A Truncated Kringle Domain of Human Apolipoprotein(a) Inhibits the Activation of Extracellular Signal-regulated Kinase 1 and 2 through a Tyrosine Phosphatase-dependent Pathway*

Received for publication, December 12, 2003, and in revised form, February 20, 2004
Published, JBC Papers in Press, March 2, 2004, DOI 10.1074/jbc.M313633200

From the ‡Mogam Biotechnology Research Institute, Yongin-city, Kyonggi-do, 449-910, Korea and the ¶Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

Jin-Hyung Ahn‡§, Jang-Seong Kim‡§, Hyun-Kyung Yu‡, Ho-Jeong Lee‡, and Yeup Yoon‡¶

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is essential for tumor growth and metastasis. Without blood vessels, tumors are unable to grow beyond a critical size (2–3 mm³) or metastasize to another organ (1). In view of this essential requirement, inhibition of tumor-associated angiogenesis has become one of the most promising pharmacological targets in cancer treatment, and numerous angiogenesis inhibitors are currently being evaluated in clinical trials. Angiogenesis is tightly controlled by a balance between pro-angiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), and anti-angiogenic factors, such as angiostatin, endostatin, and thrombospondin-1 (2). Perturbations in this balance can trigger angiogenic cellular signals, thereby leading to inappropriate angiogenesis.

The kringle domain is a protein structure comprising ~80 amino acids with conserved triple disulfide bonds that appears to function as an independent folding unit (3). Kringle domains are found in many proteins with a surprisingly diverse array of functions such as growth factors, proteases, or coagulation factors (hepatocyte growth factor, plasminogen, prothrombin, and urokinase, etc.). They are thought to play an important role in protein-protein interactions that provide specificity to and regulation of their parent proteins. Moreover, many kringle domains have been identified as inhibitors of angiogenesis (4). Based on these findings, kringle have been suggested to constitute the first example of a conserved architecture that specifically inhibits blood vessel growth.

Of the kringle-containing proteins, apolipoprotein(a) (apo(a)) contains the largest numbers of kringle domains, with as many as ~52 kringle (5). It consists of tandemly repeated kringle domains that closely resemble plasminogen kringle 4 (KIV), followed by sequences that are homologous to the kringle 5 (termed KV) and protease domains of plasminogen (6). The KIV(375) domains of apo(a) can be classified into 10 types (designated types KIV-1 to KIV-10), based on amino acid sequence (7). Although the physiological and biochemical roles of apo(a) kringle domains remain to be elucidated, there exists some evidence suggesting that they can inhibit angiogenesis in vitro (8) and suppress tumor growth in vivo (9). Recently, we have demonstrated that a recombinant peptide comprising the apo(a) kringle domains (rhLK68) that contains KIV-9, KIV-10, and KV inhibits bFGF-stimulated endothelial cell mitogenesis and migration in vitro and suppresses the growth of human lung and colon tumors in nude mice (10). However, its mechanism of action was not clearly elucidated.

This study was performed to explore the molecular mechanism of rhLK68-mediated angiogenesis inhibition. We examined the effects of rhLK68 on the extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway(s), because of the critical role these cascades play in angiogenesis. We demonstrate here that rhLK68 inhibits the activation of ERK1/2, but not PI3-K/Akt, induced by bFGF, VEGF, and HGF. In addition, we present data suggesting that protein-tyrosine phosphatases may play an important role in the inhibition of ERK signaling by rhLK68.

