAT GGA AAA CTG GGG ACT G-3′, and 3′-primer, 5′-CCT CGA GCA GCA ACT TTG GCT TTT C-3′. PCR products were digested by *Eco*RI and *Xho*I and inserted into pGEX-4T-2. GST-tagged PILSAP constructs were expressed in BL21 *E coli* and GST-tagged proteins were purified by glutathione–sepharose 4B.

PCR-based mutagenesis

PCR-based mutagenesis was performed on murine PILSAP cDNA contained in the expression vector pcDNA4 (Invitrogen) by PCR using Pyrobest DNA polymerase (Takara, Ohtsu, Japan). To construct the PILSAP mutants (E343A and E365A), the region of the cDNA to be mutated was amplified with an upstream oligonucleotide containing a unique restriction enzyme site (for E343A, *Eco*065I [5′-CAT GGT GAC CAG GTT CCC GAA CCA CTG GTG AGC CAG TGC ATG-3′]; for E365A, *Tth*111I [5′-TCT CTG GAC TGA GTC TGA TTT GCT GGT AAT GAA TGT CAG TGG-3′]) and a downstream oligonucleotide containing a restriction site (for E343A, *Kpn*I [5′-GAT GAC GAT AAG GTA CCT AGG ATC CAG TGT GGT GGA-3′]; for E365A, *Eco*065I [5′-CTG GTC ACC ATG GAA TGG TGG AAT GAC CTT TGG CTC AAT GCA GG-3′]). After purification of the resulting PCR fragment, the insert containing the mutation was substituted for the corresponding nonmutated region of the full-length PILSAP cDNA in pcDNA4.

Transfections, immunoprecipitation, and Western blot analysis

ODN or leucinthiol (LT) treatment was carried out as described previously.[3,6] Expression vector transfection was performed using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Efficiency of the transient transfection was about 20% to 30% of the cells in the preliminary experiment using reporter gene. After transfection, cells were serum starved for 24 hours in 0.1% FCS/α-MEM. Subsequently, cells were stimulated with VEGF and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (2 mM sodium orthovanadate, 50 mM NaF, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Immunoprecipitation was performed using anti-PDK1, anti-S6K, or anti-myc antibody as the primary antibody and samples were incubated for 2 hours at 4°C then with protein A–sepharose (Amersham Bioscience) for 2 hours at 4°C. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hi-Bond ECL; Amersham Bioscience). The membranes were blocked for 1 hour in 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.1% Tween 20, and 1% bovine serum albumin (BSA). In the same buffer, membranes were probed with primary antibodies for 1 hour. Subsequently, filters were washed 3 times in Tris-buffered saline (TBS) containing 0.1% BSA (wt/vol) and 0.05% Tween 20 (vol/vol) and incubated for 1 hour with horseradish peroxidase (HRP)–conjugated secondary antibodies. After being washed 3 times with TBS, immunoreactive proteins were visualized by the enhanced chemiluminescence (ECL) method using an ECL Western blotting detection kit (Amersham Bioscience).

Subcellular fractionation of PILSAP

COS-7 cells transiently transfected with PILSAP expression vector were suspended in buffer A (30 mM HEPES, pH 7.4; 2 mM EDTA; 5 mM EGTA [ethyleneglycotetraacetic acid]), homogenized in a Dounce-homogenizer, and centrifuged for 10 minutes at 800*g*. The resulting cell lysates were loaded onto a sucrose cushion (40% sucrose in buffer A) and centrifuged at 100 000*g* for 60 minutes at 4°C. The supernatant was collected as the cytosolic fraction, the interface as the plasma membrane (PM) fraction, and the pellet as the microsomal fraction. The PM fraction was pelleted by centrifugation at 100 000*g* for 30 minutes at 4°C and solubilized in solubilization buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.5% Triton X-100; 1 mM PMSF). Protein concentration was determined by use of the dendritic cell (DC) protein assay (Bio-Rad, Hercules, CA). Forty micrograms of each fraction was subjected to Western blot analysis.

DNA synthesis

MSS31 cells (3×10^3) were seeded in a 96-well multi-titer plate. Cells were transfected with ODNs and serum starved for 24 hours in α-MEM

containing 0.1% FCS. Cells were then stimulated with VEGF (50 ng/mL) while bromodeoxyuridine (BrdU) was added simultaneously. After 12 hours, incorporated BrdU was quantified with a BrdU enzyme-linked immunosorbent assay (ELISA) chemiluminescence kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cell cycle analysis

MSS31 cells (2×10^5) were plated on a 60-mm dish and treated with ODNs or LT. After a 24-hour incubation, cells were stimulated with VEGF (50 ng/mL) for another 24 hours. Then, cells were trypsinized and fixed in 70% ethanol. After being washed with phosphate-buffered saline (PBS), cells were treated with RNAse (5 mg/mL) for 30 minutes at room temperature. Subsequently, cells were treated with propidium iodide (50 μg/mL) for 30 minutes at 4°C. The cell cycle was analyzed using a fluorescence-activated cell sorter (FACS) Caliber system (Becton Dickinson, San Jose, CA) and ModFit LT software (Verity Software House, Topsham, ME).