Most proangiogenic factors exert their biological effects primarily by activating extracellular signal-regulated kinase (ERK) and phosphoinositol 3-kinase (PI3-K)/Akt signaling pathways. These pathways appear to play a critical role in endothelial cell migration, because selective inhibition of either ERK or PI3-K/Akt signaling almost completely prevented endothelial cell migration. Recently, we demonstrated that a truncated kringle domain of human apolipoprotein(a), termed rhLK68, inhibits endothelial cell migration in vitro. However, its mechanism of action was not well defined. In this study, we determined the effects of rhLK68 on ERK1/2 and PI3-K/Akt signaling pathways to explore the molecular mechanism of rhLK68-mediated inhibition of endothelial cell migration. Treatment with rhLK68 inhibited ERK1/2 phosphorylation but did not influence Akt activation. Interestingly, an inhibitor of protein-tyrosine phosphatase, sodium orthovanadate, dose-dependently reversed both rhLK68-induced dephosphorylation of ERK1/2 and decreased migration of endothelial cells, whereas rhLK68 showed no significant effects on MEKs phosphorylation. In conclusion, these results indicate that inhibition of endothelial cell migration by rhLK68 may be achieved by interfering with ERK1/2 activation via a protein-tyrosine phosphatase-dependent pathway.
**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Human LK68—** Recombinant human LK68 (rhLK68), which comprises cryptic kringle fragments of apo(a) containing KIV-9, KIV-10, and KIV, was expressed and purified from *Escherichia coli* as described previously (10). Briefly, rhLK68 expressed as an inclusion body was collected, refolded in vitro, and purified using lysine-Sepharose column chromatography. The bacterial endotoxin level was determined with the *Limulus* amebocyte lysate assay kit (Chromogenix AB, Mölndal, Sweden), and the rhLK68 preparation used in this study contained bacterial endotoxin at less than 0.4 ng/mg protein.

**Reagents and Antibodies—** Recombinant human bFGF, produced from *Saccharomyces cerevisiae* was purchased from Upstate Biotechnology, Inc. Recombinant human VEGF, and HGF, both of which were produced from *Spodoptera frugiperda*, were purchased from Calbiochem (San Diego, CA). Sodium orthovanadate (OV) was purchased from Sigma. U0126, LY294002, rabbit antibodies against phospho-ERK1/2 and ERK1/2, and phospho-mitogen activated protein kinase kinase (MEK1/2) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit antibodies against phospho-Akt and Akt were purchased from BD Biosciences Pharmingen (San Diego, CA). Mouse anti-actin was purchased from Oncogene Research Products (Boston, MA). Peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from KPL (Gaithersburg, MD).

**Cell Culture—** Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD). HUVECs within passages 3–6 were used for all experiments. HUVECs were maintained in 1.5% gelatinized 24-well plates in endothelial cell growth medium-2 (Clonetics). For studies on the cell migration and cell signaling stimulated by angiogenic factors, endothelial cell basal medium-2 supplemented with 1% fetal bovine serum was used.

**Wound Migration Assay—** The effect of rhLK68 on HUVEC migration stimulated by angiogenic factors including bFGF, VEGF, and HGF was assayed in a monolayer denudation assay as previously described (10). Confluent HUVECs were wounded by scraping, washed twice with phosphate-buffered saline to remove cellular debris, and incubated in endothelial cell basal medium-2 supplemented with 1% fetal bovine serum for 24 h, following which rhLK68 proteins (1 or 3 μg) were added. In some experiments, U0126 (1 μM), LY294002 (1 μM), or OV was also added as indicated. After 30 min, growth factors such as bFGF (3 ng/ml), VEGF (10 ng/ml), or HGF (10 ng/ml) were added to the monolayer and incubated at 37 °C under 5% CO₂ for 8 h. The control cultures were incubated in endothelial cell basal medium-2 plus 1% fetal bovine serum without growth factors. The cells that migrated into the denuded area were photographed with an Olympus C-3030 digital camera, and their numbers were quantified.

**Western Blot Analysis of Cell Signaling Molecules in HUVECs—** The effects of rhLK68 on activation of ERK or Akt were analyzed by Western blot analysis of endothelial cell extracts with antibodies to their active and phosphorylated forms. HUVECs were cultured in endothelial cell growth medium-2 until confluency, washed, and grown in endothelial cell basal medium-2 supplemented with 1% fetal bovine serum. After 24 h, the medium was replaced with fresh low serum medium with or without rhLK68 (1 or 3 μg). Occasionally, U0126, LY294002, or sodium orthovanadate was added. After a 30-min incubation, bFGF (3 ng/ml), VEGF (10 ng/ml), or HGF (10 ng/ml) was added and incubated for a further 10 or 20 min to determine the level of phospho-MEK1/2 or phospho-Akt, respectively. The level of phospho-MEKs and/or PI3-K was determined by Western blot analysis of endothelial cell extracts using rabbit antibodies against phospho-MEK1/2 or phospho-Akt, respectively. The level of phospho-ERKs was determined after incubation for 15 min with VEGF or for 30 min with bFGF or HGF. The cells were washed with phosphate-buffered saline and lysed with lysis buffer containing 20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor mixture. The lysates were quantified for protein concentration and separated on 4–20% precast SDS-PAGE gels. Control and rhLK68-treated lysates were subjected to Western blot analysis using rabbit anti-phospho-MEK1/2, anti-phospho-ERK, or anti-phospho-Akt. Equal protein loading was verified using anti-actin, anti-ERK1/2, or anti-Akt antibodies. Detection of immunoreactive bands was carried out using an ECL system (Amersham Biosciences).

**Immunostaining and Immunofluorescence Microscopy—** HUVECs (50,000 cells/cm² and 5,000 cells/cm²) were cultured on glass coverslips in endothelial cell growth medium-2 to obtain confluent and subconfluent cultures at the time of bFGF stimulation, respectively. After serum starvation, the HUVECs were treated with either 1 μM U0126 or 1 μM rhLK68 in the presence or absence of OV (10 μM) for 30 min. The cells were then treated with 5 ng/ml bFGF for 30 min. For immunostaining, the cells were washed with Tris-buffered saline, fixed with 4% paraformaldehyde on ice for 10 min, and permeabilized by treatment with Tris-buffered saline containing 0.4% Triton X-100. The coverslips were then treated with blocking buffer (3% bovine serum albumin in Tris-buffered saline) for 1 h at room temperature and incubated overnight at 4 °C with a rabbit antibody against phospho-ERK (Cell Signaling Technology) in blocking buffer. The unbound proteins were removed by washing, and the cells were subsequently incubated with fluorescein isothiocyanate-labeled secondary antibody (Kirkegaard & Perry Laboratories) for 1 h. The coverslips were then washed three times with Tris-buffered saline containing 0.05% Tween 20 and examined using a fluorescence microscope (Model Axioskop-2; Carl Zeiss, Jena, Germany).

**RESULTS**

**RhLK68 Inhibits HUVEC Migration Stimulated by bFGF, VEGF, or HGF—** To examine the effects of rhLK68 on HUVEC migration stimulated by angiogenic factors such as bFGF, VEGF, or HGF, an *in vitro* wound migration assay was employed as described above. HUVECs migrated significantly into the wound area in response to bFGF, VEGF, and HGF in a dose-dependent manner. When determined with the same concentration of proteins (10 ng/ml), HGF produced the highest level of HUVEC migration, followed by bFGF and VEGF (data not shown). Because bFGF induced similar level of HUVEC migration at concentrations of either 3 or 10 ng/ml, we used 3
experiments.

shown are representative of three separate and independent phospho-ERK (upper panels) or anti-ERK (lower panels). The results shown are representative of three separate and independent experiments.

ng/ml bFGF in the subsequent experiments. Exposure to rhLK68 inhibited the migration stimulated by bFGF, VEGF, or HGF in a dose-dependent manner (Fig. 1). The effects of a specific inhibitor of MEKs, U0126, and a specific PI3-K inhibitor, LY294002, on the migration of endothelial cells were also determined. As shown in Fig. 1, either U0126 or LY294002 almost completely blocked bFGF-, VEGF-, and HGF-induced migration of HUVECs. These results suggest that rhLK68 is an inhibitor of endothelial cell migration stimulated by multiple angiogenic factors and that both ERK and PI3-K/Akt signaling pathways may play an important role in the migration of endothelial cells.