S6 kinase activity

MSS31 cells were treated with ODNs or LT and serum deprived for 24 hours. Then, cells were stimulated with VEGF (50 ng/mL) for 20 minutes and lysed in RIPA buffer. Cell lysates (200 μg) were incubated with anti-S6K antibody for 2 hours at 4°C. Then, immune complexes were incubated with protein A–sepharose (Amersham Bioscience) for 2 hours at 4°C and precipitated. Immunoprecipitates were washed with RIPA buffer and were resuspended in 15 μL of kinase buffer (50 mM MOPS [3-[*N*-Morpholino]propanesulphonic acid], pH 7.2; 1 mM dithiothreitol [DTT]; 5 mM $MgCl_2$) containing 0.5 μg of S6 kinase peptide substrate (RRRLSSLRA) and 5 μCi (0.185 MBq) [α-^{32}P] adenosine triphosphate (ATP; Amersham Bioscience). Reactions proceeded for 30 minutes at 30°C and were stopped by the addition of SDS-PAGE sample buffer. The phosphorylated peptide was separated by 20% SDS-PAGE and visualized by autoradiography.

CDK activity

MSS31 cells were treated with ODNs or LT and serum deprived for 24 hours. The cells were then stimulated with VEGF (50 ng/mL) for 12 hours and lysed in RIPA buffer. Cell lysates (200 μg) were incubated with anti-CDK antibody for 2 hours at 4°C. Immune complexes were incubated with protein A–sepharose for 2 hours at 4°C and precipitated. Immunoprecipitates were washed with RIPA buffer and resuspended in 15 mL kinase buffer (50 mM HEPES, pH 7.2; 1 mM DTT; 5 mM $MgCl_2$) containing 0.5 μg purified Rb and 5 μCi (0.185 MBq) [α-^{32}P] ATP. Reactions proceeded for 30 minutes at 30°C and were stopped by the addition of SDS-PAGE sample buffer. The phosphorylated peptide was separated by 8% SDS-PAGE and visualized by autoradiography.

PDK1 activity

MSS31 cells were treated with ODNs or LT and serum deprived for 24 hours. Then, cells were stimulated with VEGF (50 ng/mL) for 20 minutes and lysed in RIPA buffer. Cell lysates (200 μg) were incubated with anti-PDK1 antibody for 2 hours at 4°C. Immune complexes were then incubated with protein A–sepharose for 2 hours at 4°C and precipitated. Immunoprecipitates were washed with RIPA buffer and were resuspended in 15 μL of kinase buffer (50 mM MOPS, pH 7.2; 1 mM DTT; 5 mM $MgCl_2$) containing 0.5 μg of purified GST-S6K and 5 μCi (0.185 MBq) [α-^{32}P] ATP. Reactions proceeded for 30 minutes at 30°C and were stopped by the addition of SDS-PAGE sample buffer. The phosphorylated substrate was separated by 8% SDS-PAGE and visualized by autoradiography.

In vitro cleavage of PDK1 by PILSAP

Purified PDK1 (1 μg) and PILSAP (1 μg) were incubated in Tris-HCl (50 mM, pH 7.5) at 37°C for 30 minutes. Reactions were terminated by the addition of SDS-PAGE sample buffer and samples were analyzed by Western blotting. For the determination of the PDK1 cleavage site, PDK1 was incubated with PILSAP for 1 hour and products were separated by SDS-PAGE. After transferred polyvinylidene difluoride (PVDF) membrane (Bio-Rad), membrane was stained with Coomasie Brilliant Blue and the cleaved PDK1 band was excised. The N-terminal sequence of the band was determined by an HP G1005A protein sequencing system (Hewlett Packard, Palo Alto, CA).

Aminopeptidase activity

Aminopeptidase activity was determined as described previously.[3] Briefly, transfected HEK293 cells were homogenized by ultrasonication, and the resulting lysates were centrifuged for 15 minutes at 10 000*g* at 4°C. Aminopeptidase activity was measured using a fluorogenic substrate, leucyl-4-methylcoumaryl-7-amide (Leu-MCA; Peptide Institute, Osaka, Japan).

Mouse angiogenesis model

A mouse angiogenesis model using 4-week-old male C57BL/6 mice was prepared as described previously.[3,10] Briefly, 300 μL of growth factor–reduced Matrigel in liquid form containing VEGF (100 ng/mL) plus heparin (32 U/mL) at 4°C was injected into the abdominal subcutaneous tissue (n = 5). In gene transfer experiments, a hemagglutinating virus of Japan (HVJ)–envelope vector (GenomONE; Ishihara Sangyo, Osaka, Japan) originally described by Kaneda et al[11] was used. HVJ-envelope vector contains foreign DNA in the inactivated HVJ particle and introduces foreign DNA through the fusion to plasma membrane. One arbitrary unit (AU) of HVJ-envelope vector, containing 20 μg of the PILSAP expression vector, was mixed in the Matrigel prior to the injection. On day 6 after injection, mice were killed and gels were recovered. The gels were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Three-micrometer sections of the gels were subjected to hematoxylin and eosin staining and subjected to immunohistochemical analysis. Fixed samples were incubated with blocking buffer (PBS containing 1% BSA) and then incubated with primary antibody (anti–green fluorescent protein [anti-GFP]) in PBS containing 0.1%BSA. After washing, samples were incubated with alkaline phosphatase (AP)–conjugated anti–rabbit immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA). Immunoreactive proteins were visualized by means of a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma).