RhLK68 Inhibits bFGF-, VEGF-, or HGF-stimulated Phosphorylation of ERK1/2, but Not Akt, in HUVECs—The possible involvement of ERK1/2 or Akt signaling pathways in endothelial cell migration prompted us to examine the effects of rhLK68 on the activation state of ERK1/2 and Akt. The angiogenic factors bFGF, VEGF, and HGF induced ERK1/2 phosphorylation in a time-dependent manner. In cells treated with bFGF or HGF, the phosphorylation of ERK1/2 was evident as early as 5 min, reached a maximum value at 15–20 min, and decreased thereafter. On the contrary, VEGF appeared to induce ERK activation more rapidly than bFGF or HGF, because phosphorylation of ERK1/2 increased to a peak level within 10 min of VEGF stimulation (data not shown). Interestingly, preincubation of either 1 or 3 μM rhLK68 inhibited the phosphorylation of ERK1/2 stimulated by bFGF, VEGF, or HGF, and this inhibition was dose-dependent (Fig. 2). In addition to the ERK signaling pathway, Akt signaling was also induced by treatment with bFGF, VEGF, or HGF. All of these angiogenic factors stimulated Akt phosphorylation maximally at 20 min after treatment. In contrast to the inhibition of ERK activation by rhLK68, however, rhLK68 did not influence Akt phosphorylation stimulated by bFGF, VEGF, or HGF (Fig. 3). To determine whether there was any cross-talk between these two signaling pathways, the activities of either MEK1/2 or PI3-K were selectively inhibited using U0126 or LY294002, respectively, and the effect on ERK1/2 and Akt phosphorylation was investigated. In cells activated by bFGF, VEGF, or HGF, U0126 inhibited the phosphorylation of ERKs but not Akt, whereas LY194002 inhibited Akt phosphorylation but did not influence ERKs (Figs. 2 and 3), indicating that the ERK and PI3-K/Akt signaling pathways act independently and without any significant cross-talk. RhLK68-mediated dephosphorylation of ERKs appears to be specific to endothelial cells, because phorbol 12-myristate 13-acetate-induced phosphorylation of ERKs in THP-1 human acute monocytic leukemia cells was not affected by this peptide (data not shown). Collectively, these results indicate that rhLK68 may exert its function by specifically interfering with the activation of ERKs, but not Akt, in endothelial cells. Moreover, they suggest that rhLK68 may target common ERK signaling pathways that are induced by multiple angiogenic factors.

Protein-tyrosine Phosphatases May Play an Important Role in rhLK68-mediated Inhibition of ERK Phosphorylation and Endothelial Cell Migration—Inhibition of ERK phosphorylation can be achieved either through the down-regulation of MEKs, which are responsible for the addition of phosphates to ERKs, or by the up-regulation of protein phosphatases that dephosphorylate activated ERKs. To explore the molecular mechanism involved in rhLK68-induced ERK dephosphorylation, we examined the effects of rhLK68 on MEK activation. Although bFGF, VEGF, and HGF induced the phosphorylation of MEKs, this signaling event was not affected by rhLK68 treatment (Fig. 4), thus indicating that dephosphorylation of ERKs by rhLK68 may be mediated by a protein phosphatase-
dependent mechanism. Because ERK1/2 requires the MEK1/2-mediated dual phosphorylation of threonine and tyrosine residues in the TEY motif of the activation loop (11), the inactivation of fully phosphorylated ERK1/2 requires the activities of serine/threonine and/or protein-tyrosine phosphatases. In contrast, full activation of Akt requires the phosphorylation of both the Thr-308 and Ser-473 residues; thus only serine/threonine phosphatase activities are required for Akt dephosphorylation. Taken together with the finding that rhLK68 inactivates ERK1/2 but not Akt, we hypothesized that protein-tyrosine phosphatases may be involved in ERK1/2-specific dephosphorylation by rhLK68. To examine this hypothesis, HUVECs were preincubated with a general protein-tyrosine phosphatase inhibitor, OV, or OV, or HGF in the presence of either 1 or 3 μM rhLK68. OV treatment recovered rhLK68-induced ERK dephosphorylation in a dose-dependent manner (Fig. 5). In cells stimulated with bFGF, VEGF, or HGF, treatment with 10 nM OV had little effect on rhLK68-induced ERK dephosphorylation. However, a higher concentration of OV (10 μM) resulted in a level of ERK phosphorylation that was nearly identical to cells stimulated by OV, and stimulated with bFGF, VEGF, or HGF, treatment with 10 μM OV had little effect on rhLK68-induced ERK dephosphorylation. These results indicate that inhibition of protein-tyrosine phosphatases by treatment with 10 μM OV can completely compensate for rhLK68-induced dephosphorylation. Given that rhLK68 inhibits endothelial cell migration by interfering with the phosphorylation of ERKs, we performed a wound migration assay to examine whether OV can also reverse the rhLK68-mediated inhibition of endothelial cell migration. As shown in Fig. 6, treatment with increasing concentrations of OV (0.01–10 μM) restored the rhLK68-mediated inhibition of HUVEC migration in a dose-dependent manner. This effect was more pronounced in cells treated with 1 μM rhLK68 than that in cells treated with 3 μM, reflecting differences in the migration inhibitory activities of 1 and 3 μM rhLK68. Collectively, these results indicate that rhLK68 inhibits the phosphorylation of ERKs and subsequent endothelial cell migration stimulated by multiple angiogenic factors, possibly via a protein-tyrosine phosphatase-dependent mechanism.

RhLK68 May Inhibit the Phosphorylation of ERKs Preferentially in Migrating Cells than Contact-inhibited Cells—Despite the strong correlation between the level of ERK phosphorylation and the corresponding degree of cell migration, we found that cell migration was somewhat similar even when the extent of ERK dephosphorylation was significantly higher in cells treated with U0126 than rhLK68 (Figs. 1 and 2). Moreover, although OV restored completely the rhLK68-mediated dephosphorylation of ERKs, it could not compensate the decreased cell migration completely (Figs. 5 and 6). To explain this lack of correlation between ERK activity and the rate of migration, we raised a hypothesis that those treatments may affect differentially the phosphorylation of ERKs between migrating and nonmigrating cells. To address this possibility, immunostaining for phospho-ERK was performed using confluent or sparse cells, representing two distinct cell populations in the wound migration assay system employed in the present study: one contact-inhibited cells and one migrating cells—respectively. When compared with saline-treated cells (Fig. 7A), bFGF significantly induced the phosphorylation of ERKs in both confluent and sparse cells (Fig. 7B). However, the ERK phosphorylation was less apparent in confluent cells in comparison with that found in sparse cells. Similarly, inhibition of ERK phosphorylation by cell-cell contact was also reported by Lampugnani et al. (12) in VEGF-treated HUVECs. U0126 sig-

![Fig. 4. Effect of rhLK68 on the phosphorylation of MEKs. HUVECs were preincubated with rhLK68 (1 or 3 μM) for 30 min and were stimulated with bFGF (A, 3 ng/ml), VEGF (B, 10 ng/ml), or HGF (C, 10 ng/ml) for 10 min. Total cellular proteins were analyzed by SDS-PAGE and subsequent Western blotting using anti-phospho-Akt (upper panels) antibodies. Actin (lower panels) was used as a loading control. The results shown are representative of three separate and independent experiments.](image)