Measurements were taken of the hemoglobin contents of the Matrigel for quantification of angiogenesis. Briefly, the Matrigel was weighed and homogenized in distilled water (10 μL/mg wet weight). After centrifugation at 5000*g* for 5 minutes, the hemoglobin concentration in the supernatant was determined by a hemoglobin B-test kit (Wako, Osaka, Japan).

Tumorigenesis assay

Twenty-four hours after the injection of Matrigel mixed with HVJ-envelope vector containing LacZ, wild-type (Wt), or mutated PILSAP (E343A) vector, B16F10 cells (10^5 cells in 100 μL sterile PBS) were inoculated on top of the Matrigel. B16F10 is a highly metastatic cell line established from B16 melanoma cells.[12] Fourteen days after the inoculation, tumors were excised, fixed in 4% paraformaldehyde, and embedded in paraffin. For microvessel quantification, 5 μm of sections were blocked with 2% goat serum and 2% BSA in PBS for 30 minutes at room temperature and incubated with anti-VWF antibody (DAKO) overnight at 4°C. After 3 washes in PBS, slides were incubated with rhodamine-conjugated anti–rabbit IgG (Vector Laboratories) for 1 hour at room temperature, washed, and counterstained with DAPI (4,6 diamidino-2-phenylindole; Molecular Probe, Eugene, OR). Stained microvessels were identified by fluorescence microscopy (OLYMPUS, Tokyo, Japan) counted at × 20 magnification in 10 random fields from the tumor. All animal studies were reviewed and approved by the Committee for humane animal study at our institute.

Results

Role of PILSAP in the cell cycle progression of ECs

We previously reported that PILSAP AS-ODN and LT treatment is able to suppress VEGF-stimulated EC proliferation and migration.[3,6] Here we examined the mechanism by which PILSAP regulates the EC proliferation stimulated by VEGF. We confirmed that PILSAP AS-ODN specifically inhibited the expression of PILSAP, whereas LT potently inhibited the PILSAP enzymatic activity.[3] We then used PILSAP AS-ODN and LT for the following experiments.

We found that PILSAP AS-ODN and LT inhibited VEGF-stimulated DNA synthesis (Figure 1A) and as a result there was a decrease of cells in the S phase (Figure 1B). This indicates that PILSAP was involved in G_1/S progression in cell cycle. The direct effectors of G_1/S progression are CDKs, which form complexes with cyclins and phosphorylate Rb, thereby dissociating E2 promoter binding factor (E2F) from Rb. To examine the CDK activities, we performed immune-complex kinase assays with a fragment of the Rb protein as the substrate. As shown in Figure 2A-B, PILSAP AS-ODN or LT treatment decreased the VEGF-stimulated activation of CDK4 and CDK6. We also confirmed the Rb phosphorylation by Western blotting. The amount of phosphorylated Rb protein was again decreased by treatment with PILSAP AS-ODN or LT (Figure 2C).

Involvement of PILSAP in the activation of S6K

We further examined the upstream intracellular signaling molecules. S6K lies upstream of the CDKs and regulates the G_1/S transition.[13,14] As shown in Figure 3A, PILSAP AS-ODN or LT treatment clearly decreased the VEGF-stimulated activation of S6K. In our previous study, PILSAP AS-ODN inhibited angiogenic effects of both VEGF and basic fibroblast growth factor (bFGF).[3]

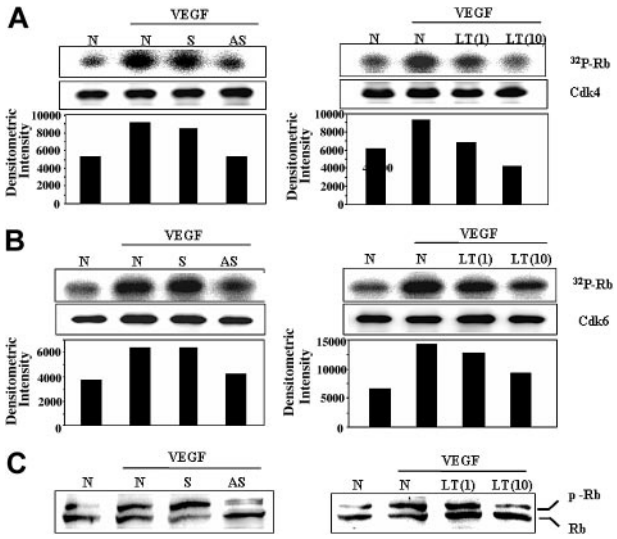

Figure 2. PILSAP is required for CDK activity and Rb phosphorylation upon VEGF stimulation. After treatment with ODNs or with LT, MSS31 cells were serum starved for 24 hours and then stimulated with VEGF (50 ng/mL) for 12 hours. (A) CDK 4 and (B) CDK 6 activities were analyzed by immune-complex kinase assays. The densitometric intensities were determined and normalized with those of the total protein level. (C) Phosphorylation state of the Rb protein was analyzed by immunoblotting.

Here we observed that PILSAP AS-ODN or LT inhibited bFGF-stimulated S6K activation as well (data not shown). Thus, PILSAP plays a crucial role in S6K activation of ECs in response to representative angiogenic growth factors.

Since S6K is known as a downstream target of PI3K, we analyzed the phosphorylation status of PI3K. As shown in Figure 3B, neither PILSAP AS-ODN nor LT affected the activation of the PI3K in response to VEGF stimulation. Akt, another downstream target of PI3K, plays an important role in angiogenesis by

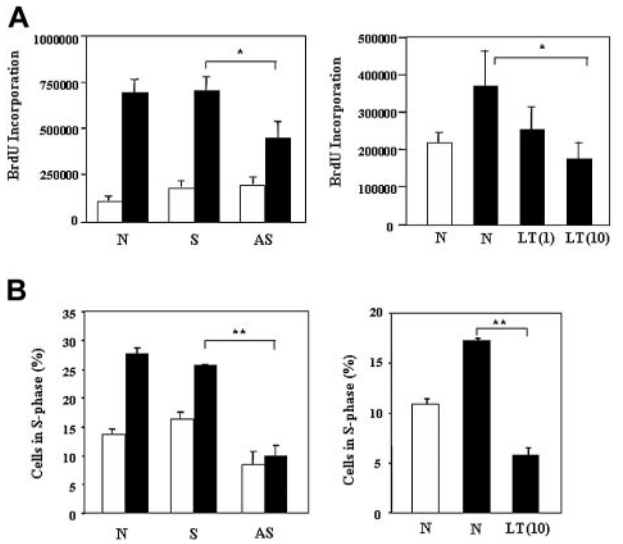

Figure 1. PILSAP is required for DNA synthesis and G1/S transition upon VEGF stimulation. (A) Serum-starved MSS31 cells were unstimulated (□) or were stimulated with VEGF (■) (50 ng/mL) for 12 hours. Thereafter, BrdU incorporation was analyzed. (Left) N indicates no ODN; S, PILSAP S-ODN; and AS, PILSAP AS-ODN. (Right) N indicates no LT; LT(1), LT 1 nM; and LT(10), LT 10 nM. (B) Serum-starved MSS31 cells were stimulated with VEGF (50 ng/mL) for 18 hours. Thereafter, cell cycle was analyzed by flow cytometry. Data indicates the percentage of cells in the S phase. (Left) N indicates no ODN; S, PILSAP S-ODN; and AS, PILSAP AS-ODN. (Right) N indicates no LT; LT(1), LT 1 nM; and LT(10), LT 10 nM. Error bars indicate SDs. (n = 4; $*P < .01$; $**P < .005$).

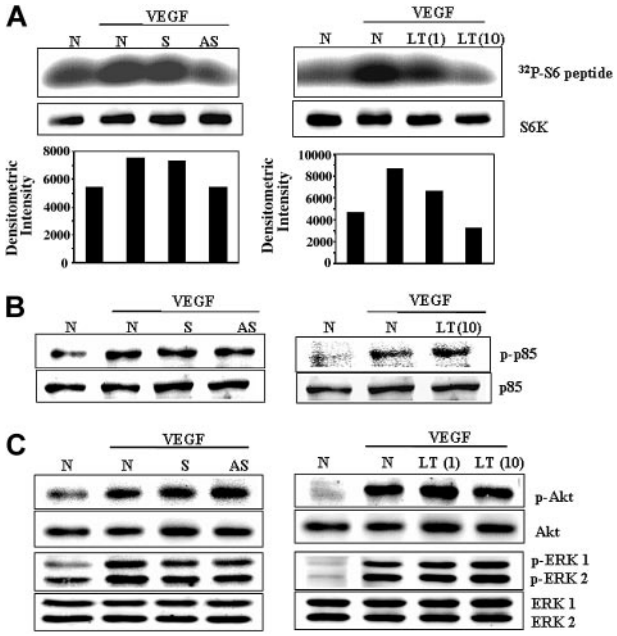

Figure 3. PILSAP is required for the activation of S6K upon VEGF stimulation. After treatment with ODNs or with LT, MSS31 cells were serum starved for 24 hours and then stimulated with VEGF (50 ng/mL) for 15 minutes. (A) S6K activities were analyzed by immune-complex kinase assay. The densitometric intensities were determined and normalized with those of the total protein level. (B) Akt and (C) ERK1/2 were analyzed by immunoblotting.