![Fig. 5. Inhibition of rhLK68-mediated ERK dephosphorylation by OV. HUVECs were preincubated in the presence or absence of either rhLK68 (1 or 3 μM) or OV for 30 min as indicated. The cells were stimulated with bFGF (A, 3 ng/ml), VEGF (B, 10 ng/ml), or HGF (C, 10 ng/ml) and lysed, and the proteins were separated by SDS-PAGE. The phosphorylation of ERKs was determined by Western blotting using anti-phospho-ERK (upper panels) or anti-ERK (lower panels). Relative amounts of phospho-ERKs normalized to total ERKs are indicated between the upper and lower panels. The level of ERKs in unstimulated cells was considered as 1.0. The results shown are representative of three separate and independent experiments.](image)
significantly inhibited the phosphorylation of ERKs induced by bFGF in both cell populations (Fig. 7C). On the other hand, although rhLK68 inhibited the phosphorylation of ERKs in confluent cells, its inhibitory effects were significantly higher in sparse cells (Fig. 7D). OV restored the rhLK68-mediated ERK dephosphorylation in both cell populations (Fig. 7E). Similar results were also observed in VEGF- or HGF-treated cells (data not shown). These results were consistent with the results obtained from Western blot analysis and indicate that rhLK68 may inhibit the phosphorylation of ERKs induced by angiogenic factors preferentially in migrating cells than in contact-inhibited cells.

**DISCUSSION**

During angiogenesis, quiescent endothelial cells become activated by pro-angiogenic factors and are induced to migrate, proliferate, and differentiate to form a new lumen-containing vessel. Because the migration of endothelial cells is a critical step in angiogenesis, the inhibition of endothelial cell motility would be expected to affect the angiogenic process. Although a number of angiogenesis inhibitors have been reported to inhibit endothelial cell migration, angiogenesis, and consequently the growth of angiogenesis-dependent malignancies, their mechanism of action is not well defined. In the migration of endothelial cells, the ERK (13, 14) and PI3-K/Akt (15, 16) signaling pathways have been reported to play critical roles. In this study we have consistently shown that selective inhibition of either ERK or PI3-K/Akt signaling, using U0126 or LY294002, almost completely prevented endothelial cell migration. Furthermore, although cross-talk between these adjacent signaling pathways has been reported previously (17), here activation of ERK and PI3-K/Akt signaling induced by bFGF, VEGF, or HGF appeared to occur without any cross-talk, because PI3-K inhibitors had no effect on ERK but abolished Akt phosphorylation, whereas MEK inhibitors significantly inhibited ERK activation without affecting Akt phosphorylation.

Of these two signaling pathways, rhLK68 selectively inhibited ERK1/2 phosphorylation induced by bFGF, VEGF, or HGF, without influencing the phosphorylation of Akt. In addition, endogenous angiogenesis inhibitors such as the 16-kDa fragment of prolactin (18), platelet factor 4 (19), and plasminogen kringle 5 (20) have also been reported to inhibit the phosphorylation of ERKs and endothelial cell migration. Therefore, inhibition of ERK phosphorylation appears to be an important mechanism of action employed by angiogenesis inhibitors. Despite the importance of ERK and PI3-K/Akt signaling pathways, inhibition of ERK or Akt signaling does not always seem to be required to inhibit endothelial cell migration. Angiostatin, for instance, has been reported to inhibit the phosphorylation

![Effect of OV on the rhLK68-mediated inhibition of HUVEC migration.](image1)

![Differential effects of rhLK68 on the phosphorylation of ERKs between confluent and sparse cells.](image2)
of ERKs (21). However, Eriksson et al. (22) reported contradictory results demonstrating that angiostatin inhibits endothelial cell migration without affecting intracellular signaling including the ERK and Akt pathways. Recently, Wajih and Sane et al. (23) demonstrated that angiostatin selectively inhibits HGF-induced phosphorylation of both ERKs and Akt by interfering with the binding of HGF to their receptor, c-Met, but does not affect bFGF- or VEGF-induced phosphorylation of ERKs and Akt. These findings suggest that, in addition to ERK and PI3-K/Akt signaling, additional signaling cascades are likely to be involved in endothelial cell migration.