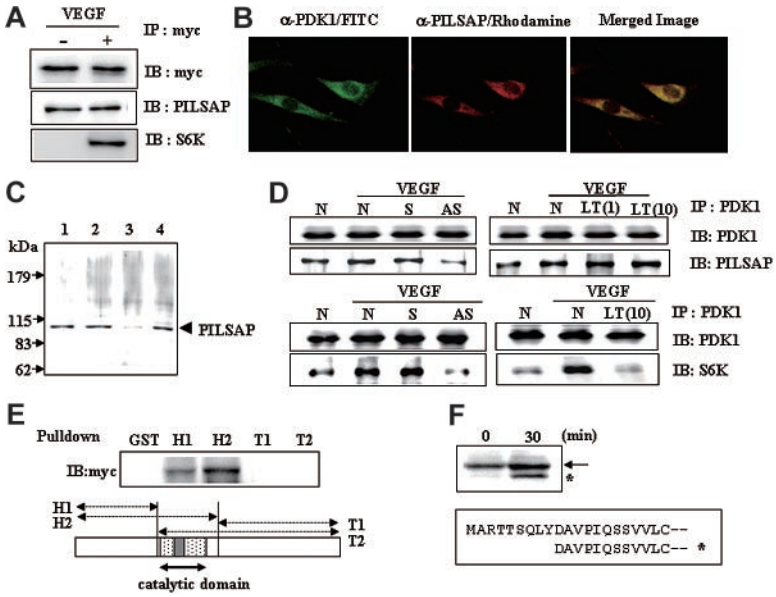

Figure 4. PILSAP binds and modifies PDK1 allowing the association of S6K. (A) MSS31 cells were transfected with an expression plasmid encoding myc-tagged PDK1. Cells were treated with or without VEGF (50 ng/mL). Cell lysates were immunoprecipitated (IP) with anti-myc antibody (Ab). Immunoprecipitates were resolved and immunoblotted (IB) with the indicated antibodies. PDK1 binds under basal conditions, and S6K associates with these proteins upon VEGF stimulation. (B) MSS31 cells were transfected with the PDK1 expression vector and stained with an anti-His (PDK1) or anti-PILSAP antibody. Immunofluorescent staining of PDK1 (green) and PILSAP (red) was performed. Cells were observed by confocal microscopy (LSM410; Carl Zeiss, Jena, Germany). Objective lens: Plan Neofluor 20×; camera: 35 mm Contax 167MT; software: LSM3.98. (C) COS-7 cells were transfected with the PILSAP expression vector and the subcellular localization of PILSAP was analyzed as described in "Materials and methods." Lane 1 shows the total; lane 2, cytosolic fraction; lane 3, membrane fraction; and lane 4, microsomal fraction. (D) After treatment with ODNs or with LT, MSS31 cells were serum starved for 24 hours and then stimulated with VEGF (50 ng/mL) for 15 minutes. Cell lysates were immunoprecipitated with an anti-PDK1 Ab. Precipitates were then immunoblotted with anti-PDK1 and anti-PILSAP Abs or with anti-PDK1 and anti-S6K Abs. (E) HEK293 cells were transfected with a PDK1-myc expression vector and cell lysates were incubated with purified GST-tagged PILSAP constructs (H1, H2, T1, T2) for pull-down analysis. (F) Purified PILSAP and PDK1 were incubated for 30 minutes at 37°C and subjected to immunoblotting of PDK1. The N-terminal amino acid sequence was determined. *The cleaved PDK1.

regulating the survival, migration, and NO synthesis in ECs.[15-17] Neither PILSAP AS-ODN nor LT affected the activation of Akt in response to VEGF stimulation (Figure 3C). The classic MAP kinase, ERK1/2, plays an important role in cell proliferation by inducing cyclin D1.[18] As shown in Figure 3C, treatment with PILSAP AS-ODN or LT did not affect the activation of ERK1/2 in response to VEGF stimulation. Thus, PILSAP is involved rather selectively in the activation of S6K.

PDK1 as a substrate of PILSAP

An important question is the mechanism by which PILSAP is able to modulate the activation of S6K. PDK1 transmits the signal from PI3K to S6K and plays a critical role in the phosphorylation of the threonine 229 of S6K.[19] The general mode of action of metalloproteinases, including the aminopeptidases, is to bind substrates in order to cleave them.[20] We examined whether PILSAP could bind PDK1 or S6K. We transiently transfected the PDK1 gene and performed immunoprecipitation followed by Western blotting. We found that the binding of PILSAP and PDK1 was observed under basal conditions, and S6K associated and made ternary complex with PDK1 and PILSAP when cells were stimulated with VEGF (Figure 4A). The colocalization of PILSAP and PDK1 was shown by immunocytochemical analysis as well (Figure 4B). Subcellular fractionation analysis showed that PILSAP localized in cytosolic and microsomal fractions (Figure 4C).

We then examined the binding of endogenous proteins. PILSAP was co-immunoprecipitated with PDK1 under basal conditions (Figure 4D). The down-regulation of PILSAP by AS-ODN decreased the binding with PDK1, whereas the inhibition of aminopeptidase activity by LT did not (Figure 4D). In contrast, the association of S6K was augmented by the VEGF stimulation; PILSAP AS-ODN and LT equally inhibited this augmented association of S6K (Figure 4D). Thus, although the aminopeptidase activity was not required for the binding of PILSAP and PDK1, it required the association of S6K upon VEGF stimulation. We determined the PDK1-binding region of PILSAP. Pull-down analysis revealed that H1 and H2 but not T1 and T2 of PILSAP bound with PDK1 (Figure 4E). Thus, PDK1 primarily binds to N-terminus noncatalytic region (H1). We then tested whether PILSAP could cleave PDK1 as a substrate. Purified PILSAP and PDK1 were mixed in a test tube. After a 30-minute incubation, a new band appeared below the authentic PDK1 (Figure 4F asterisk). The determination of the amino acid sequence revealed that the new band was a truncated form of PDK1, which had lost 9 amino acids from the N-terminus (Figure 4F).

Mutant PILSAP as a dominant-negative molecule

For further investigation, we generated mutant PILSAP lacking in aminopeptidase activity. The consensus HEXXH(18X)E motif is highly conserved in zinc metalloproteinases, and 2

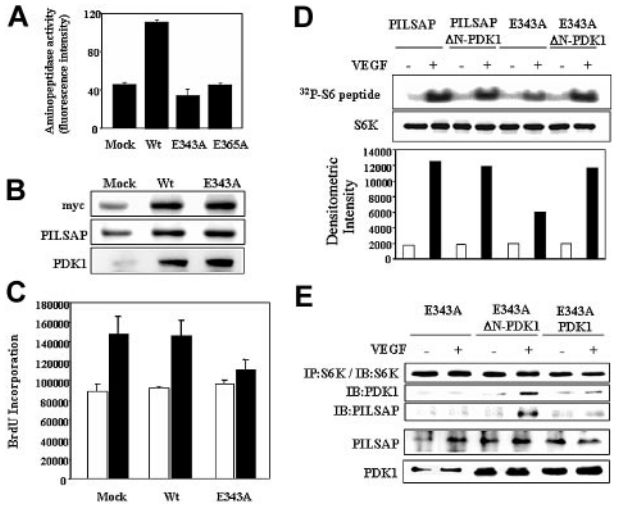

Figure 5. Mutant PILSAP acts as a dominant-negative molecule. (A) HEK293 cells were transfected with a Wt or mutant PILSAP expression plasmid and aminopeptidase activity was measured using Leu-MCA as the substrate. Error bars indicate SDs. (B) MSS31 cells were transfected with a Wt or E343A expression vector and immunoprecipitated with an anti-myc antibody. Thereafter, pull-down analysis was performed using myc-tagged Wt and E343A. (C) Mock, Wt, or E343A transfected MSS31 cells were serum starved and stimulated with or without VEGF (50 ng/mL) for 12 hours. Incorporated BrdU was measured by a chemiluminescent BrdU ELISA. Error bars indicate SDs. (D) MSS31 cells were transfected with the indicated combination of expression vectors (wild-type PILSAP, E343A, and ΔN-PDK1). S6K was immunoprecipitated and kinase activities were measured using S6 peptide as the substrate. The densitometric intensities were determined and normalized with those of the total protein level. (E) MSS31 cells were transfected with the indicated combination of expression vectors (E343A, ΔN-PDK1, and wild-type PDK1) and were stimulated with or without VEGF (50 ng/mL). Association of S6K, PDK1 including ΔN-PDK1, and PILSAP including E343 was analyzed by immunoprecipitation followed by Western blotting. Total PILSAP and PDK1 were shown by Western blotting in the bottom 2 panels.

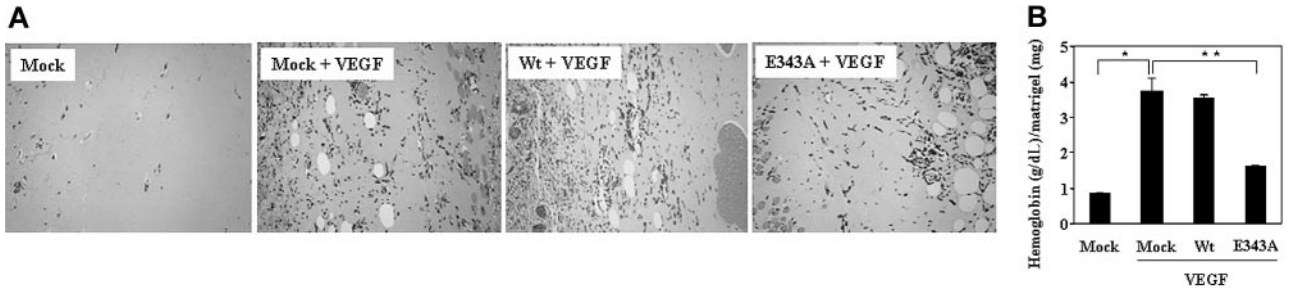

Figure 6. Mutant PILSAP inhibits angiogenesis in vivo. A mock or E343A expression plasmid was incorporated into the HVJ-envelope vector and mixed with Matrigel. Matrigel was subcutaneously injected into the abdomen of C57BL/6 mice. Sections were observed using an Olympus BH2 microscope and a high-resolution CCD color camera. Objective lens: SPlan 20×; camera: CCD Digital3 CCD camera KS630; software: Image-Processing (FLOVEL, Tokyo, Japan). The hemoglobin content of the Matrigel was determined (n = 4; *$P < .01$; **$P < .005$). Error bars indicate SDs.