Because of the critical importance of ERK1/2 in cellular signaling, the activities of ERKs must be tightly regulated, and this may be achieved by the balanced activities of MEKs and/or various types of ERK-specific protein phosphatases. Although the mechanisms of ERK activation have been well characterized, much less is known about its subsequent inactivation. Given that rhLK68 inhibits endothelial cell migration stimulated by multiple angiogenic factors and does not affect the activation of MEKs, it appears unlikely that rhLK68 affects events upstream of ERK activation, such as the binding of angiogenic factors to their receptors, receptor tyrosine kinase activation, and subsequent signaling cascades leading to activation of MEKs. In contrast to ERK activation, which can be accomplished solely by MEKs, the presence of multiple protein phosphatases involved in ERK inactivation indicates that these enzymes may play an important role in the regulation of ERK activity. Considering that dephosphorylation of either the phosphothreonine or phosphotyrosine residue is sufficient for its total enzymatic inactivation (24), ERK1/2 deactivation could occur through the action of all classes of protein phosphatases including serine/threonine protein phosphatase PP2A (25, 26), protein-tyrosine phosphatase PTP-SL (27), and HePTP (28) or through the dual specificity mitogen-activated protein kinase phosphatases (29). Of these options, protein-tyrosine phosphatases seem to play a significant role in rhLK68-induced ERK dephosphorylation and decreased migration of endothelial cells, because these events are restored by treatment with a tyrosine phosphatase inhibitor, sodium orthovanadate, in a dose-dependent manner. This notion is further supported by the findings that full inhibition of serine/threonine phosphatase using FK506 produces only a partial recovery of rhLK68-phosphorylation (30). Similarly, endostatin has been reported to activate the serine/threonine phosphatase PP2A, and this, in turn, perturbs the phosphorylation of endothelial nitric-oxide synthase that is essential for endothelial cell migration and survival (31). Moreover, pharmacological inhibition of protein phosphatase activity has been shown to stimulate the motility of endothelial cells and nonmammalian tumor cells (32, 33). Recently, DEPI/CD148, a membrane-associated tyrosine phosphatase that is up-regulated by confluent and is located at intercellular junctions, has been reported to play a crucial role in developmental vascular organization through the regulation of endothelial proliferation and endothelium-pericyte interactions (34). It also contributes to vascular endothelial cadherin-induced inhibition of VEGF receptor-2 activation (12) and can dephosphorylate HGF receptor c-Met (35), suggesting that this phosphatase has a general role in regulating signaling from growth factor receptors. Collectively, these results suggest that protein phosphatases may play an important role in the regulation of angiogenesis. Therefore, further elucidation of complete phosphatase regulatory networks and their specific substrates may provide more insights into the molecular mechanisms of action of angiogenesis inhibitors, including rhLK68, and/or provide useful molecular target(s) for the rational design of anti-angiogenic therapy.

RhLK68 appears to block a common angiogenic signaling pathway triggered by various angiogenic factors including bFGF, VEGF, and HGF. This is an important and clinically beneficial aspect in blocking tumor angiogenesis. Because cancer masses consist of heterogeneous populations of tumor cells that constantly undergo genetic mutations, they are very likely to switch their angiogenic factors. Indeed, an early tumor may secrete one or two angiogenic factors, whereas a large progressive tumor can produce many angiogenic factors (36). Angiogenesis inhibitors that target only one particular angiogenic factor may therefore evade drug resistance problems. Therefore, broad spectrum inhibitors that block common angiogenic pathways could bypass drug resistance and may be therapeutically effective against multiple cancer types. In conclusion, we demonstrate in the present study that rhLK68 is a potent inhibitor of endothelial cell migration stimulated by multiple angiogenic factors and that this inhibition may be mediated through the perturbation of ERK1/2 signaling via a protein-tyrosine phosphatase-dependent pathway.

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