glutamate residues are essential for catalytic activity.[20] Each of the glutamate residues in this motif was substituted to alanine (E343A and E365A). As shown in Figure 5A, aminopeptidase activity was lost in both mutant proteins. We used the mutant E343A in the following experiments. Pull-down analysis showed that wild-type (Wt) PILSAP and E343A associated with PDK1 (Figure 5B). We transiently transfected MSS31 cells with mock, Wt, or E343A expression vector. Whereas VEGF stimulated DNA synthesis in mock and Wt transfectants, it greatly reduced the stimulatory effect in an E343A transfectant (Figure 5C). VEGF-stimulated activation of S6K was markedly impaired in cells transfected with E343A cDNA (Figure 5D). Thus, mutant PILSAP acts as a dominant-negative molecule. However, when cells were cotransfected with E343A and a modified PDK1 truncated of 10 amino acids from the N-terminus (ΔN-PDK1) expression plasmids, ΔN-PDK1 reduced the inhibitory effect of E343A on S6K activation (Figure 5D). The association of S6K, PDK1, and PILSAP in the VEGF-stimulated condition was evident when MSS31 cells were cotransfected with E343A and ΔN-PDK1 expression vectors, whereas this association was not observed when MSS31 cells were transfected with E343A expression vector or with E343A and wild-type PDK1 expression vectors (Figure 5E). These results indicate that cotransfection of ΔN-PDK1 gene negates the dominant-negative effect of E343A and thus further confirm the importance of N-terminal truncation of PDK1 for the association and activation of S6K.

Application of mutant PILSAP for the control of angiogenesis

Since E343A acted in a dominant-negative manner, we tested whether E343A was applicable for the control of angiogenesis. We used the HVJ-envelope vector system in our in vivo experiments (see "Materials and methods"). A control experiment revealed that more than 80% of the ECs that invaded Matrigel were transfected when the GFP gene was enveloped by an HVJ-envelope vector and applied to the Matrigel plug experiment (data not shown). We then applied the E343A gene to this system. As shown in Figure 6A-B, E343A significantly inhibited VEGF-stimulated angiogenesis in the Matrigel.

Finally, we tested the effect of mutant PILSAP on the growth of tumor. We used B16F10 cells for this analysis. Transient transfection of E343A gene in B16F10 cells did not affect the DNA synthesis in vitro (Figure 7A). We then inoculated B16F10 cells on top of the Matrigel layer 24 hours after the Matrigel injection and analyzed tumor growth on 3 different Matrigel layers (ie, containing the LacZ, wild-type PILSAP, or E343A gene). As shown in Figure 7B, the in vivo growth of melanoma on the Matrigel containing the E343A gene was noticeably retarded. Macroscopic inspection of tumor indicated that tumors on Matrigel containing LacZ or the wild-type PILSAP gene had robust feeder vessels, whereas tumors on Matrigel containing the E343A gene had substantially smaller vessels (data not shown). Microscopic analysis further revealed that microvessel density in tumors on Matrigel containing E343A was observably less than that in tumors on

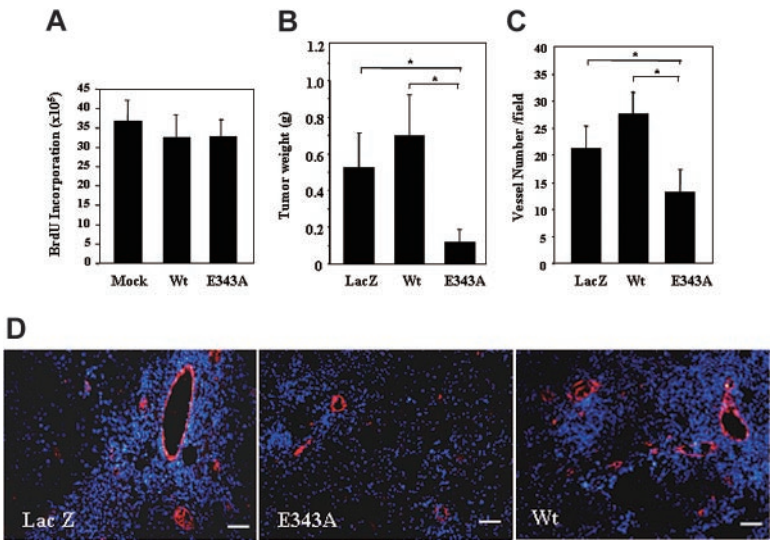

Figure 7. Mutant PILSAP inhibits tumor growth and angiogenesis in vivo. (A) B16F10 cells were transiently transfected with a mock, Wt, or E343A expression plasmid. Thereafter, B16F10 cells were stimulated with 10% FCS for 12 hours and the in vitro–incorporated BrdU were measured by a chemiluminescent BrdU ELISA (n = 6). (B) A mock, Wt, or E343A expression plasmid was incorporated into the HVJ-envelope vector and mixed with Matrigel. Matrigel was subcutaneously injected into the abdomen of C57BL/6 mice. Twenty-four hours after Matrigel injection, B16F10 cells were inoculated subcutaneously on top of the Matrigel. Fourteen days after inoculation, tumors were excised and weighed (n = 4; *$P < .01$). (C) The number of microvessels in the tumor tissue was counted (n = 4; *$P < .01$). Error bars indicate SDs. (D) Tumor sections were stained with anti-VWF antibody (red) and nuclei were counterstained with DAPI (blue). Scale bar: 100 μm. Sections were observed using fluorescence microscopy (Olympus IX71 microscope and Color View Soft Image System). Objective lens: LCPlan Fl 20×; camera: CCD Color Camera System's ColorView II; software: analySIS Docu (Soft Imaging System, Münster, Germany).

Matrigel containing either LacZ or wild-type PILSAP (Figure 7D). Quantitative analysis revealed that microvessel density was significantly decreased in tumors on Matrigel containing E343A (Figure 7C).

Discussion

Although a variety of intracellular signaling molecules such as PI3K, Akt, and MAPKs have been reported to be involved downstream of the VEGF-VEGFR signal transduction pathway,[2] the function of S6K has not been adequately determined. However, there are at least 3 reports that have indicated that S6K is required for mitogenic activity in ECs.[21-23] The present study confirmed the importance of S6K and further revealed that PILSAP plays an indispensable role in the activation of S6K in VEGF-stimulated cell cycle progression in ECs. As PILSAP is an aminopeptidase, PILSAP would be expected to modulate cell function by catalyzing its physiologic substrates. However, the substrates of PILSAP have yet to be determined. Our present results suggest that PILSAP binds PDK1 through its N-terminus noncatalytic region and removes 9 amino acids from the N-terminus of PDK1, which allows the subsequent S6K activation.

PDK1 is a 63-kDa serine/threonine kinase that consists of an N-terminal kinase domain containing a hydrophobic PDK1-interacting fragment (PIF)–binding pocket and a C-terminal pleckstrin homology (PH) domain.[24] PIF-binding pocket localizes between 115 and 155 amino acid residues in its N-terminus.[24] Once S6K is phosphorylated in its hydrophobic motif by an as yet uncharacterized kinase upon the activation of PI3K, phosphorylated S6K binds to the PIF-binding pocket of PDK1.[24] This in turn allows PDK1-mediated activation of S6K.[24] However, it has been obscure whether the modification of PDK1 is required for this process. Here we showed for the first time that PILSAP and PDK1 bound under basal conditions. Moreover, S6K associated with PDK1/PILSAP complex upon VEGF stimulation, but specific elimination of the expression of PILSAP or inhibition of its enzymatic activity abrogated this association of S6K. Although the modification of endogenous PDK1 remains to be established, PILSAP cleaved 9 N-terminal amino acids of PDK1 in a test tube. In addition, E343A acted as a dominant-negative molecule, but simultaneous expression of ΔPDK1 could overcome the dominant-negative effect of E343A. From these results, we assume that the 9 N-terminal amino acids of PDK1 block the PIF-binding pocket, and cleavage of these 9 N-terminal amino acids by PILSAP allows the association of S6K upon VEGF stimulation. To the best of our knowledge, this is the first demonstration that an intracellular signaling molecule is modified by an aminopeptidase to express its activity. However, such N-terminal modification is likely to not be unique to PDK1. Other intracellular signaling molecules such as Raf-1 and TGF-β activating kinase 1 (Tak-1) contain N-terminal inhibitory domains.[25-27] Therefore, we propose that the results obtained in the present study may serve as a guidepost for further research into aminopeptidase-related intracellular signaling.

Although PDK1 activates both S6K and Akt, VEGF-stimulated activation of Akt was not influenced by PILSAP (Figure 3B). This apparent discrepancy might possibly be accounted for as follows. The mechanism by which PDK1 activates Akt is distinct from S6K, as PDK1 does not directly bind to Akt.[28] PDK1 contains a PH domain in its C-terminus region, whereas Akt contains the same domain in its N-terminus region. Once PI3K is activated, phosphatidyl-inositol-3, 4, 5-triphosphate (PIP3) is generated on the inner surface of the plasma membrane. The PH domains of PDK1 and Akt guide these 2 molecules to plasma membrane PIP3 in close proximity, which in turn allows PDK1 to activate Akt.[28] Since the PH domain of PDK1 localizes in its C-terminus, it is reasonable to hypothesize that N-terminal modification of PDK1 by PILSAP does not affect the targeting of PDK1 to the plasma membrane. While this remains to be borne out by experimental confirmation, it would account for the discordance between S6K and Akt.

The activation of S6K requires hierarchical phosphorylation of threonines in positions 389 and 229.[19] PDK1 is responsible for the phosphorylation of threonine in 229, whereas the mammalian target of rapamycin (mTOR), a downstream target of Akt,[29,30] is responsible for the phosphorylation of threonine at 389.[19] Thus, in terms of the activation of S6K, mTOR might be thought to be another target of PILSAP. However, this may not be the case. mTOR is shown to activate S6K as well as protein phosphatase 2A (PP2A).[31,32] We observed that neither PILSAP AS-ODN nor LT was able to affect VEGF-stimulated PP2A activity (data not shown). This observation rather strongly suggests the activity of mTOR is not, in fact, affected by PILSAP.

We have previously demonstrated that the specific elimination of PILSAP expression inhibits angiogenesis in vivo.[3] As mutant PILSAP E343A acts as a dominant-negative molecule, we employed the E343A gene for the inhibition of angiogenesis in the present study. Transfection of the E343A gene in ECs in Matrigel by means of HVJ-envelop vector inhibited angiogenesis. Moreover, when B16F10 cells were inoculated on top of Matrigel containing HVJ-envelop vector/E343A, tumor growth and angiogenesis were significantly inhibited. B16F10 cells did not express PILSAP (data not shown), and E343A did not affect the growth of B16F10 cells in vitro (Figure 7A). Antiangiogenic therapy offers several potential advantages as an approach to cancer treatment.[33] Our present results suggest endothelial PILSAP to be a molecular target of considerable potential for the inhibition of tumor angiogenesis.

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